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Curcumol Suppresses Triple-negative Breast Cancer Metastasis by Attenuating Anoikis Resistance *via* Inhibition of Skp2-mediated Transcriptional Addiction

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Abstract. *Background/Aim: Triple-negative breast cancer (TNBC) is a unique subtype that lacks expression of several conventional biomarkers and has a higher incidence of lymph node invasion and distal metastasis among all breast cancers. Anoikis resistance is the fundamental reason behind tumor cells' survival without their attachment to the extracellular matrix and metastasis to distal organs. Therefore, finding novel anti-cancer drugs that can suppress anoikis resistance in cancer cells is critical for patients with TNBC. Materials and Methods: Curcumol, a natural compound, was used to assess whether it can inhibit the anoikis resistance and affects cell mortality and motility of IV2-1 TNBC cells. Results: Curcumol suppressed anoikis resistance and inhibited TNBC cell survival in suspension. Additionally, these anti-cancer effects induced by curcumol could be related to the YAP1/Skp2 molecular pathway. Conclusion: Curcumol is an effective Skp2-targeted therapy that attenuates anoikis resistance and metastasis in TNBC cells.*

Breast cancer is a common type of cancer and one of the leading causes of death among women worldwide (1, 2). Among all breast cancers, triple-negative breast cancer (TNBC) that lacks the expression of estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2, has the highest metastatic and recurrence rates, high incidence of drug resistance and worst prognosis, and is, thus, the most difficult subtype for treatment (3). Most deaths in women with TNBC are due to the metastatic behavior of TNBC and not because of the primary tumor growth (4). Therefore, identification of biomarkers and therapeutic targets related to TNBC cell invasion and metastasis is crucial for the diagnosis, prognosis, and drug development in this clinical setting.

During the metastatic process, tumor cells detach from their primary sites, invade blood or lymphatic vessels, and having survived the blood flow they extravasate and finally proliferate in distal organs. When losing contact with the extracellular matrix or neighboring cells, tumor cells might undergo anoikis (anchorage-dependent programmed cell death) (5). Disruption of the cytoskeleton, which accompanies cell-matrix dissociation, may also contribute to anoikis induction by releasing pro-apoptotic factors, such as Bim, or death receptors, such as Fas, from a sequestered state (6). Interestingly, tumor cells can acquire anoikis resistance through genetic mutations or overexpression of antiapoptotic proteins (7). Resistance to anoikis enables the tumor cells to escape apoptosis and allows anchorage-independent cell growth (8). Therefore, anoikis resistance is one of the most important features of metastatic cancer (9, 10). Additionally, upregulation of several genes involved in epithelial-mesenchymal transition (EMT), such as Zeb1 and Skp2, is also an important indicator of anoikis resistance (11, 12). Although previous studies have suggested that Skp2 can promote anoikis resistance contributing to invasion and

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metastasis in several cancer types (12-14), whether Skp2 also plays an essential role in regulating the anoikis phenomenon in TNBC remains unclear.

Curcumol, a natural compound derived from the Curcuma longa plant, shows therapeutic activity and has beneficial effects. The antitumoral effect of curcumol has been proposed due to its anti-inflammatory, antioxidant, pro-apoptotic, and anti-cell adhesion properties, as well as its role in inducing cell cycle arrest (15-18). Curcumol suppresses the migration and invasion ability of breast cancer cells by regulating the NFκB-dependent expression of metalloproteinase MMP-9 *via* blockade of the JNK/Akt signaling pathway (19). Curcumol treatment also inhibits proliferation and induces apoptosis in MDA-MB-231 cells by activating the tumor suppressor protein p73 and the pro-apoptotic protein PUMA (20). Additionally, the pro-apoptotic effects of curcumol on non-small cell lung cancer and nasopharyngeal carcinoma through the deactivation of pro-survival signaling, such as ERK and Akt, have also been identified (16, 21). Despite all this data, little is known about the suppressive effect of curcumol on anoikis resistance and its underlying mechanism in TNBC cells. Therefore, the aim of this study is to investigate the potential of curcumol in preventing TNBC progression by blocking cancer cell metastasis.

Materials and Methods

Cell lines. Several human TNBC cancer cells, such as MDA-MB-231 (ATCC, Manassas, VA, USA) and IV2 (IV2-1 and IV2-2) cells (22) were used in this study. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin (all Gibco, Life Technologies, Carlsbad, CA, USA) under a humidified atmosphere with 5% CO_2 at 37˚C.

Cell proliferation assay. About 1×10^4 cells were seeded per well in 24-well plates and incubated for 24 h. Following treatment with different doses of curcumol (ChemFaces, Wuhan, Hubei, China) (0-50 μg/ml) for 24, 48 or 72 h, the Cell Counting Kit-8 (CCK-8) (Enzo Life Sciences, Inc., USA) was added to each well for an additional 4 h incubation at 37˚C. The optical density of the medium was then determined by absorbance at 450 nm (Infinite 200 pro, Tecan Group Ltd., Männedorf, Switzerland). The experiments were repeated in triplicate.

