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ORIGINAL PAPER

Curcumin suppresses malignant behaviors of ovarian cancer through regulation of tumor-associated macrophages

Xi Li[1] · Lingzi Su[2] · Chen Qian[1] · Wenchao Qiu[1] · Lin Tao[1] · Zhaowei Guo[1] · Jun Shi[1] · Chaoqin Yu[3]

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Abstract

Curcumin, a natural polyphenol with established anti-tumor properties, has shown therapeutic potential in ovarian cancer. However, its mechanisms, particularly through modulation of tumor-associated macrophages (TAMs) in the tumor microenvironment, remain unexplored. This study aimed to elucidate how curcumin suppresses ovarian cancer progression by regulating TAM polarization. Primary TAMs isolated from ascites of ovarian cancer patients were co-cultured with SKOV3/OVCAR-3 cancer cells. Curcumin was administered at varying doses (5–80 μM) to assess its direct effects on cancer cell viability and its indirect effects via TAM modulation. Epithelial-mesenchymal transition (EMT), migration, invasion, and cytokine profiles were analyzed using CCK-8, flow cytometry, RT-PCR, Western blot, and functional assays. High-dose curcumin (40–80 μM) directly inhibited cancer cell proliferation. In contrast, low-dose curcumin (5–20 μM) suppressed TAM-induced malignant behaviors: it reduced M2 polarization ($CD206^+$ TAMs decreased by 54.89% to 32.14%, $p < 0.01$) while increasing M1-associated cytokines (IL-12↑, IL-1β↑) and decreasing M2 markers (IL-10↓, TGF-β↓). TAM-conditioned medium primed with 20 μM curcumin significantly attenuated cancer cell migration (scratch closure: 65% vs. 85% in TAM-only group, $p < 0.01$), invasion, and EMT (E-cadherin↑, N-cadherin↓, Vimentin↓). Our study uncovered the mechanism of the anti-tumor effect of curcumin in low doses related to the regulation of TAMs, which might provide novel insight into the treatment of ovarian cancer.

Keywords Curcumin · Ovarian cancer · Tumor-associated macrophages · M1/M2 polarization · Epithelial-mesenchymal transition

Xi Li and Lingzi Su contributed equally to this work.

✉ Jun Shi
shijundoctor@163.com

✉ Chaoqin Yu
chqyu81@gmail.com

Xi Li
lixilixi1992@163.com

Lingzi Su
susulingzi@yeah.net

Chen Qian
chenqianjoyce@163.com

Wenchao Qiu
qyuzijiang@163.com

Lin Tao
doreentao23@sina.com

Zhaowei Guo
terrices@126.com

[1] Department of Traditional Chinese Medicine, Shanghai Fourth People's Hospital Affiliated to Tongji University, Shanghai 200434, China

[2] Department 1 of Oncology, Yueyang Hospital of Integrated Traditional Chinese and Western Medicine, Shanghai University of Traditional Chinese Medicine, Shanghai 200437, China

[3] Department of Gynecology of Traditional Chinese Medicine, Changhai Hospital, Naval Medical University, Shanghai 200433, China

Introduction

Ovarian cancer is one of the most common malignant tumors in the female genital system, with the highest mortality. In 2015, the number of new cases was 52.1 thousand, while the number of deaths was as high as 22.5 thousand, indicating ovarian cancer seriously threatening the health of women [1]. Tumor cytoreductive surgery and the following adjuvant chemotherapy with platinum and taxane have been regarded as standard strategies in the treatment of ovarian cancer since the 1970s. Despite the improvement of short-term effects in the treatment of ovarian cancer, 75% of patients with advanced ovarian cancer recurred tumors in two years, with a 5-year survival rate as low as 30.6% [2, 3]. As a result, better therapeutic strategies against ovarian cancer are urgent in exploration.

So far, most of the treatments for ovarian cancer target the intervention of cancer cells. However, the mutation of cancer cells leads to the failure of most of those treatments. In recent years, researchers have demonstrated that the micro-environment of cancer cells is important in the survival and function of cancer cells [4]. In tumor microenvironment, tumor-associated macrophages (TAMs) are one of the most studied groups of cells. TAMs are referred to as macrophages invading tumor tissues, holding 30%-50% immune cells [5]. TAMs are mainly derived from monocytes in bone marrow, accumulating in tumor tissues via various kinds of cytokines, such as the chemokine (C–C motif) ligand 2 (CCL2) [6]. TAMs have been proven to polarize towards two different subtypes, including the pro-inflammatory classic activation of macrophage (M1 subtype) and the anti-inflammatory alternative activation of macrophage (M2 subtype). The regulation of TAM polarization might provide a new pathway in the treatment of cancers.

