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Curcumin induces apoptosis in human neuroblastoma cells via inhibition of AKT and Foxo3a nuclear translocation

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Abstract

Neuroblastoma (NB) is one of the most frequent extracranial solid tumors in children. It accounts for 8–10% of all childhood cancer deaths, and there is a need for development of new drugs for its treatment. Curcumin (diferuloylmethane), a major active component of turmeric (Curcuma longa), has been shown to exert anti-tumor activity on NB, but the specific mechanism by which curcumin inhibits cancer cells proliferation remains unclear. In the present study, we investigated the anti-proliferative effect of curcumin in human LAN5 NB cells. Curcumin treatment causes a rapid increase in reactive oxygen species and a decrease in the mitochondrial membrane potential—events leading to apoptosis activation. Furthermore, curcumin induces decrease in haet shock protein (Hsp)60 and hexokinase II mitochondrial protein levels and increase in the pro-apoptotic protein, bcl-2 associated death promoter (BAD). Moreover, we demonstrate that curcumin modulates anti-tumor activity through modulation of phosphatase and tensin homolog deleted on chromosome 10 and consequential inhibition of the survival Akt cell-signaling pathway. Inhibition of Akt causes its translocation into the cytoplasm and import of Foxo3a into the nucleus where it activates the expression of p27, Bim, and Fas-L pro-apoptotic genes. Together, these results take evidence for considering curcumin as a potential therapeutic agent for patients with NB.

Keywords: neuroblastoma, curcumin, oxidative stress, apoptosis, signaling pathway

Abbreviations: AML, Acute Myeloid Leukemia; ANOVA, Analysis of Variance; Akt (PKB), protein kinase B; BAD, bcl-2 associated death promoter; BSA, Bovine Serum Albumine; DCFH-DA, dichlorofluorescein diacetate; DTT, Dithiothreitol; 5′-FU, 5′ Fluoracil; HSP, Haet Shock Protein; HKII, hexokinase-II; $\Delta\Psi$m, mitochondrial membrane potential; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; NF-κB, nuclear factor kappa B; PAGE, Poly Acrilamide Gel Electrophoreses; PBS, Phosphate Buffered Saline; PCR, Polymerase Chain Reaction; PICT (GLTSCR2), Glioma Tumor Suppressor Candidate RegionGene 2; PTEN, phosphatase and tensin homolog deleted on chromosome 10; PTP, permeability transition pore; ROS, reactive oxygen species; SDS, Sodium Dodecyl Sulfate; TBST, Tris Buffered Saline plus Tween-20; TRITC, Tetramethyl rodamine-5(and6) isothiocyanate

Introduction

Neuroblastoma (NB) is an aggressive childhood cancer of the peripheral nervous system arising from neural crest sympathoadrenal progenitor cells. Children with localized NB and favorable tumor genomic characteristics have an excellent overall survival probability, with little or no cytotoxic therapy. However, approximately 50% of patients with NB have a clinically aggressive form of the disease with overall survival rates of less than 40% [1,2]. Although high-risk NB accounts for only 4% of all pediatric cancer diagnoses, it is responsible for 12% of pediatric cancer deaths [3], and new therapeutic approaches are desirable. Chemotherapeutic agents usually utilized in cancer treatment are toxic not only to tumor cells but also to normal cells, so these agents produce major side effects. In addition, these agents are highly expensive and thus not affordable for most. Traditional medicines are generally free of the deleterious side effects and usually inexpensive. Medicines derived from plants have played a pivotal role in the health care of many ancient and contemporary cultures even if, in some countries, the use of medicinal plants is often associated with witchcraft and superstition, because people did not have the scientific insight to explain and predict the curative action of plants [4–8]. Over the last two decades, however, successful attempts to better understand molecular mechanisms of action of some natural products have stimulated interest in their therapeutic use in modern medical settings. Extensive research over the last half-century has revealed several important functions of curcumin. This natural compound is a diphenolic extracted from the rhizome of turmeric (Curcuma longa), and is a prominent candidate for treating cystic fibrosis, anti-inflammatory, neurodegenerative, and malarial diseases in addition to cancer [9].

The anti-cancer potential of curcumin stems from its ability to suppress proliferation of a wide variety of malignant cell types, as well as tumor initiation, promotion, and metastasis, presumably due to its myriad of biological properties [9]. Moreover, extensive recent researches in cell cultures and in rodents have revealed that curcumin can sensitize tumor to different chemotherapeutic agents including doxorubicin, 5′ Fluoracil (5-FU), paclitaxel, vincristine, melphalan, butyrate, cisplatin, celecoxib,

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vinorelbine, gemcitabine, oxaliplatin, etoposide, sulfinosine, thalidomide, and bortezomib [7,10]. The cellular and molecular mechanisms underlying curcumin-mediated apoptosis, which seem to depend and diverge in different cell lines and in NB cells, have not been well defined. Some studies have showed that curcumin induced cell cycle arrest and apoptosis by p53 activation [11]. Curcumin significantly suppresses nuclear factor kappa B (NF-κB)-mediated radioprotection and modulates expression of apoptosis-related genes in human NB cells [12]. Moreover, it has been demonstrated that curcumin's effect is improved if it is entrapped into nanoparticle drug delivery systems, enhancing activation of Hsp70 and reducing NF-kB activity in several NB cell lines, such as LAN5, NB1691, CHLA-20, and SK-N-AS [13,14].

Apoptosis is, often, a consequence of reactive oxygen species (ROS) generation. Cell metabolism generates potentially harmful ROS. At moderate levels, ROS act as second messenger for different cellular functions [15]. At the same time, a variety of mechanisms protect cells against ROS excess. However, chronic and/or abrupt increases in ROS levels above a physiological threshold may trigger cell death by interfering with normal cellular mechanisms. Several reports suggest that curcumin can potentially increase the oxidative stress within cells by enhancing the reactivity of ROS like superoxide and hydrogen peroxide. Curcumin, indeed, may promote the one-electron reduction (i.e., protonation) of superoxide to form the hydroperoxyl radical [16,17], which is believed to be more reactive than superoxide in biological systems [18]. Furthermore, curcumin may also promote the reduction of transition metals such as iron (i.e., the reduction of ferric ($Fe3+$) iron to ferrous ($Fe2+$) iron) that can catalyze Fenton chemistry and generate the highly reactive hydroxyl radical from hydrogen peroxide [19–22].

The tumor suppressor protein gene, phosphatase and tensin homolog deleted on chromosome10 (PTEN), is one of the most frequently mutated genes in human cancer. PTEN is a non-redundant, plasma-membrane lipid phosphatase that antagonizes the highly oncogenic pro-survival PI3K/Akt signaling pathway [23,24]. Akt promotes cell survival pathway by inactivating certain pro-apoptotic mediators such as transcription factors of the forkhead (FOXO) family [25,26] or the Bcl2 antagonist, Bad protein. FOXO transcription factors are key players in cell death/life pathways. In neurons, FOXO has been involved mostly in cell death processes such as those because of trophic deprivation [27] or excess ROS [28].

The aim of this study was to identify mechanisms and molecules involved in curcumin-mediated apoptosis in NB LAN5 cells.