Cell wound healing assay. Different experimental groups of cells were seeded in 2-well silicone inserts (Ibidi GmBH, Gräfelfing, Germany) and incubated in culture medium with different doses of curcumol (0-50 μg/ml) at 37˚C in a 5% CO_2 incubator for 24-48 h before removing the inserts. Images were captured using an optical microscopy (CKX53, Olympus Co. Ltd, Tokyo Japan) at 0 and 8 h after removing the inserts and the experiments were repeated in triplicate.

Cell invasion assay. The Cell invasion assay were performed as previously described (23). Briefly, cells were cultured in suspension condition for 24 h with different doses of curcumol (0-50 μg/ml). After that, $0.5\text{-}1\times10^3$ cells were seeded in the Matrigel coated inserts with serum-free medium and placed in the upper chamber of the 8 μm transwell (Corning, NY, USA). DMEM containing 10% FBS was plated in the bottom chamber. Following an 8 h incubation, cells were fixed, rinsed and stained with Giemsa solution (Sigma, St. Louis, MO, USA). Cell invasion assays were run in triplicate.

Immunofluorescent (IFC) staining. Cells were first fixed with 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA), washed and permeabilized with 0.2% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) and 1% BSA in PBS, and then incubated with primary antibodies: YAP1 (GTX129151) and TEAD4 (GTX108750), in PBS with 5% BSA overnight at 4˚C. After that, cells were washed with PBS, incubated with a secondary antibody (GTX213110-01) in PBS with 5% BSA at room temperature for 1.5 h, and stained with 4',6-diamidino-2-phenylindole (DAPI) (ab104139, Abcam, Cambridge, MA, USA). Immunofluorescent images were captured using a fluorescent microscopy (IX-81, Olympus Co. Ltd, Tokyo Japan).

Quantitative real-time polymerase chain reaction (q-PCR). Total RNA extraction and q-PCR protocols were performed as previously described (24). In brief, synthesized cDNA was used as a template for PCR amplification using primers for human Skp2 (forward: 5'-CTGTCTCAAGGGGTGATTGC-3', and reverse: 5'-TTCGATAGGTC CATGTGCTG-3'), and human GAPDH (forward: 5'-AAGGCTGGG GCTCATTTGC-3', and reverse: 5'- GCTGATGATCTTGAGGCT-3') (Genomics, New Taipei City, Taiwan). Quantitative real-time PCR was performed with the Roche LightCycler 480 II system (Basel, Switzerland) following the manufacture's protocols. Skp2 gene expression was determined as follows:

ΔCT=CT (target gene) – CT (GAPDH) and

ΔΔCT=ΔCT (experimental group) – ΔCT (control group)

Western blotting. Antibodies against Bim (#2933), Atk (#4691), phosphor-(p) Akt (#4060), Skp2 (#2652), p-YAP1(#13008), and β-actin (#3700) were purchased from Cell Signaling Technology (Beverly, MA, USA). Vimentin (GTX100619), ZEB1(GTC105278), YAP1 (GTX129151), TEAD4 (GTX108750) were purchased from GeneTex International (Irvine, CA, USA).

Chromatin immunoprecipitation assay. Cells were harvested and chromatin immunoprecipitation assay was performed as described previously (25). Antibodies against YAP1 (ab52771) and TEAD4 (ab58310) was purchased from Abcam (Cambridge, MA, USA). Each PCR assay was carried out in a 15 μl reaction volume by using the eluted immunoprecipitated DNA. The Skp2 promoter chip sequences for YAP1/TEAD4 binding were: i) forward: 5'-AGGAT GAGGGACAGCTGGTAT-3' and ii) reverse: 5'-ACCCTAATCCTG GCAAAGCTA-3' (Genomics). DNA fragments were amplified by using the PCR machine.

Skp2 and survival analysis of triple-negative breast cancer patients. Skp2 gene expression analyses contained a data set from 833 breast cancer samples consisting of: i) 114 normal tissues, ii) 566 luminal breast tumors, iii) 37 HER2+ breast tumors, and iv) 116 triple-negative breast tumors generated by the UALCAN database (http://ualcan.path.uab.edu/) in The Cancer Genome Atlas (TCGA). The relationship between Skp2 expression and TNBC patients'

survival was analyzed through the Kaplan–Meier survival analyses, which were performed using the online database (www.kmplot.com).

Statistical analysis. Statistical differences between control and experimental groups were compared using the two-tailed Student's *t*-test, and a *p*-Value<0.05 was considered statistically significant. All statistical data were analyzed using the SPSS software (19.0, IBM Corp., Armonk, NY, USA).