Curcumin is a kind of liposoluble polyphenol extracted from some traditional Chinese medicine of Gingeraceae and Araceae [7–9]. Curcumin has been proven to function in anti-oxidation, anti-bacterium, anti-inflammation, analgesia, and improvement in wound healing [7–9]. It has been demonstrated that curcumin could alleviate the severity of ovarian cancer by inhibiting cancer cell invasion, inducing autophagy and apoptosis, and suppressing the function of cancer stem cells (CSC) [10–13]. These findings suggest that curcumin may represent a promising therapeutic strategy for the treatment of ovarian cancer.

In this study, we used immunomagnetic bead sorting to obtain TAMs from the abdominal dropsy of ovarian cancer patients and created a TAMs-ovarian cancer cells co-cultural system. Epithelial-mesenchymal transition (EMT), cell migration, and invasion were studied to analyze the malignant behaviors of ovarian cancer cells. We aimed to explore the effects of curcumin on ovarian cancer and its underlying mechanisms.

Methods

Reagents

Curcumin (HPLC > 94%) was purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA) and kept at – 20 ℃ [14]. The details of the primary antibodies used for Western blot analysis targeting E-cadherin, N-cadherin, Vimentin, and GAPDH are provided below.

Cell culture and treatment

The Central Lab of Changhai Hospital, Navy Medical University provided SKOV3 and OVCAR-3 cells. TAMs were obtained from ascitic fluid of ovarian cancer patients as previously described [15, 16]. Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS; Gibco, USA) and 1% penicillin–streptomycin solution, and incubated at 37 °C in a 5% CO_2 atmosphere. For specific experimental groups, cells were treated with curcumin at varying concentrations for subsequent analysis. Short tandem repeat (STR) profiling was performed on SKOV3 and OVCAR-3 cell lines to confirm their identity and data has been provided in the supplementary file S1.

Cell viability assay

Cells were seeded in a 96-well plate at a density of 1.0×10^5 cells per well and incubated overnight. Subsequently, the cells were exposed to the indicated treatments. Cell viability was assessed using a CCK-8 assay (Dojindo Molecular Technologies, Japan) [17].

Flow cytometry

Cells were washed three times with cold PBS, and surface staining was performed at room temperature for 30 min using anti-human CD86 antibodies and/or anti-human CD206 antibodies (eBioscience, San Diego, CA, USA) [18]. We typically use 1×10^6 cells in a test. Results were analyzed using a FACSCalibur flow cytometer by FlowJo software (Tree Star Inc., Ashland, OR, USA).

Western blot

Cells were harvested and lysed in a lysis buffer. The protein concentration was determined and separated using SDS-PAGE and electro-transferred to nitrocellulose membranes (Millipore, Billerica, MA, USA) [19]. Immunoblotting was

conducted using the primary antibodies targeting E-cadherin (1:1000, Santa Cruz Biotechnology, Dallas, TX, USA), N-cadherin (1:1000, Abcam, Cambridge, MA, USA), vimentin (1:1000, Abcam, Cambridge, MA, USA) and GAPDH (1:5000, Beyotime Biotechnology, Shanghai, China). Membranes were then incubated with an IRDye800CW-conjugated secondary antibody (Rockland; Gilbertsville, PA, USA). Images were obtained by the Odyssey infrared imaging system (LI-COR Bioscience, Lincolin, NE, USA).

Realtime PCR (RT-PCR)

Total RNA was isolated from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). First-strand cDNA synthesis was performed with PrimeScript RT Master Mix (Takara, Otsu, Shiga, Japan). The relative gene expression was quantified using the $2^{\Delta\Delta CT}$ method, with β-Actin serving as the internal reference. RT-PCR was conducted using a Real-Time PCR System and the Fast Start Universal SYBR Green Master (Roche, Basel, Switzerland) [20]. The primers used are listed in Table 1.

Enzyme-linked immunosorbent assay (ELISA)

After stimulation, the cell culture supernatant was collected, and the levels of proinflammatory cytokines, including IL-10 and IL-12, were measured using commercially available ELISA kits (R&D Systems, New York, NY, USA) according to the manufacturer's instructions [21].