Materials and methods

Cell cultures and treatment

LAN5 NB cells were plated onto 96-well plates at a density of 6×10^4 cells per well and cultured with RPMI 1640 medium (Celbio srl, Milan, Italy), supplemented with 10% fetal bovine serum (Gibco-Invitrogen, Milan, Italy) and 1% antibiotics (50 μg ml of penicillin and 50 μg/ml of streptomycin) and anti-mycotic agent (SIGMA). Cells were maintained in a humidified 5% CO_2 atmosphere at $37 \pm 0.1°C$. LAN5 cells were treated with growing concentration (5–10–15–20 μM) of curcumin (SIGMA) for 24 h. In time course experiment, curcumin was utilized at 15 μM for 3, 5, and 24 h. In the experiment using α-tocopherol, 15 μM of curcumin and 50 μM of α-tocopherol were used . The treated cultured cells and the controls were morphologically analyzed by microscopy inspection. The images were obtained using an Axio Scope 2 microscope (Zeiss, USA) and captured using an Axiocam (Zeiss, USA) digital camera interfaced with a computer.

Cell viability

Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (Promega Italia, S.r.l., Milan, Italy). MTS was utilized according to the manufacturer's instructions. After cell treatments, 20 μl of the MTS solution was added to each well, and the incubation was continued for 4 h at 37°C, 5% CO_2. The absorbance was read at 490 nm on the Microplate reader WallacVictor 2 1420 Multilabel Counter (PerkinElmer, Inc. Monza, Italy). Results were expressed as the percentage of MTS reduction in the control cells.

ROS generation and mitochondrial membrane potential analysis

To assess ROS generation, the cells (6×10^4) were incubated in 96-well black microtiter plate. Afterward, cells were incubated with 1 mM dichlorofluorescein diacetate (DCFH-DA) in phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, and 8 mM Na3PO4; pH, 7.4) for 10 min at room temperature in the dark. After washing with PBS, the cells were analyzed by fluorescent microplate reader (WallacVictor 2 1420 Multilabel Counter; PerkinElmer, Inc.) capable of reading 480 nm (excitation) and 530 nm (emission). The mitochondrial membrane potential ($\Delta\Psi m$) was measured directly using a MitoProbe JC-1 Assay kit (Molecular Probes, Eugene, OR, USA). After treatment, the cells were incubated with 2 mM 5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) fluorescent dye in PBS for 30 min at 37°C. Fluorescence emission shift of JC-1 from red (590 nm) to green (529 nm) was evaluated by fluorimeter (Microplate reader WallacVictor 2 1420 Multilabel Counter; PerkinElmer, Inc.).

Apoptosis assays

A terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (Promega) was performed according to the manufacturer's instructions. After curcumin treatment, the cells were fixed in 4% paraformaldehyde

with PBS for 30 min. The samples were then washed with PBS, permeabilized with 0.2% Triton X-100 in PBS for 5 min, rinsed with PBS, and incubated with a TUNEL reaction mixture (enzyme and nucleotides) in a humidified atmosphere at 37°C for 1 h. Staining was obtained using a peroxidase substrate, hydrogen peroxide, and the stable chromogen diaminobenzidine. After washing three times in PBS, the samples were analyzed using a Zeiss Axio Scope microscope. For nuclear staining, cells were incubated with Hoechst 33258 (5 µg/mL). The samples were analyzed using a DHL fluorescent microscope (Leica).

Protein extraction and Western blotting

Proteins were obtained from total extract or from cytoplasmic and nuclear extracts of untreated (control) or treated with curcumin LAN5 cells. Cytoplasmic and nuclear extracts were obtained by using NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Thermo Scientific) according to the manufacture's instructions. The pellets of all these samples were lysed in ice cold solubilizing buffer (50 mM Tris–HCl pH 87.4, 150 mM NaCl, 0.5% Triton X-100, 2 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM Dithiothreitol (DTT), 0.1% sodium dodecyl sulfate (SDS) with protease inhibitor; Amersham Biosciences (Milan, Italy) and phosphatase inhibitor cocktail II; Sigma-Aldrich, Milan, Italy). Protein samples (20 µg) were electrophoretically separated using 10% SDS-poly acrilamide gel electrophoreses (PAGE) gel and transferred onto nitrocellulose filters for immunoblotting. After blocking in 3% bovine serum albumine (BSA) in tris buffered saline plus tween-20 (TBST), the Western blot was incubated with anti-phosphorylated Akt (S473) (1:500; Invitrogen), anti-Akt (1:1000; Cell Signaling), anti-phosphorylated Foxo 3a (S253) (1:500; Cell Signaling), anti-Foxo 3a (1:1000; Cell Signaling), anti-hexokinase-II (HKII) (1:1000; Cell Signaling), anti-Hsp60 (1:1000; Cell Signaling), anti-BAD (1:1000; Cell Signaling), anti-Lamin B (1:1000; Cell Signaling), and anti-β-actin (1:1000; Sigma-Aldrich). Primary antibodies were detected using the ECL chemiluminescence kit (Amersham Biosciences, Milan, Italy) according to the manufacturer's instructions and using secondary antibodies conjugated with horseradish peroxidase (1:2000; Cell Signaling, Boston, USA). Band intensities were analyzed with a gel documentation system (Bio-Rad, Milan, Italy), and expression was adjusted to β-actin expression. The protein levels were expressed as densitometry and percentage of controls.

Immunofluorescence

LAN5 cells (5×10^5) were cultured on Lab-Tek II Chambered Coverglass (Nunc) and treated as mentioned above. After washing in PBS, the cells were fixed in 4% paraformaldehyde for 30 min and kept at 4°C. After incubation with 3% BSA/PBS for 1 h, the cells were then immunostained with anti-phosphorylated Akt or anti-Foxo3a

(1:100 Santa Cruz Biotechnology) antibody at 4°C overnight. After washing in PBS, the samples were incubated with anti-rabbit tetramethyl rodamine-5(and6) isothiocyanate (TRITC) conjugated secondary antibody (1:300 SIGMA). For nuclear staining, cells were incubated with Hoechst 33258 (5 µg/mL). The samples were analyzed using a DHL fluorescent microscope (Leica). Fluorescence intensity was measured using LeicaQFluoro program.

Quantitative real-time polymerase chain reaction (PCR)

Total RNA was extracted using RNeasy Mini Kit (Qiagen). Two nanograms of RNA was used to synthesize the first-strand cDNA using RT First Strand Kit (Qiagen). Synthesized cDNAs were amplified using RT^2 SYBR Green/ROX qPCR Mastermix (Qiagen) and StepOne Real-Time instrument (Applied Biosystem). Gene expression validation was performed using RT2 qPCR Primer Assay for human Foxo3a, FasL, Bim, p27, and actin (SABiosciences). The experiment was performed on a StepOne Real-Time instrument (Applied Biosystem). Gene expression was normalized to actin. The data were analyzed using the comparative Cycle Threshold (CT) method.

Statistical analysis

All experiments were repeated at least three times, and each experiment was performed in triplicate. The results are presented as mean SD. Statistical evaluation was conducted using analysis of variance (ANOVA), followed by Student's t-test for analysis of significance. Results with a *P* value of less than 0.05 were considered statistically significant.