Results

IV2-1 cells are more resistant to anoikis compared to MDA-MB-231 cells. We first compared anoikis resistance between MDA-MB-231 and IV2 TNBC cells under suspension culture conditions. Previous studies have indicated that Bim expression is related to anoikis (6). In our study, the lowest expression of Bim protein was found in IV2-1 cells compared to MDA-MB-231 cells (Figure 1), suggesting that IV2-1 cells could be more resistant to anoikis. Previous studies have indicated that IV2-1 and IV2-2 cells have a high potential to induce metastasis (26, 27), and our results were consistent with their findings. Thus, we used IV2-1 cells as the most appropriate model to perform our subsequent experiments in this study.

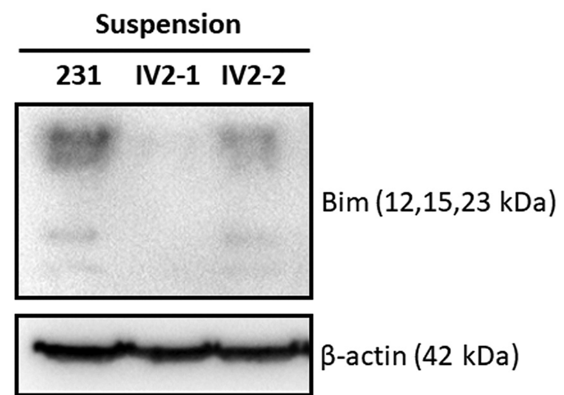

Figure 1. *IV2 cells are more resistant to anoikis compared to MDA-MB-231 cells. MDA-MB-231 and IV2 TNBC cells were cultured suspension for 24 h. Western blot analysis showed that Bim expression in IV2 cells (IV2-1 and IV2-2) were lower compared to MDA-MB-231 cells.*

Curcumol suppresses the viability of anoikis-resistant TNBC cells under suspension culture conditions. To determine the effects of curcumol on attached and suspended TNBC cells, we cultured IV2-1 cells under these conditions for 24 h, and then treated them with 25-50 μg/ml curcumol for 24-72 h. As shown in Figure 2A, about 25% of cell death could be induced in attached cells after treating them with 25-50 μg/ml curcumol for 72 h; however, there was no significant cell death induction by curcumol by 48 h of treatment. Similar results have been seen in a previous study by Ning *et al*. (19). Interestingly, when we cultured IV2-1 cells in suspension for 24 h followed by treatment with 25-50 μg/ml curcumol for 24 h, about 40%-50% cell death was induced (Figure 2B). Our western blot results also showed that p-Akt protein expression decreased with increasing doses of curcumol when cells were in suspension (Figure 2C). Therefore, curcumol has the potential to inhibit the cell growth of anoikis-resistant TNBC cells in a short duration of treatment under suspension conditions.

Curcumol inhibits cell migration and invasion of anoikis-resistant TNBC cells. Our results suggested that curcumol could inhibit cell growth in IV2-1 cells in suspension. Next, we aimed to verify whether curcumol could also inhibit cell motility of IV2-1 cells, by assessing their migration and invasive capacity. As shown in Figure 3, pre-treatment with 25-50 μg/ml curcumol for 48 h significantly inhibited cell migration of attached IV2-1 cells (Figure 3A) and invasion of suspended IV2-1 cells (Figure 3B). Additionally, several EMT-related proteins such, as Vimentin and ZEB1, were also decreased upon pre-treatment with 25-50 μg/ml curcumol for 48 h (Figure 3C). Collectively, curcumol not only inhibited the migration of attached cells but also inhibited the invasion of suspended IV2-1 cells.

Curcumol-dependent decrease of Skp2 expression by curcumol inhibits anoikis resistance is transcriptionally regulated through YAP1/TEAD4 in TNBC cells. As seen above, we found that curcumol had the potential to inhibit anoikis-resistant IV2-1 cell invasion (Figure 3B). Previous studies have indicated that Skp2 is related to anoikis resistance and cell proliferation in several types of cancer cells (12, 19, 28); however, whether the same applies for the anoikis-resistant TNBC cells through curcumol remains unclear. As shown in Figure 4A, we found that when we treated suspended IV2-1 cells with 25-50 μg/ml curcumol Skp2 expression decreased but Bim expression increased. Therefore, curcumol could inhibit anoikis resistance by regulating the Skp2 and Bim signaling pathway in IV2-1 TNBC cells.

To confirm that Skp2 mRNA expression could be downregulated by curcumol we next used qPCR. As shown in Figure 4B, curcumol inhibited the mRNA expression of Skp2 in IV2-1 cells. Previous studies have indicated that the effector of the Hippo signaling pathway, YAP1, could participate in the regulation of Skp2 transcription (29-31). Here, we found that curcumol induced the downregulation of Skp2 and YAP1 proteins expression but promoted the phosphorylation of YAP1 (Figure 4A). To further address whether recruitment of YAP1 at the Skp2 promoter is required for the down-regulation of Skp2 in TNBC cells when treating with curcumol, we performed a chromatin immunoprecipitation (ChIP) assay. As shown in Figures 4C and D, YAP1 and