Immunofluorescence analysis

Following stimulation, cells were washed with PBS and fixed in 4% paraformaldehyde for 15 min. After fixation, cells were incubated with 1% bovine serum albumin in PBS and stained with 4',6-diamidino-2-phenylindole (DAPI; Life Technologies) for 2 min. Colocalization was assessed using a confocal laser scanning microscope (Fluoview FV1000; Olympus, Tokyo, Japan [22].

Cell scratch test

A cell scratch assay was performed to assess the migratory ability of cells [23]. Cells were seeded into 6-well plates and uniformly scraped using a 200 μL pipette tip to create wounds before transfection (NC or si-TINCR; pcDNA3.1 or TINCR). Each well was washed three times with PBS to remove any floating cells. The initial wound distance (0 h) and the distances traveled by cells at 24 and 48 h post-scratching were recorded microscopically at 100 × magnification for each group. ImageJ software was used to quantify the scratch area.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 8.0 software (GraphPad Prism, San Diego, CA, USA). All results were described as means ± standard error of the mean (SEM). as $p < 0.05$.

Comparisons between multiple sample groups were performed using a one-way analysis of variance (ANOVA). The LSD test was used for pairwise comparisons when the assumption of homogeneity of variance was met, while Dunnett's T3 test was applied when this assumption was not met. An independent samples t-test was used to assess the homogeneity of variance between the two groups, and a Welch's t-test was applied when homogeneity of variance was not assumed. Statistical significance was defined as $P < 0.05$.

Table 1 Sequences of RT-PCR primers

Gene	Forward (5'–3')	Reverse (5'–3')
β-Actin	GTGACAGCAGTCGGTTGGA	AGTGGGGTGGCTTTTAGGA
IL-1	ATGATGGCTTATTACAGTGGCAA	GTCGGAGATTCGTAGCTGGA
IL-12	GCCTTCACCACTCCCAAAAC	ATGGTAAACAGGCCTCCACT
TNF-α	AGGACCAGCTAAGAGGGAGA	CCCGGATCATGCTTTCAGTG
TGF-β	GTAGCTCTGATGAGTGCAATGAC	CAGATATGGCAACTCCCAGTG
CCL-23	GAAGCATCCCGTGTTCACTC	TTCCTGGTCTTGATCCGTGT
CD-206	AACGGACTGGGTTGCTATCA	CCCGATCCCTTGTAGAGCAT
CXCR-2	GCATCAGTGTGGACCGTTAC	GGCTGGGCTAACATTGGATG
E-cadherin	ACGCATTGCCACATACACTC	GGTGTTCACATCATCGTCCG
N-cadherin	ATATTTCCATCCTGCGCGTG	GTTTGGCCTGGCGTTCTTTA
Vimentin	AGCTAACCAACGACAAAGCC	TTGCGTTCAAGGTCAAGACG

Results

Effects of curcumin on SKOV3, OVCAR-3 and TAMs

To assess the effect of curcumin on cell viability, SKOV3, OVCAR-3, and TAMs were treated with different concentrations of curcumin for varying durations. Cell viability was evaluated using the CCK-8 assay. Treatment with 80 μM curcumin for 48 h significantly inhibited cell proliferation in both SKOV3 (Fig. 1A) and OVCAR-3 cells (Fig. 1B). In contrast, no significant inhibitory effect on cell proliferation was observed in OVCAR-3 cells treated with 40 μM curcumin. Furthermore, curcumin concentrations of 0, 5, 10, and 20 μM did not show any notable effect on cell viability in either SKOV3 (Fig. 1A) or OVCAR-3 cells (Fig. 1B) after 24 or 48 h of treatment. To eliminate the direct inhibitory effects of curcumin on cell proliferation, tumor-associated macrophages (TAMs) were isolated as described above and treated with curcumin at concentrations of 5, 10, and 20 μM (Fig. 1C). No significant changes in TAM cell viability were observed after 24 or 48 h of stimulation. Since minimal differences were noted between the 24 and 48 h treatments, the 48-h stimulation was chosen for further analysis. Flow cytometry was used to assess the proportions of CD206 + (M2)