Results

Curcumin inhibits cell viability and induces apoptosis in LAN5 NB cells

Deregulated cell proliferation is a hallmark of cancer cells. To determine the anti-tumor activity of curcumin in NB cell line, LAN5 cells were treated with different concentrations of curcumin for 24 h and assessed for cell viability and morphological assay. Curcumin treatment induced a drastic alteration on the cell morphology (Figure 1A) and an inhibition of cell viability in a dose-dependent manner (Figure 1B). At the maximum concentration (20 µM), curcumin significantly reduced the percentage of viable LAN5 cells to 50% (Figure 1B). Since morphological changes in NB cells, that is, a significant number of NB cells started rounding up, were observed after curcumin treatment, we investigate whether these changes were due to apoptosis activation. Cells were treated with increasing doses of curcumin for 24 h, and apoptosis was visualized using TUNEL assay (Figure 1C, D). The proportion of apoptotic cells significantly increased through exposure to 20 µM of curcumin (50%), compared with that of non-treated control cells. Moreover, the presence of
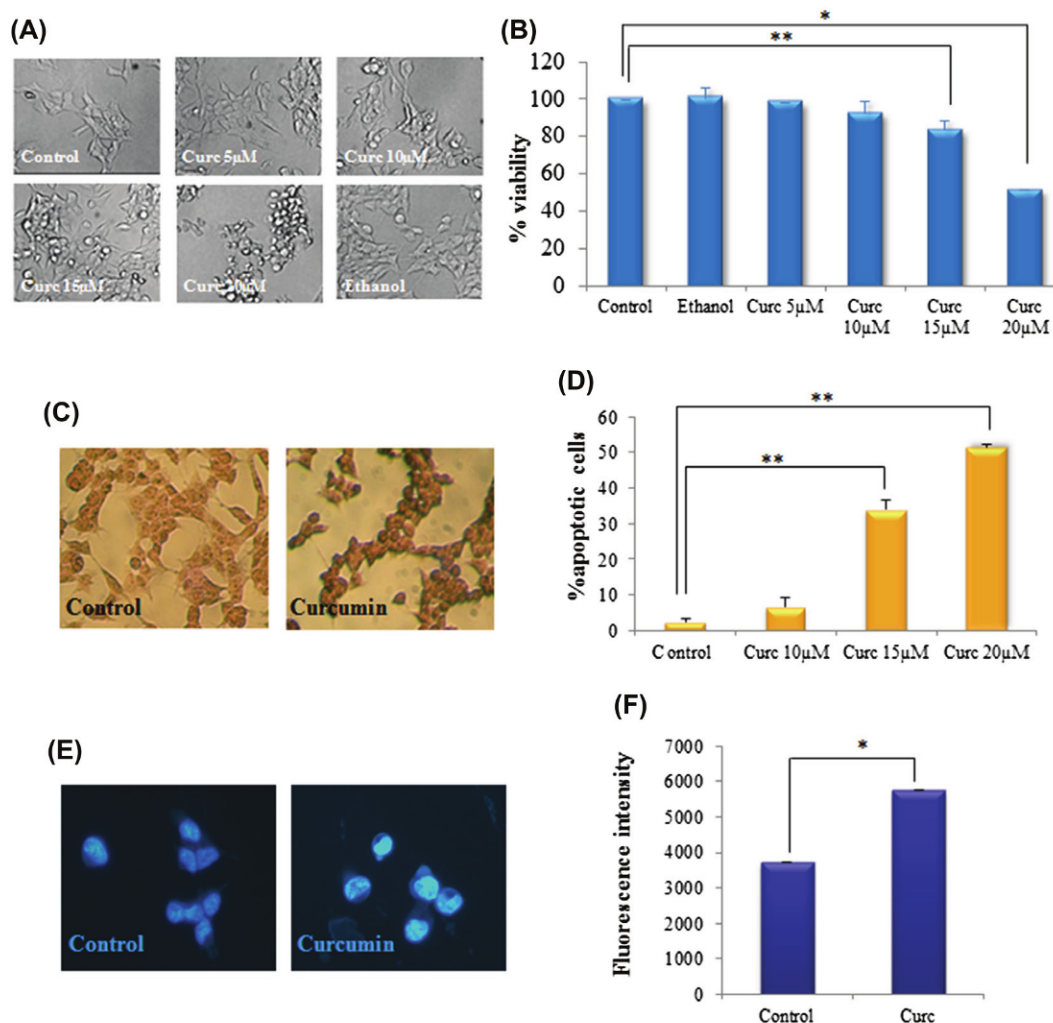
Figure 1. Curcumin induces toxicity in dose-dependent manner. A) Representative morphological images of LAN5 (6×10^4 cells/well) cells that were untreated (Control), or treated with curcumin (Curc) at different concentrations for 24 h. B) LAN5 NB cells were untreated (Control) or treated with curcumin as described above and submitted to MTS assay. The viability is expressed as the percentage of MTS reduction in the control cells. C) Apoptosis was verified using TUNEL assay. D) Percentage of apoptotic cells after incubation with 10, 15, and 20 μM of curcumin. E) Fragmentation of the nuclei was evidenced using Hoechst 33258. F) Measure of the fluorescence intensity in the nuclei. All the experiments were repeated three times. *$P < 0.05$, **$P < 0.01$, vs. indicated groups.

apoptosis was confirmed by Hoechst 33258 staining of the nuclei. Intensive blue staining revealed the presence of DNA nicks (Figure 1E). Thus, the total cell population decreased with this treatment indicating that curcumin exhibits anti-tumor activity.

Effects of curcumin on ROS production and mitochondrial membrane potential

Oxidative stress is one of the main causes leading to cell death. We evaluated whether curcumin induces apoptosis as a consequence of ROS activation. Intracellular ROS accumulation, produced by curcumin addition in LAN5 cells, was measured using the fluorescent probe DCFH-DA, in which the emitted fluorescence is directly proportional to the concentration of hydrogen peroxide inside the cell. Fluorimetric analysis showed that fluorescence intensity, corresponding to the intracellular ROS presence, increased in proportion to the curcumin concentrations, after 24 h of treatment (Figure 2A). These results were also