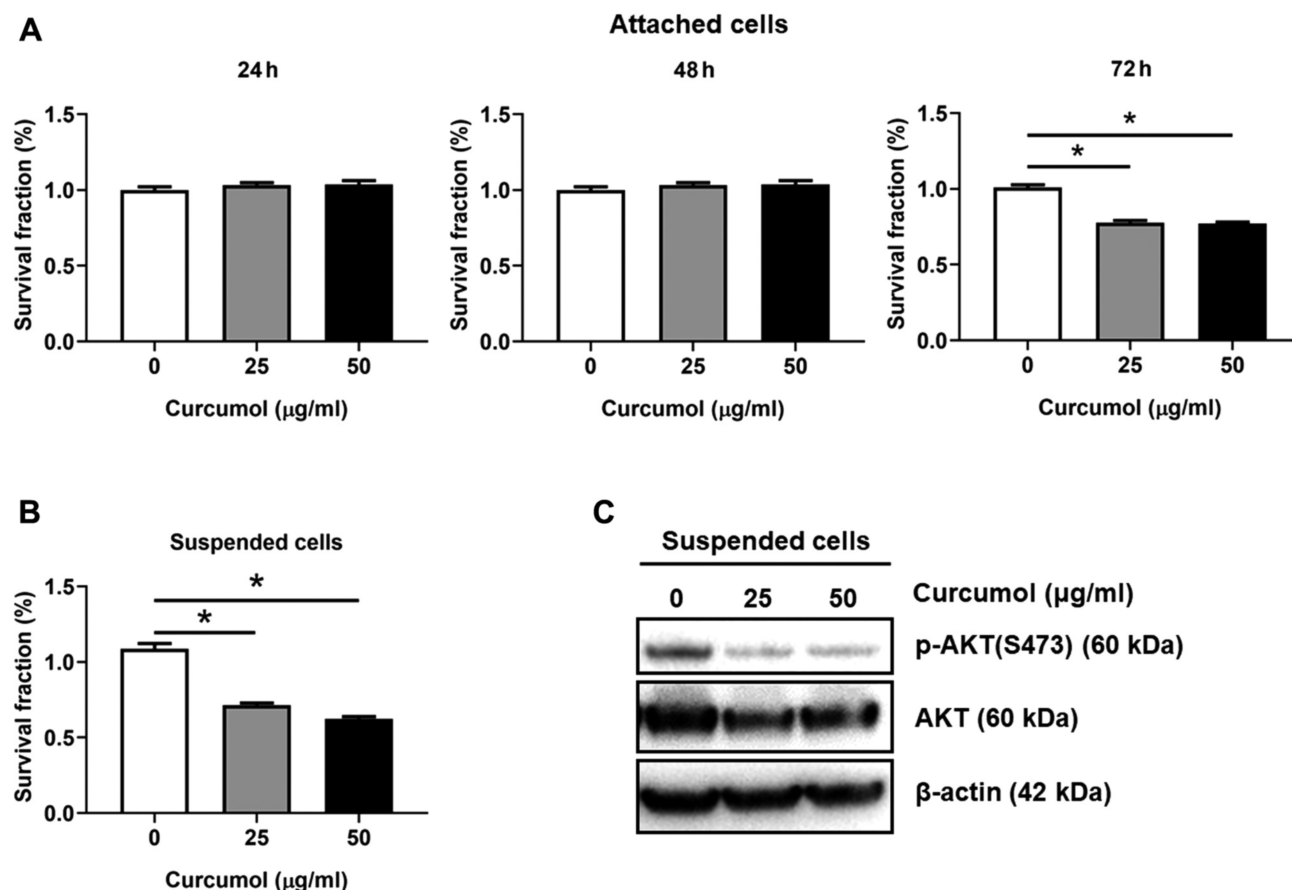

Figure 2. *Suspended TNBC cells are more sensitive to curcumol compared to attached cells. (A) Long-term treatment of curcumol (25-50 μg/ml for 72 h) significantly induced IV2 cell death when cells were attached. (B) Short-term treatment of curcumol (25-50 μg/ml for 24 h) significantly induced IV2 cell death when cells were in suspension. Cell viability experiments were performed using CCK-8. (C) Short-term treatment of curcumol significantly decreased p-Akt expression in IV2 cells in suspension. *$p<0.05$.*

TEAD4 bind to the Skp2 promoter but this interaction is blocked by curcumol (50 μg/ml). In addition, our IFC staining showed that curcumol also decreased YAP1 and TEAD4 expression in IV2 cells. Therefore, curcumol inhibited Skp2 expression in TNBC cells in a transcriptionally regulated manner through YAP1/TEAD4 signaling.

Skp2 is overexpressed in TNBC samples and is associated with poor prognosis of TNBC. We next sought to assess whether Skp2 could be a prognostic biomarker for TNBC patients. From the UALCAN database by TCGA breast cancer samples, we found that Skp2 expression was significantly higher in TNBC tissues compared to those of normal or other breast cancer subtypes (Figure 5A). Additionally, survival data of TNBC patients analyzed using the online Kaplan-Meier plotter database also showed that high Skp2 expression positively correlated with a relapse-free survival, suggesting that Skp2 had the potential to be a prognostic biomarker for TNBC (Figure 5B). Taken together, these findings suggest that Skp2 not only regulates the anoikis resistance of TNBC cells but can also play an important role in TNBC tumorigenesis.