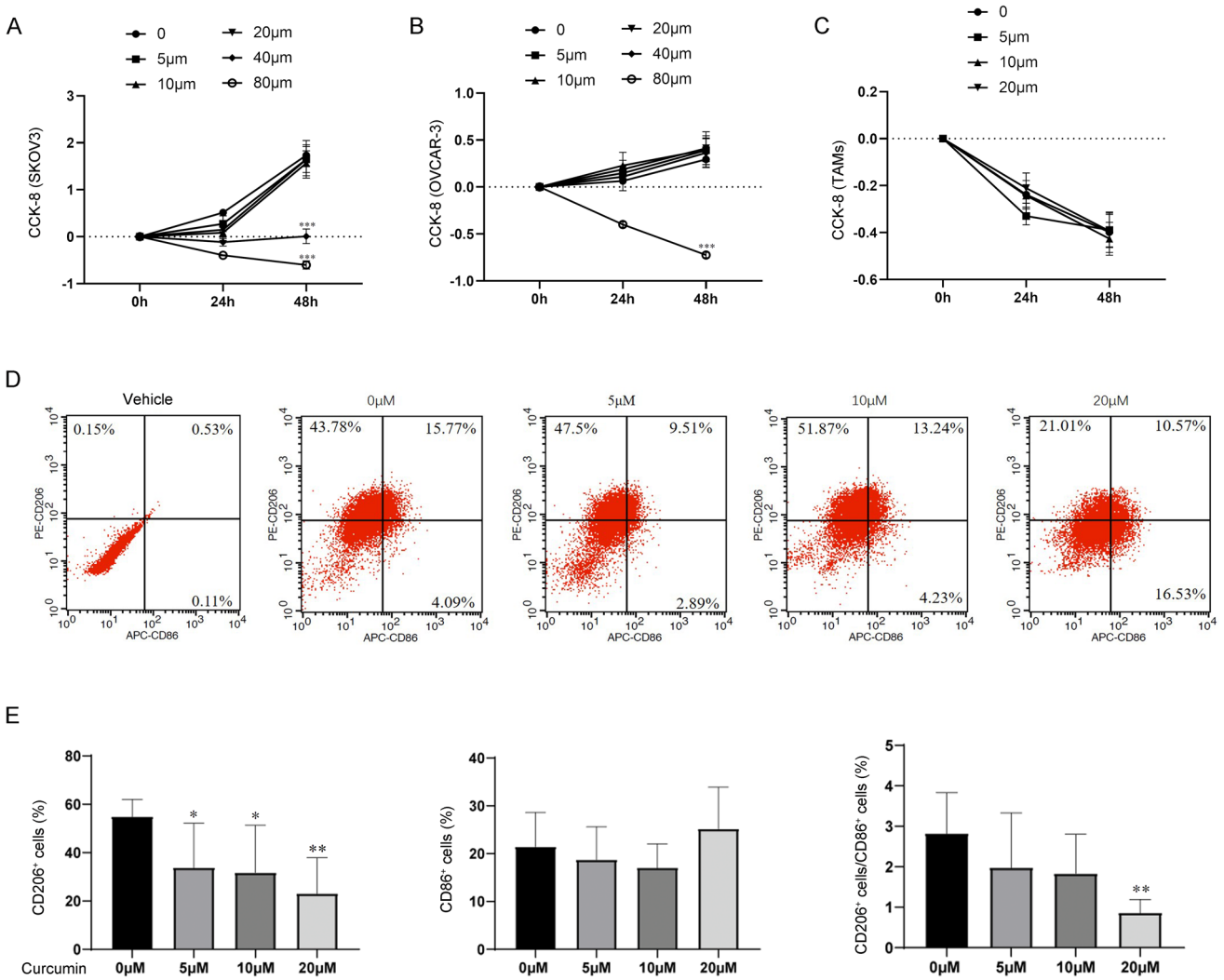


Fig. 1 Effects of curcumin on SKOV3, OVCAR-3 and TAMs. **A** Curcumin was administrated to SKOV3 cells for 24 h or 48 h at different doses. Qualitative analysis of cell viability values via CCK-8 (n = 3 per group). **B** Curcumin was administrated to OVCAR-3 cells for 24 h or 48 h at different doses. Qualitative analysis of cell viability values via CCK-8 (n = 3 per group). **C** TAMs were obtained and curcumin was administrated for 24 h or 48 h at different doses. Qualitative analysis of cell viability values via CCK-8 (n = 3 per group). **D**, **E** TAMs were obtained and curcumin was administrated for 48 h at different doses. Qualitative analysis of the expression of CD206 and CD86 in TAMs via Flow cytometry (n = 6 per group). *P < 0.05, **P < 0.01, ***P < 0.001. Note: In all figures, the terms "SKOV3" and "OVCAR-3" refer to untreated (vehicle) conditions for the respective cell lines unless otherwise specified