confirmed by immunohistochemistry experiment, in which the curcumin treatment induces an increase in fluorescence compared with the control (Figure 2B). ΔΨm is an important parameter of mitochondrial function and used as cell health indicator. A significant loss in ΔΨm depletes cells of energy with subsequent cell death cascade. We measured ΔΨm using the potential sensitive dye, JC-1. At physiological membrane potential, JC-1 forms red fluorescent aggregates. Therefore, undamaged cells stained with JC-1 exhibit a red fluorescence, detectable by fluorimetric analysis. On the other hand, unhealthy cells result in a breakdown of ΔΨm and a prevalent increase of green fluorescence. As shown in Figure 2C, curcumin induced a rapid increase in green fluorescence and a reduction in the red fluorescence at all the tested concentrations indicating that a decrease in the ΔΨm occurred. The result was also visible through macroscopic fluorescence observation (Figure 2D). Curcumin exhibits its apoptotic activity via stress oxidative activation and mitochondrial dysfunction.
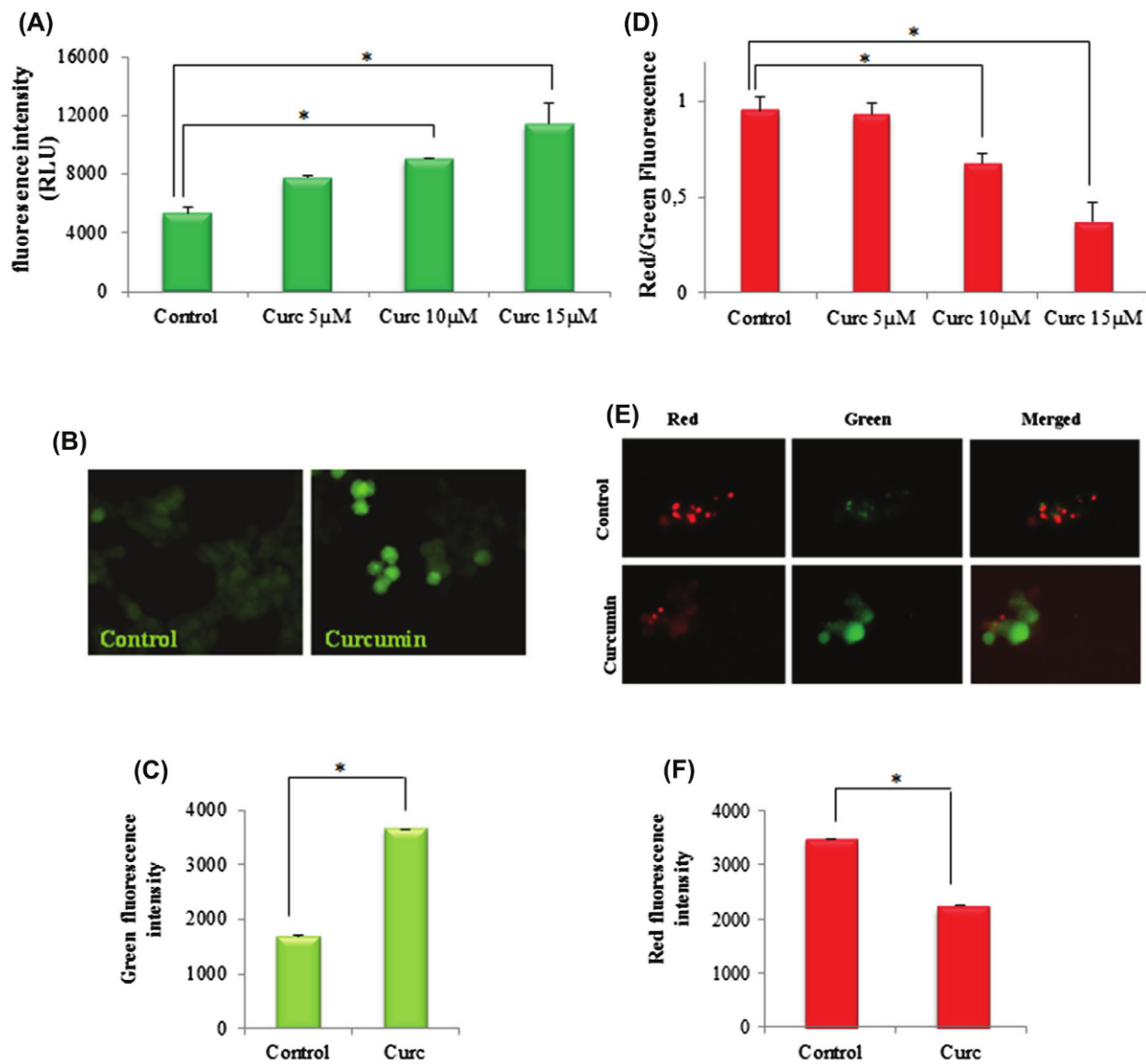
Figure 2. Curcumin induces ROS generation in NB cells in dose-dependent manner. A) LAN5 (6×10^4 cells/well) cells were untreated (Control) or treated with different concentrations of curcumin (Curc) for 24 h. After this treatment, the cells were submitted to DCFH-DA assay and fluorescence was measured. B) Representative fluorescent images of untreated (control) and curcumin (15 μM)-treated LAN5 cells. LAN5 cells untreated (Control) or treated with different concentrations of curcumin (Curc) were submitted to JC-1 assay. D) The histogram represents the obtained red/green fluorescence ratio. E) Representative red and green fluorescent images of untreated (control) and curcumin (15 μM)-treated LAN5 cells after JC1 assay. C, F) Measure of the fluorescence intensity in the cells. The experiments were repeated three times. *$P < 0.05$, **$P < 0.01$, vs. indicated groups.

Curcumin modulates mitochondrial proteins expression

To confirm that curcumin induces apoptotic activity through mitochondrial damage, the expression levels of some mitochondrial proteins, such as Hsp60, HKII, and Bad, were examined. In particular, we analyzed Hsp60—an important constitutive mitochondrial protein considered a marker of mitochondrial integrity. During oxidative stress, mitochondrial integrity is disrupted through the opening of a large channel referred as the permeability transition (PT) pore that produces mitochondrial depolarization, a key event to initiate the cell death cascade. The PT pore has been seen to be composed of the voltage-dependent anion channel, adenine nucleotide translocator, cyclophilin D, and HK-II proteins, although perhaps other molecular constituents are present. The opening of the PT pore favors cytochrome c release and apoptosis activation. Further, apoptosis induction can be confirmed by activation of Bad—a pro-apoptotic member of the Bcl-2 gene family. Bad may reside in the cytosol, but translocates to mitochondria following a death signal making a pro-apoptotic complex with Bcl-xL. NB cells were treated with different concentrations of curcumin and the proteins extracted were submitted to Western blot. In agreement with the previous results, a reduction in Hsp60 and HK-II levels was observed in a dose-dependent manner (Figure 3A). In contrast, the pro-apototic Bad protein was increased after curcumin treatment in a dose-dependent manner.

Curcumin induces apoptosis via PTEN/Akt/Foxo3a pathway

PTEN and Akt constitute an important pathway in regulating the signaling of multiple biological processes, such as apoptosis, metabolism, cell proliferation, a
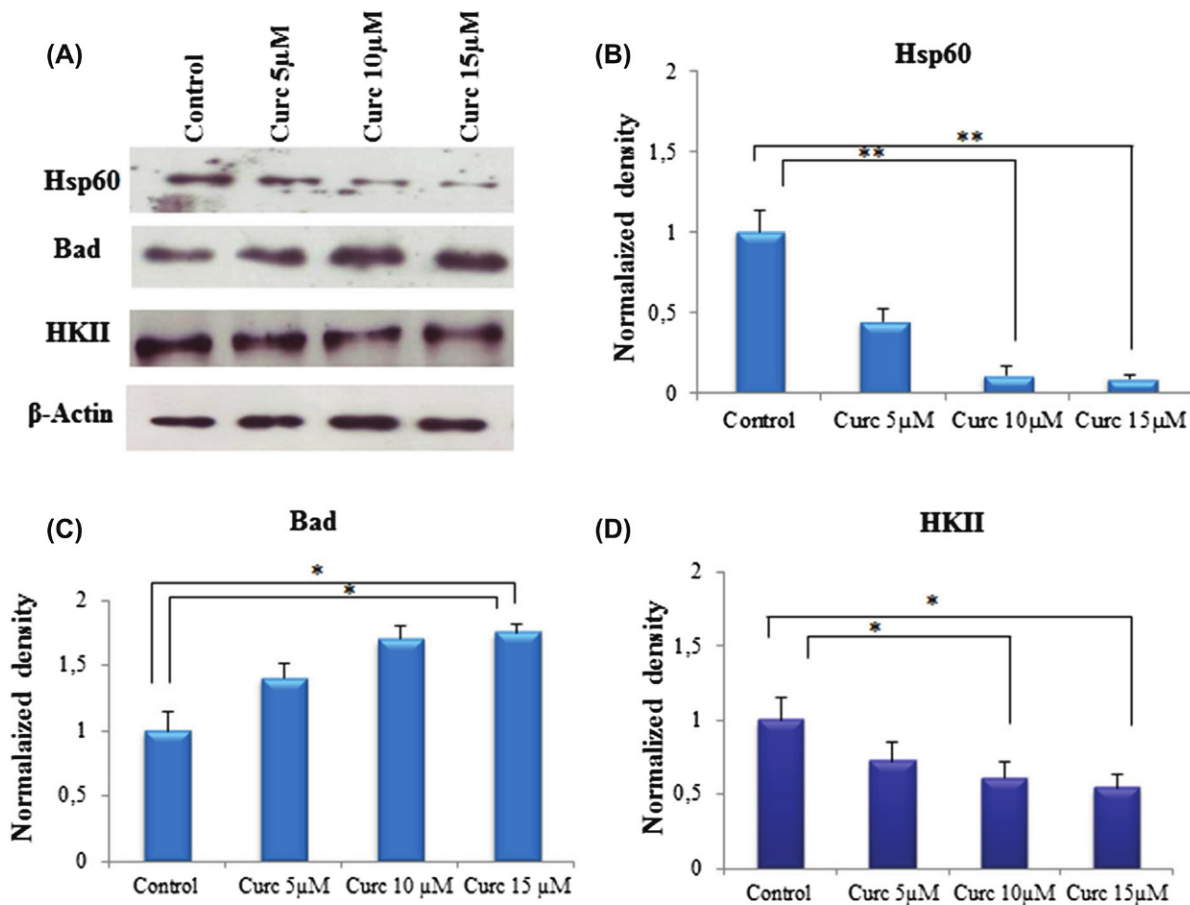
Figure 3. Curcumin affects mitochondrial proteins. Western blot of proteins (20 µg) extracted from LAN5 untreated or treated with curcumin (Curc) at different concentrations for 24 h and incubated with anti-Hsp60, anti-Bad, anti-HKII, and anti-β-actin. B-D) Quantification of immunoreactivity was performed using densitometric analysis; uniformity of gel loading was confirmed with β-actin utilized as standard. *$P < 0.05$, **$P < 0.01$, vs. indicated groups.