Discussion

Although TNBC accounts for only 15–20% of all the breast cancer types (32), several malignant characteristics, such as higher metastatic and recurrence rates, lower survival rates, and fewer therapeutic strategies remain important clinical challenges. Among these malignant characteristics, cell invasion and metastasis are the most relevant factors for TNBC-induced mortality. Metastatic TNBC cells possess the ability to resist anoikis, which is the most critical step of metastasis following intravasation into the circulation (33, 34). After screening different TNBC cells, we found that IV2-1 cells had the lowest Bim protein expression under suspension culture conditions, suggesting that IV2-1 is highly resistant to anoikis. Therefore, IV2-1 was an ideal cell

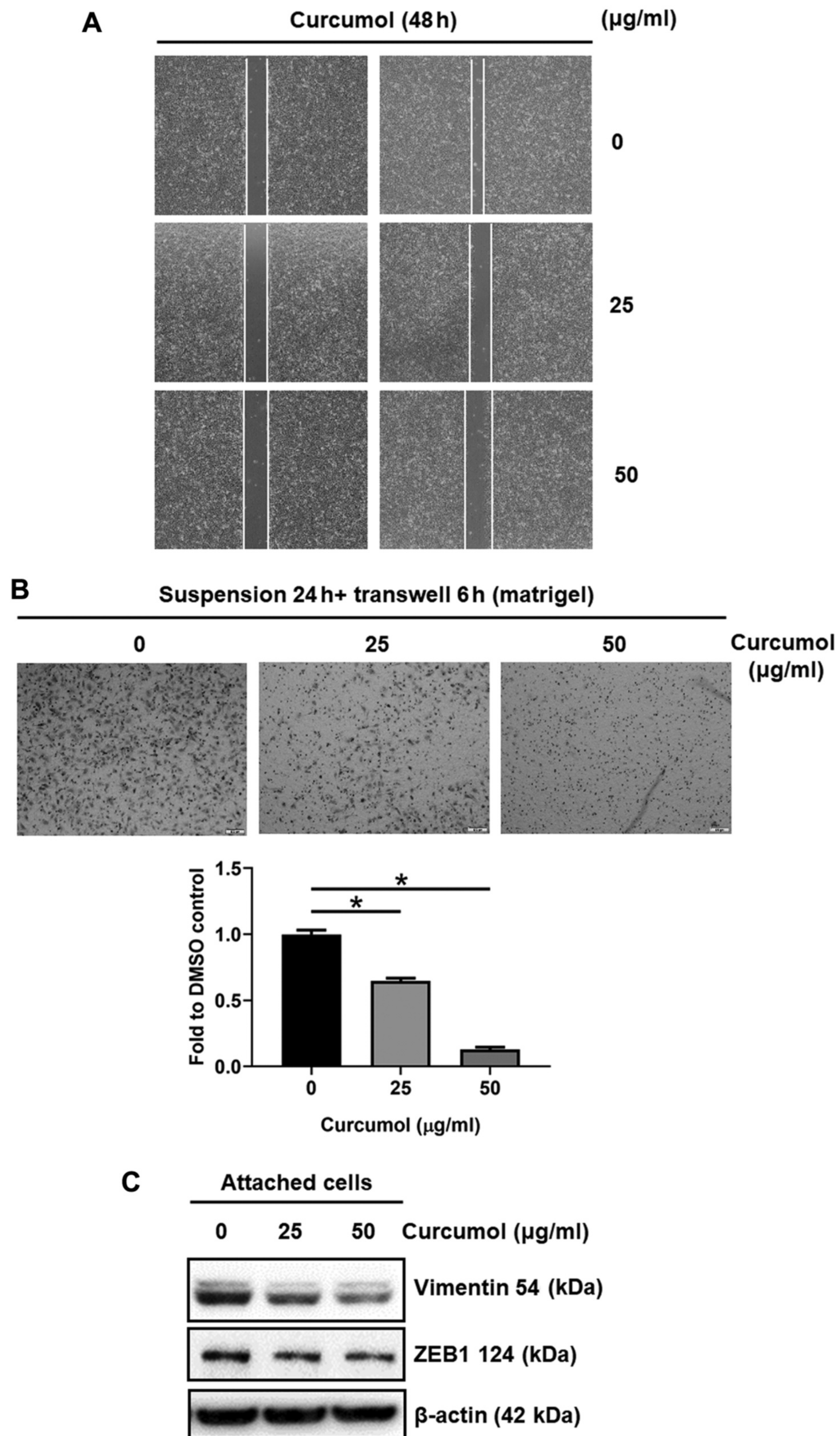

Figure 3. *Curcumol affects cell migration and invasion in TNBC cells. (A) Curcumol significantly inhibited IV2 cell migration. (B) Curcumol significantly inhibited IV2 cell invasion. (C) Curcumol decreased the expression of the EMT-related proteins, vimentin and ZEB1, in IV2 cells. *p<0.05.*