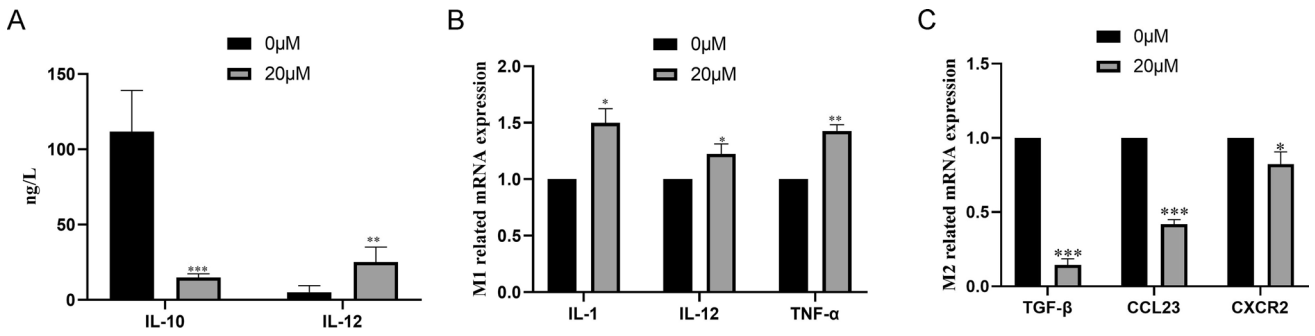


Fig. 2 Effects of curcumin on levels of M1 and M2 related cytokines. TAMs were obtained and curcumin was administrated for 48 h at the dose of 20 μM. **A** Qualitative analysis of the protein levels of M2 related IL-10 and M1 related IL-12 via ELISA (n = 6 per group). **B** Qualitative analysis of the mRNA levels of M1 related IL-1, IL-12 and TNF-α via RT-PCR (n = 3 per group). **C** Qualitative analysis of the mRNA levels of M2 related TGF-β, CCL23 and CXCR2 via RT-PCR (n = 3 per group). *P < 0.05, **P < 0.01, ***P < 0.001

and CD86 + (M1) cells (Fig. 1D, F). Curcumin treatment significantly reduced the ratio of CD206 + cells and the CD206 + /CD86 + ratio, while no significant change was observed in the proportion of CD86 + cells. Based on these findings, a 48-h treatment with 20 μM curcumin was selected for subsequent experiments.

Curcumin decreases M2 related cytokines while increasing M1 related cytokines

To further investigate the effect of curcumin on M1 and M2 macrophage subtypes, the protein levels of M2-associated IL-10 and M1-associated IL-12 were measured via ELISA in tumor-associated macrophages (TAMs) following 48-h stimulation with 20 μM curcumin (Fig. 2A). Curcumin administration significantly reduced IL-10 levels and increased IL-12 levels. Additionally, we assessed the mRNA expression of M1-related cytokines (IL-1, IL-12, TNF-α) (Fig. 2B) and M2-related markers (TGF-β, CCL23, CXCR2) (Fig. 2C) via RT-PCR. Curcumin treatment significantly decreased the mRNA levels of TGF-β, CCL23, and CXCR2 while increasing those of IL-1, IL-12, and TNF-α.