Different studies have shown that constitutively activated Akt significantly protected cells from apoptosis [29]. A direct substrate for Akt are the members of the Forkhead transcription factors (Foxo), whose phosphorylation causes the inhibition of their transcriptional activity [30]. Akt phosphorylation can promote its translocation to the nucleus, where it directly phosphorylates Foxo by inhibiting its transcriptional activity and causing its export from the nucleus to the cytoplasm, thus activating a cell survival program [31,32]. In contrast, in response to oxidative stress and consequent triggering of cell death program, activated Akt moves into cytoplasm and Foxo3a moves into the nucleus, where it activates pro-apoptotic genes. To determine whether curcumin-induced apoptosis is associated with PTEN/Akt/ Foxo3a activity, we determined the PTEN and phosphorylated Akt and Foxo 3a levels, after treatment with different concentrations (5, 10, and 15 µM) of curcumin for 24 h. PTEN expression increases in a dose-dependent manner after curcumin treatment (Figure 4A, B), whereas Akt— constitutively activated in the cells—decrease its expression level (Figure 4A, C). In contrast, as expected, an increase of Foxo3a levels was detected (Figure 4A, D). Moreover, we investigated when curcumin activates the PTEN/Akt/ Foxo3a signaling. LAN5 cells were incubated with curcumin (15 µM) for different times (3, 5, 24, and 48 h) and the expression levels of the analyzed proteins were

evaluated (Figure 5). The time course experiment revealed that phosphorylated Akt is already activated at the earliest time and its level declined after 24 h. In contrast, according to the canonical pathway, PTEN and Foxo3a increase significantly with time following curcumin exposure.

Curcumin promotes translocation of Foxo3a, which is inhibited by α-tocopherol, into the nuclei

To obtain evidence that curcumin, thereafter phospho-Akt downregulation, promoted translocation of Foxo3a into the nuclei, NB cells, treated with or without two curcumin concentrations, were separated in cytosolic and nuclear fractions. Protein extracts were immunoblotted with antibodies against anti-phospho-Akt, anti-Foxo3a, and anti-laminin B, as a nuclear control. Levels of active Akt decreased, with respect to the control, in the nuclei of curcumin-treated cells in a dose-dependent manner; in contrast, the presence of Foxo3a into the nuclei of curcumin-treated cells increased in a dose-dependent manner (Figure 6A).

To visualize phospho-Akt and Foxo3a trafficking, an immunofluorescence experiment was carried out. In agreement with the Western blot results, we revealed that, under curcumin treatment, phospho-Akt is not present in the nuclei, whereas red staining due to the presence of Foxo3a
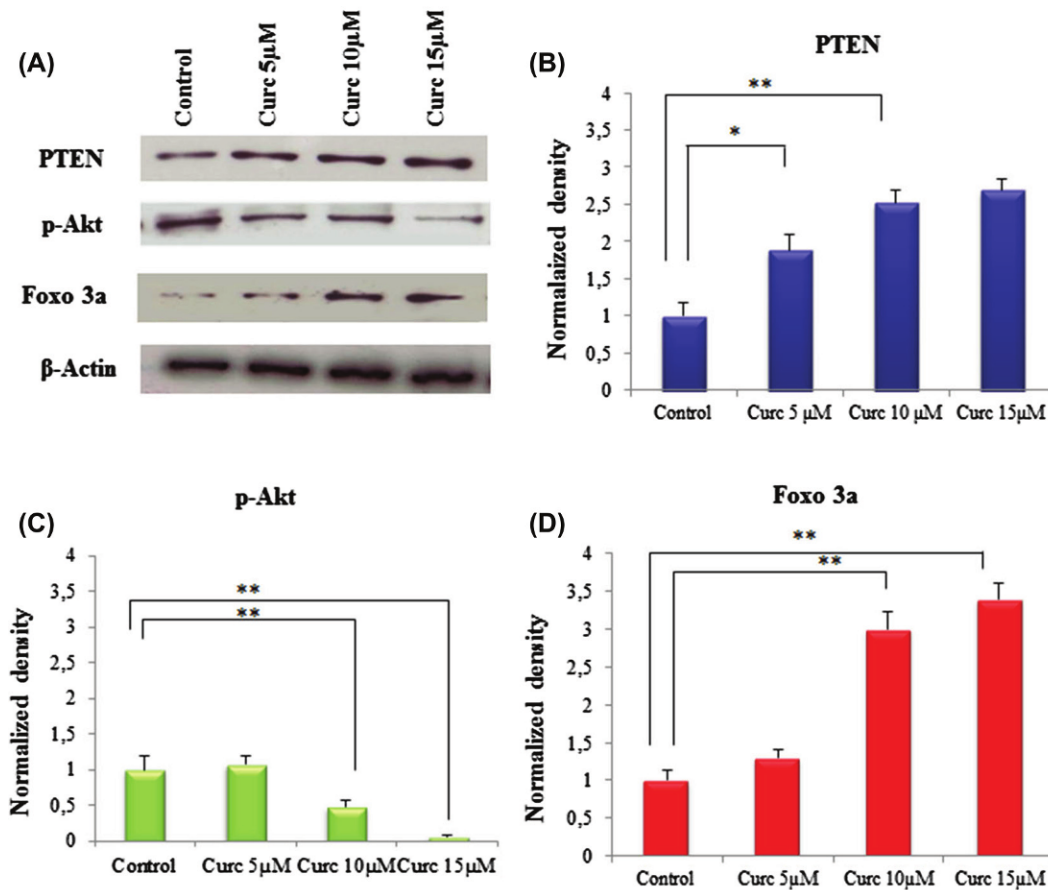
Figure 4. Curcumin modulates PTEN/Akt/Foxo3a pathway. A) Western blot of proteins (20 µg) extracted from LAN5 cells untreated (Control), or treated with different concentrations of curcumin (Curc) for 24 h and incubated with anti-PTEN, anti-phospho-Akt (p-Akt), anti-Foxo3a, and anti-β-actin. B-D) Quantification of immunoreactivity was performed using densitometric analysis; $*P < 0.05$, $**P < 0.01$, vs. indicated groups. Uniformity of gel loading was confirmed with β-actin utilized as standard.

is well observed (Figure 6D). Thus, curcumin favors movement of Foxo3a into the nucleus, where its pro-apoptotic function can be promoted. Finally, in order to confirm that Foxo3a is activated following the curcumin pro-oxidant action, we utilized α-tocopherol—a component of vitamin E. α-Tocopherol is the most abundant form of tocopherol in green leaves of many plant species and is useful as biological antioxidant due to its radical-scavenging activity [33]. Immunofluorescence experiment shows the ability of α-tocopherol to antagonize the effect of curcumin, inhibiting the movement of Foxo3a from cytoplasm to nucleus (Figure 7).