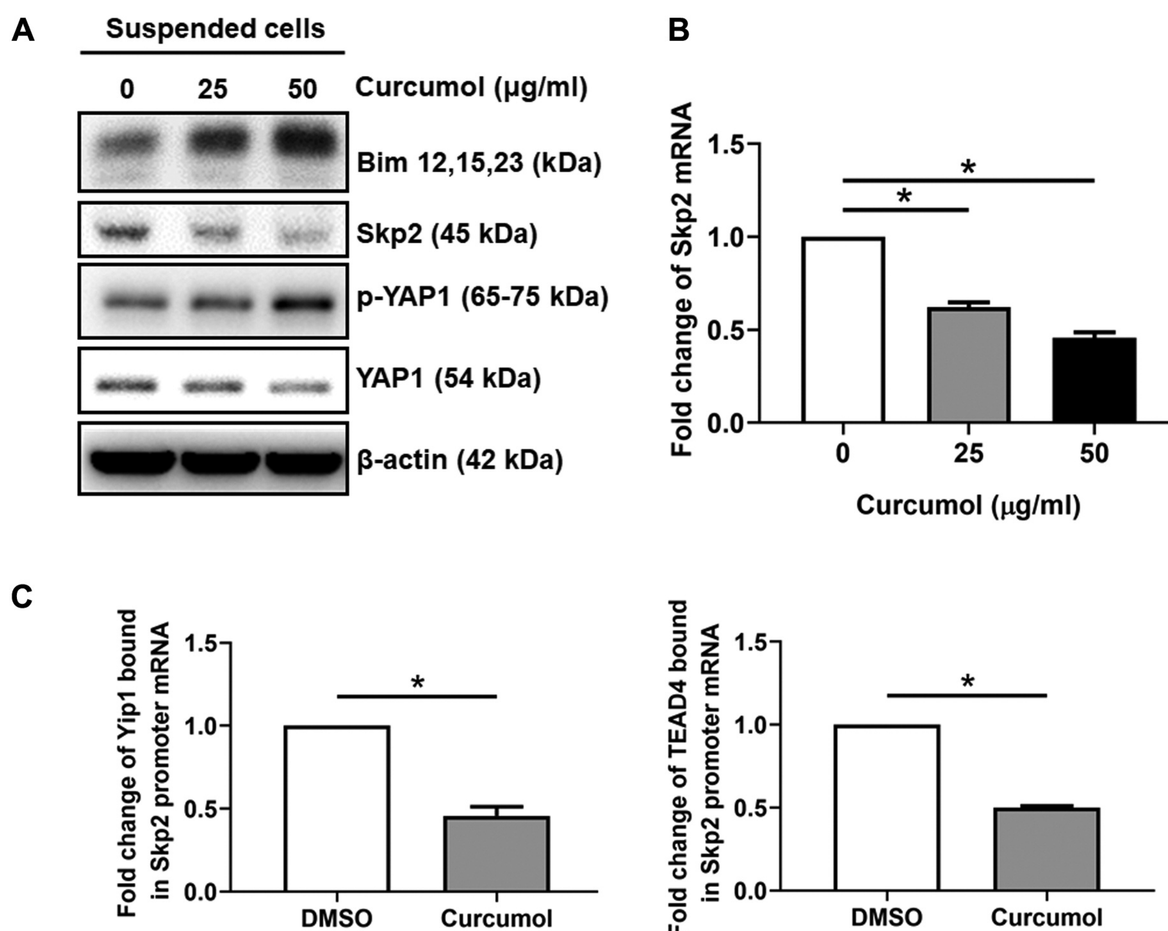

Figure 4. *Continued*

model to study anoikis resistance under suspension culture conditions.

In order to identify natural compounds that have the potential to suppress anoikis resistance in TNBC cells, we investigated the effects of curcumol on anoikis resistance in IV2-1 cells. Curcumol was recently reported to exhibit an antiproliferative effect on numerous human tumor cell lines (15-18). Previous studies have suggested that curcumol may affect cell motility by regulating EMT and MMP9 inhibition in cancer cells, as well as cell survival through the Akt-related signaling pathway (19, 21, 35). All of these experiments, however, have been conducted under attached culture conditions. Therefore, whether curcumol can also inhibit anoikis resistance in metastatic TNBC cells remains unclear. In this study, we found that curcumol could suppress the motility of attached as well as suspended IV2-1 cells. In addition, curcumol triggered cell death in IV2-1 cells.