Activated Foxo3a induces pro-apoptotic gene expression

Foxo3a, also in response to oxidative stress, translocates from cytoplasm to nucleus, where it activates a gene expression program that regulates apoptosis. Foxo factors can initiate apoptosis by triggering both transcription of Fas-L, the ligand for the Fas-dependent cell death pathway, and the pro-apoptotic Bcl-2 family member, Bim. Moreover, Foxo3a factor can promote cell-cycle arrest by upregulating the cyclin-dependent kinase inhibitor, p27 (Figure 8A). In order to check whether activation-associated nuclear translocation of Foxo3a, following curcumin exposure, triggers its target genes, we performed

quantitative real-time PCR. Foxo3a together with Bim, Fas-L, and p27 are upregulated compared to control (Figure 8B), confirming that a death program is mediated in response to curcumin treatment.

Discussion

NB is an aggressive malignancy of the nervous system and is the most common solid extracranial tumor of childhood. Children with high-risk NB have a very poor prognosis, and new treatment strategies are needed. The ability of tumor cell populations to expand in number is determined not only by the rate of cell proliferation, but also by the rate of cell death. Apoptosis is a major source of cell death; agents that trigger apoptosis/cell death, in malignant cells, are promising therapeutic candidates for cancer. Thus, signal transduction pathways involved in cell growth and apoptosis are potential targets for chemo-preventive agents. However, the signaling leading to apoptosis in mammalian cells are complex and the pro- and anti-apoptotic variations, regulating cell survival, depend on the cell type [17]. Identification of compounds with anti-cancer activity, especially natural compounds, could be a source of chemo-preventive and therapeutic agents for various human tumors. Curcumin, the principal curcuminoid of
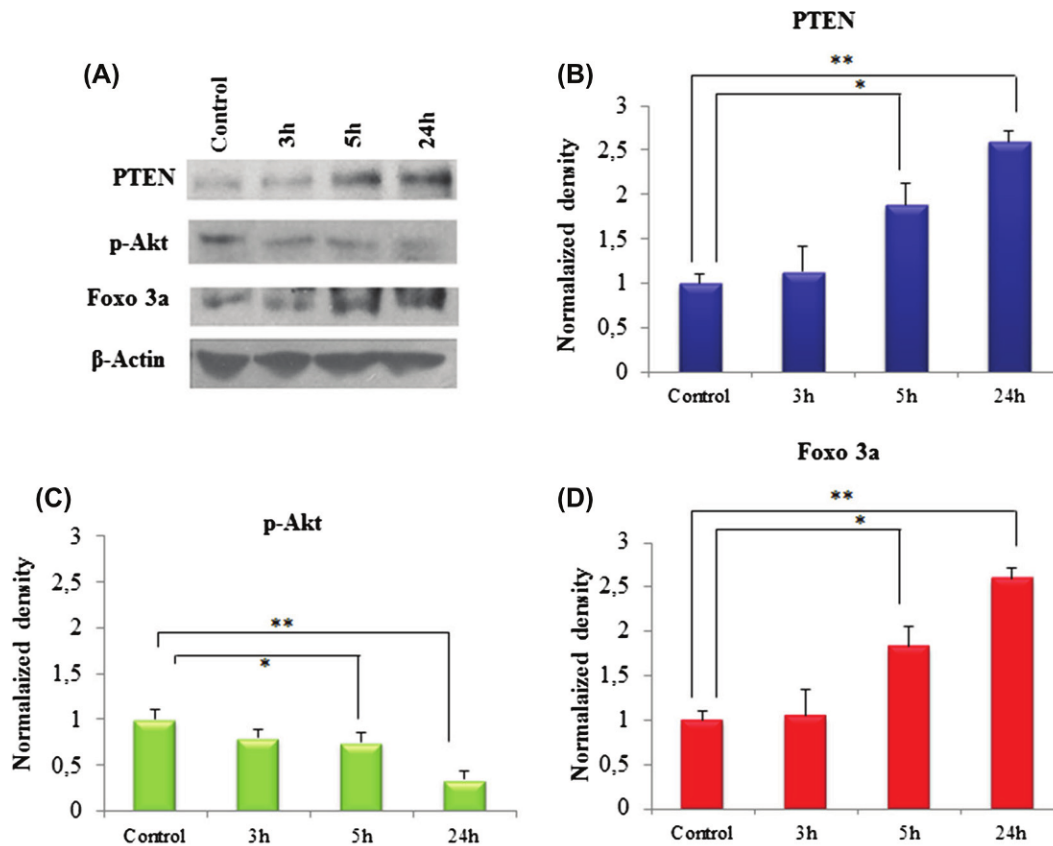
Figure 5. PTEN/Akt/Foxo3a signaling is activated early in response to curcumin. A) LAN5 cells were exposed to curcumin (15 μM) for indicated times. Total cell lysates were prepared, separated by SDS-PAGE, and transferred to nitrocellulose. The Western blot was incubated with anti-PTEN, anti-phospho-Akt (p-Akt), anti-Foxo3a, and anti-β-actin. B-D) Quantification of immunoreactivity was performed using densitometric analysis; *$P < 0.05$, **$P < 0.01$, vs. indicated groups. Uniformity of gel loading was confirmed with β-actin utilized as standard.

turmeric, inhibits carcinogenesis in various types of solid tumor and cell lines [34]. However, some studies based on DNA damage or other parameters, such as K^+ ion leakage and glutathione depletion, have demonstrated that curcumin exhibits both antioxidant and pro-oxidant activity in a concentration-dependent manner, supporting the idea that curcumin may play a conflicting dual role in carcinogenesis [35–37]. The mechanisms responsible for curcumin-mediated apoptosis seem, indeed, to depends and diverge in different cell lines, and few evidences are reported for NB cells. Some studies have revealed that curcumin induces apoptosis in HL-60 cell line (a promyelocytic leukemia type of acute myeloid leukemia (AML)) through several pathways, including the ornithine decarboxylase-dependent pathway [38], endoplasmic reticulum stress [39], and inhibition of telomerase activity [40]. A recent report suggests that curcumin inserts deep into the cellular membrane in a trans-bilayer orientation, anchored by hydrogen bonding to the phosphate group of lipids, thus inducing changes in the bilayer structure [41]. The promotion of modifications in the membrane by curcumin may have a direct effect on apoptosis by increasing the permeabilizing activity of apoptotic proteins such as Bid [42]. In the experimental model system utilized here, curcumin treatment resulted in a reduced viability and cell death induction in dose-dependent manner. Moreover,