Skp2, an F-box protein and E3 ubiquitin ligase, regulates many key cellular processes (14, 28). Skp2 overexpression has been shown to be predictive of cancer progression and is associated with a poor prognosis (28, 36-38). Higher levels of Skp2 are associated with tumor metastasis and poor survival, whereas downregulation of Skp2 results in the inhibition of tumor growth and metastasis (14, 28, 37). Additionally, overexpression of Skp2 also plays a role in suppressing anoikis in tumor cells (12). Using the UALCAN database, we found that Skp2 expression in TNBC tissues was significantly higher compared to normal breast tissues and that this high Skp2 expression was positively correlated with a short relapse-free survival of TNBC patients. In our cell model, IV2-1 cells had a high Skp2 and a low Bim expression under suspension culture conditions, suggesting that Skp2 regulated anoikis resistance in TNBC cells. Anoikis resistance was inhibited when Skp2 was downregulated following curcumol treatment. Therefore, curcumol can suppress anoikis resistance by blocking Skp2 and downstream effectors in suspended TNBC cells and has the potential to provide a therapeutic strategy for metastatic TNBC.

Previous studies have indicated that YAP1 could regulate cell ploidy and tumorigenesis through Skp2 and affect the

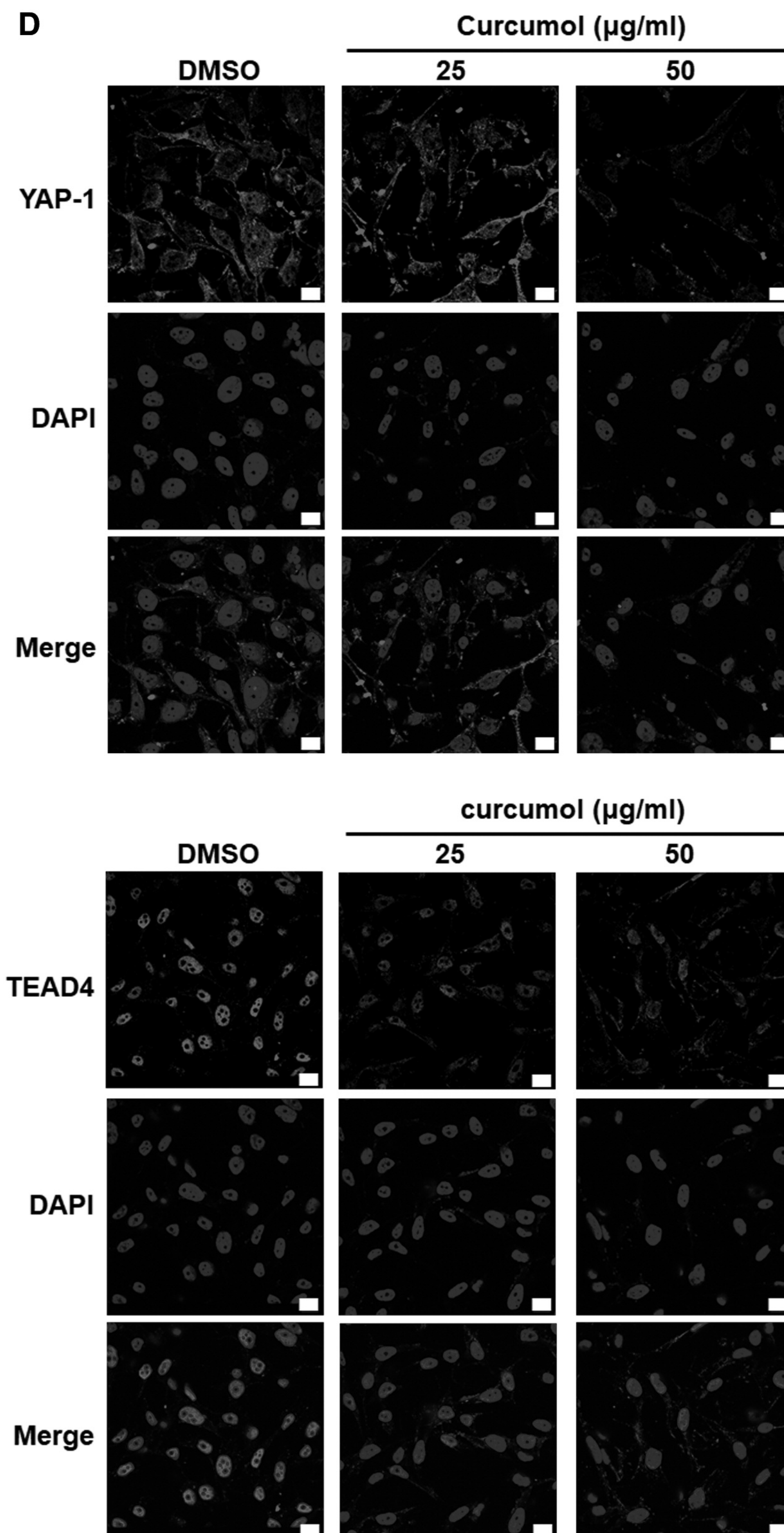

Figure 4. *Curcumol inhibits anoikis resistance through Skp2 downregulation and YAP1/TEAD4. (A) Curcumol treatment increased Bim and p-YAP1 expression but decreased Skp2 and YAP1 expression in IV2 cells. (B) Curcumol treatment decreased the mRNA expression of Skp2 in IV2 cells. (C) Binding of YAP1 and TEAD4 at the promoter of Skp2 was disrupted by curcumol. (D) Curcumol treatment reduced YAP1 and TEAD4 expression in IV2 cells. Scale bar: 10 µm. *p<0.05.*