curcumin increased ROS generation and mitochondrial dysfunction—events triggering apoptosis. These data are in agreement with studies on other cancer cells in which curcumin induced an increase of intracellular ROS levels, a rapid decrease in ΔΨm, and activation of caspase-9 and caspase-3 [43–46]. Mitochondrion plays a pivotal role in many physiological and pathological apoptotic processes. The intrinsic pathway of apoptosis involves the activation of pro-apoptotic members of the Bcl-2 family that exert their function through mitochondria. During apoptosis, the permeability of the outer mitochondrial membrane increases, the PT is opened, and some proteins, including cytochrome c, considered the most critical event to initiate the apoptotic cascade are released into the cytoplasm. In NB cells, mitochondria play a well-established role in curcumin-induced apoptosis. Curcumin reduces levels of Hsp60 and HKII and increases Bad levels, mitochondrial proteins involved in apoptotic process. Our results are in agreement with a study in which, in rat liver, curcumin induced increased mitochondrial membrane permeability, resulting in swelling, loss of membrane potential, and inhibition of ATP synthesis. These effects were mediated by the opening of the permeability transition pore [47]. Thus, curcumin induces apoptosis through ROS and mitochondrial damage-dependent mechanism. Accumulating evidence suggests that curcumin-induced apoptosis is
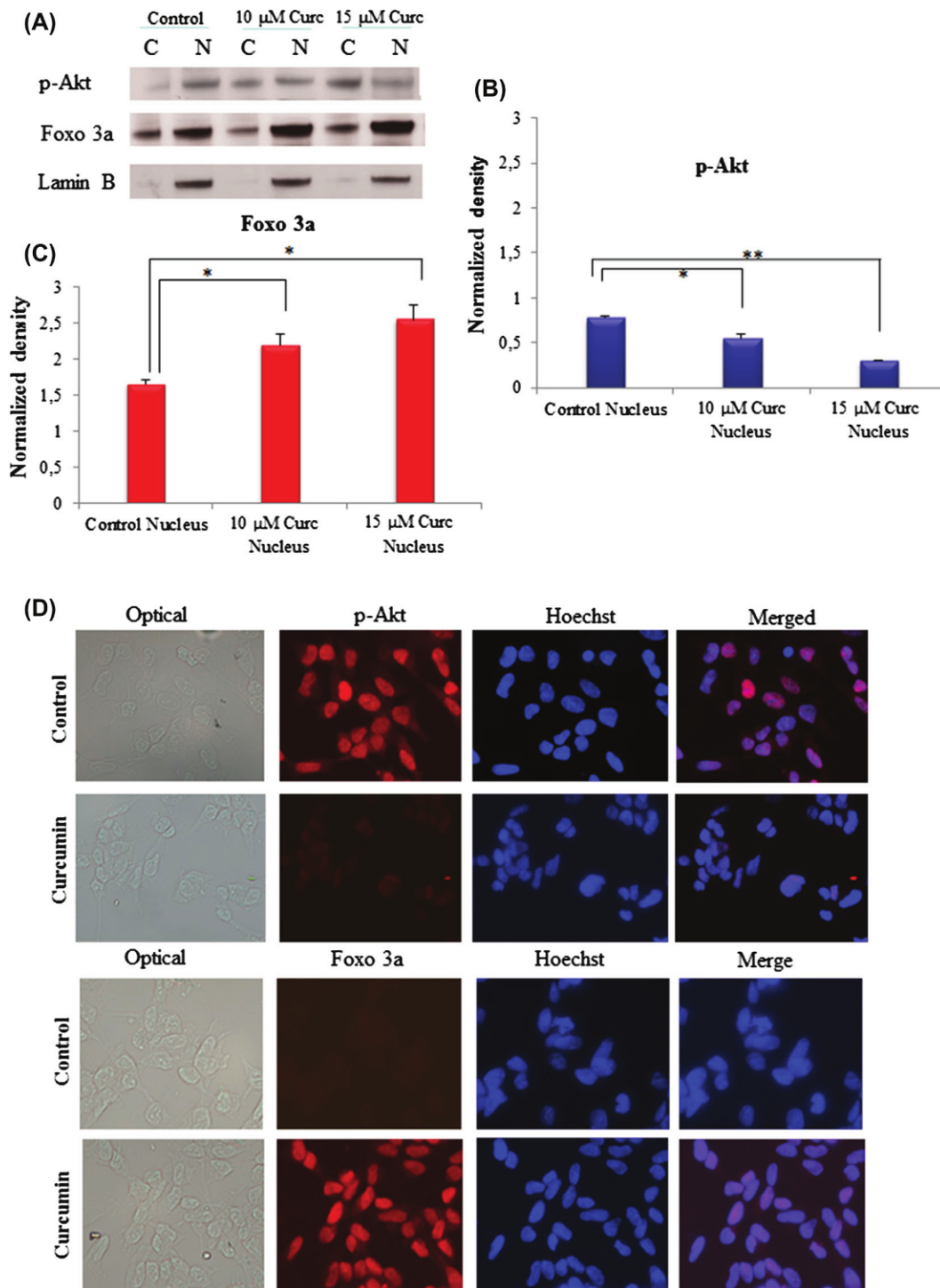
Figure 6. Nuclear–cytoplasmic shuttling of p-Akt and Foxo3a. A) Western blot of proteins (20 µg) extracted from LAN5 cells untreated (Control) or treated with different concentrations of curcumin (Curc) for 24 h and incubated with anti-phospho-Akt (p-AKT) and anti-Foxo3a (FOXO3a). The relative purity of the nuclear and cytoplasmic fractions was confirmed by probing with the nuclear marker, Lamin B. B, C) Quantification of immunoreactivity was performed using densitometric analysis; *$P < 0.05$, **$P < 0.01$, vs. indicated groups. D) Immunohistochemistry of LAN5 cells untreated (Control) or treated with curcumin, incubated with anti-phospho-Akt (p-Akt) and anti-Foxo3a (red staining) and Hoechst 33258 (blue staining), and examined by fluorescent microscopy. Merged images of anti-phospho-Akt and anti-Foxo3a with Hoechst 33258 staining are shown.

mediated both by the activation of cell death pathways and by the inhibition of different growth/proliferation pathways [48]. Curcumin has a varied range of molecular targets, supporting the concept that it acts upon numerous biochemical and molecular cascade; hence its potential

employment could reduce the possibility to develop resistance.