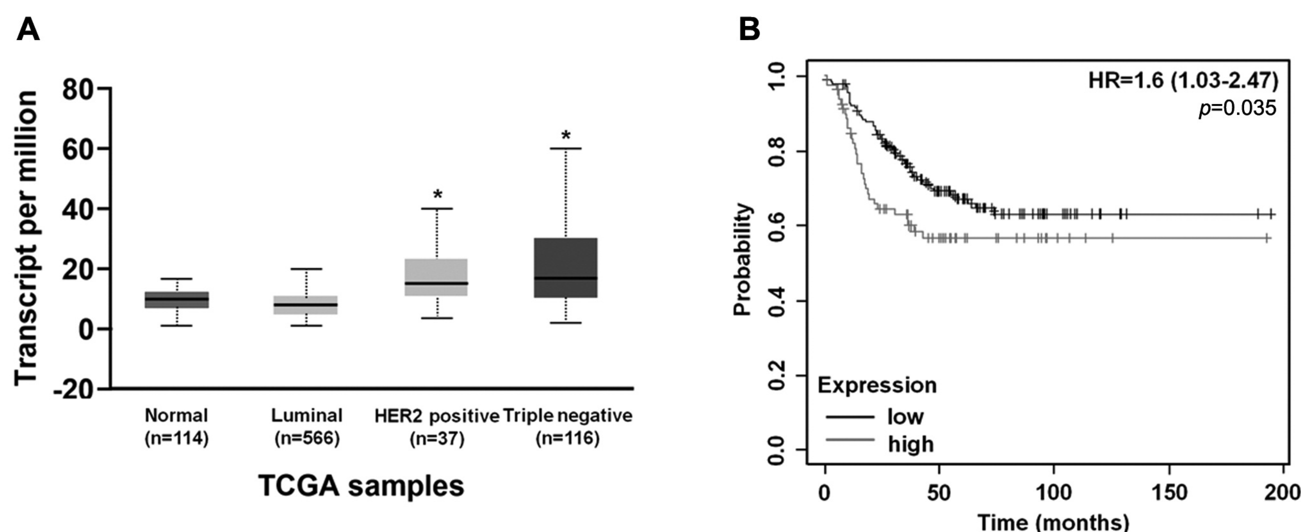

Figure 5. *Higher expression of Skp2 is associated with poor prognosis of TNBC patients. (A) Patients with TNBC have higher Skp2 expression compared to normal tissues and other breast cancer subtypes by the UALCAN database in TCGA breast cancer samples. (B) Kaplan–Meier (KM) analyses of relapse-free survival of TNBC patients from online KM plotter. Patients were stratified into 'low' and 'high' Skp2 expression based on auto select best cutoff. *$p<0.05$. HR: Hazard ratio.*

anoikis in several cancer cells (29-31). Additionally, other investigators have found that Skp2 depletion or introduction of a ubiquitination-dead mutant form of YAP1 into the cells might induce the elimination of YAP1 ubiquitination, retain YAP1 in the cytoplasm and impair its activity. (39). In our quest to assess whether the inhibition of Skp2 by curcumol in TNBC cells could also through the YAP1/Skp2 pathway, we found that curcumol could inhibit Skp2 on both mRNA and protein level. In addition, we also found that curcumol could promote the phosphorylation of YAP1 and decrease YAP1 protein expression. Additionally, TEA domain (TEAD) 4 function as a transcription factor that forms a complex with YAP1 to regulate gene transcription (40). Here, we found that YAP1 and TEAD 4 might bind to the Skp2 promoter, and this binding ability might be destroyed by curcumol. Therefore, the mechanism of curcumol inhibition of Skp2 expression may be linked to the YAP1/Skp2 interaction in TNBC cells.

In summary, the results of the present study demonstrated the direct effect of curcumol on the survival of TNBC cells by triggering inhibition of anoikis resistance. The underlying mechanism of this process possibly includes a reduction/suppression of Skp2 expression. These results indicate that curcumol may be a promising natural compound to further investigate for the development of novel cancer therapeutics, particularly for metastatic TNBC.

Conflicts of Interest

The Authors declare no conflicts of interest.

Authors' Contributions

CWL and MFH designed the study. CCL, CWH, CJK and SYF carried out all experiments and drafted the manuscript. FOY and MRP supervised all experiments performed. CCL, CWH, CJK and SYF participated in the data interpretation. CLL, CWL, and MFH prepared the final manuscript. All Authors read and approved the final manuscript.

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