PTEN, a dual protein/lipid phosphatase, is one of the most frequently mutated tumor suppressor genes in human cancer. PTEN/phosphatidylinositol 3-kinase (PI3K)/Akt
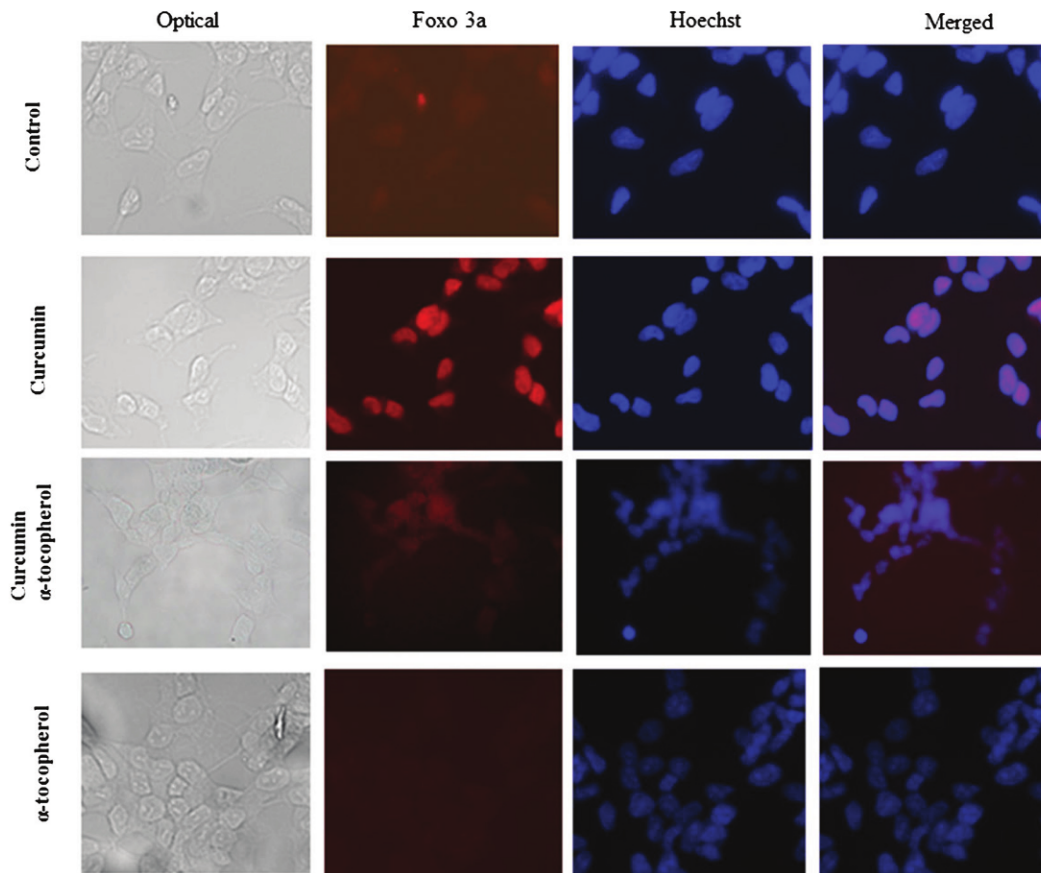
Figure 7. α-Tocopherol inhibits the translocation of Foxo3a form cytoplasm to nucleus induced by curcumin. Immunohistochemistry of LAN5 cells untreated (Control) or treated with curcumin without or with α-tocopherol or with α-tocopherol alone, incubated with anti-Foxo3a (red staining) and Hoechst 33258 (blue staining), examined by fluorescent microscopy. Merged images of anti-Foxo3a with Hoechst 33258 staining are shown.

constitute an important pathway regulating the signaling of multiple biological processes, such as apoptosis, metabolism, cell proliferation, and cell growth [49]. When acting as a tumor suppressor, PTEN antagonizes the effects of PI3K through its lipid phosphatase activity. Some evidences indicate that in NB tumor, the PI3K/PKB signaling pathway is frequently hyperactivated leading to proliferation and therapy resistance of the tumor cells.

PTEN mutations are rare (below 10%) in NB, but altered expression of protein interacting with the carboxy terminus-1 (PICT-1), a PTEN-stabilizing protein [50], and aberrant neurotrophic factor signaling may be responsible for the hyperactivation of this signaling pathway. Foxo transcription factors are negatively regulated by the PI3K/Akt signaling pathway [51]. Direct phosphorylation by PI3K/Akt inhibits transcriptional activation of Foxo
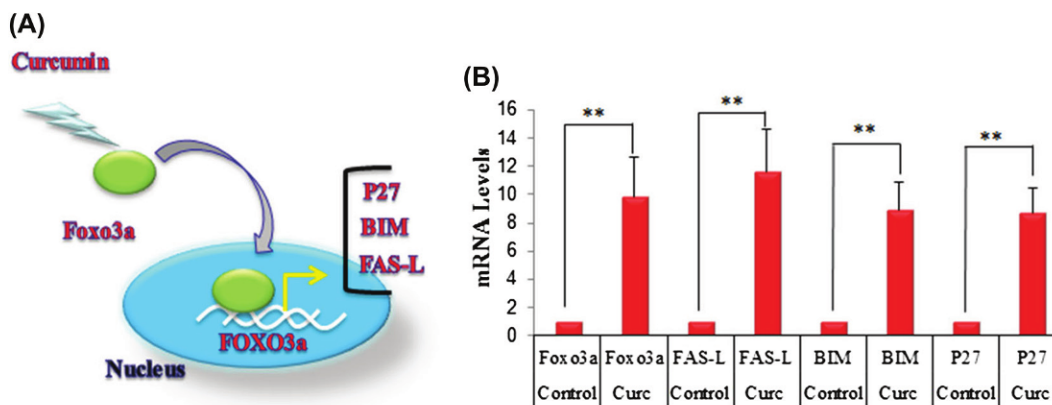

Figure 8. Curcumin modulates nuclear Foxo3a translocation and pro-apoptotic gene expression. A) Schematic representation of p27, Bim, and Fas-L activation after curcumin-induced Foxo3a nuclear translocation. B) RNAs (2 ng) were extracted from LAN5 cells treated without (Control) or with curcumin (Curc) and used to determine the Foxo3a, p27, Bim, and Fas-L mRNA levels by quantitative real-time PCR. **$P < 0.01$, vs. indicated groups.

factors, causing their export from the nucleus to the cytoplasm [26]. Further, in response to oxidative stress, Foxo factors activate a program of gene expression that regulates apoptosis [52]. Foxo factors, such as Foxo3a, can initiate apoptosis by activating transcription of FasL, the ligand for the Fas-dependent cell death pathway, and by activating the pro-apoptotic Bcl-2 family member, Bim. Moreover, Foxo factors can promote cell-cycle arrest by upregulating the cell-cycle inhibitor, p27, to induce G_1 arrest [52]. Here we give evidence that, in response to curcumin, oxidative stress is activated together with a signaling that promotes trafficking of molecules essential to activate pro-apoptotic genes. Using Western blot and immunofluorescence analysis, we demonstrated that curcumin increases PTEN levels in dose-dependent manner causing decrease of phospho-Akt and its presence into nucleus. In contrast, Foxo3a levels increase and it is imported into the nucleus; the use of α-tocopherol, a scavenger molecule, inhibits this shuttling, confirming that this effect is due to the stimulated oxidative stress. Consequently, Foxo3a induces transcription of p27, Bim, and Fas-L apoptotic target genes. It has been demonstrated that Foxo3a upregulates PTEN transcription and that PTEN/Akt/Foxo3a/Bim signaling contributes to ROS-mediated selenite-induced apoptosis [53]. Since Foxo3a activation seems to be beneficial for the treatment of the NB, use of curcumin could be a strategy to improve the therapy for this malignant disease.

On this basis, we cannot exclude a mechanism through which curcumin treatment in NB cells induces ROS production and mitochondrial damage that inhibits Akt/Foxo3a pathway, producing translocation of Foxo3a in the nucleus. Foxo3a enhances the transcription of Bim, Fas-L, and p27, which lead to apoptosis. Foxo3a probably upregulates PTEN, producing further inhibition of Akt/Foxo3a signaling and apoptosis in response to curcumin. However, whether a positive feedback loop exists between PTEN and the Akt/Foxo3a signaling pathway requires further study.

Conclusions

Curcumin induces apoptosis in NB cells through ROS-dependent modulation of PTEN/Akt/Foxo3a signaling pathway. Our results help to elucidate the molecular mechanisms underlying curcumin-induced cell death in NB cells and provide a theoretical basis for translational applications of curcumin.

Declaration of interest

The authors report no declarations of interest. The authors alone are responsible for the content and writing of the paper.

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