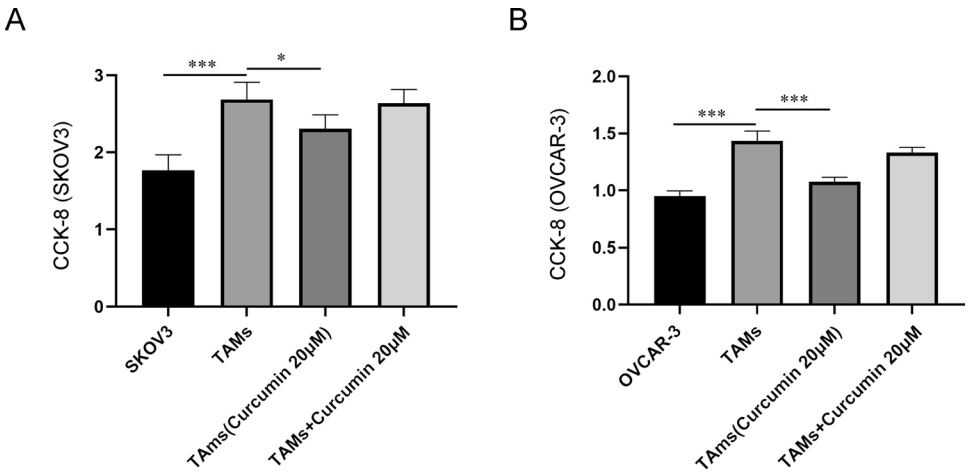
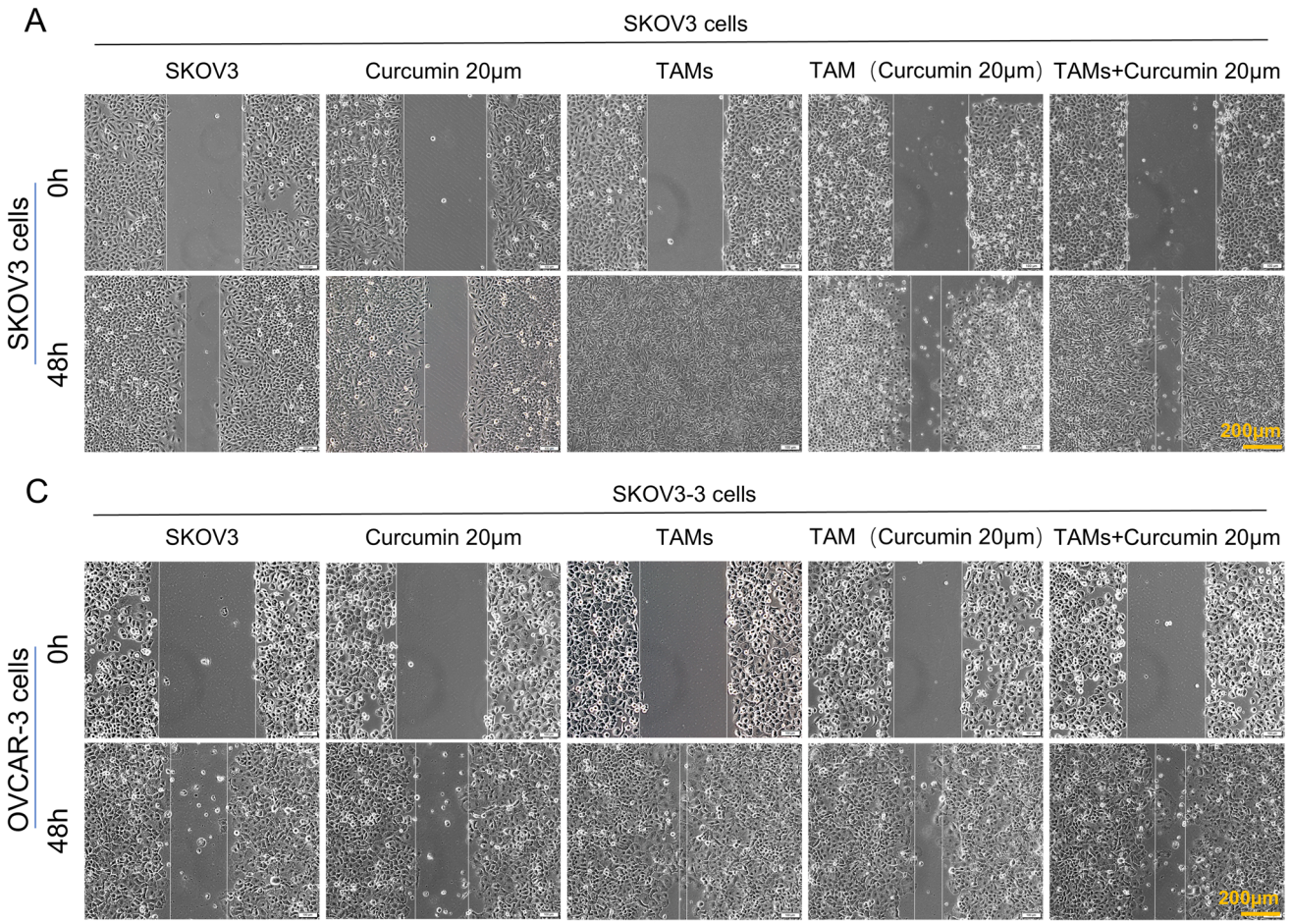


Fig. 3 Effects of curcumin on cell viability of SKOV3 and OVCAR-3 in the cultural medium of TAMs. **A** SKOV3 cells were treated for 48 h. Qualitative analysis of cell viability values via CCK-8 (n = 3 per group). SKOV3, SKOV3 cells cultured with normal medium (10% FBS and 1.5% penicillin/steptomycin solution in 1640 medium); TAMs, SKOV3 cells cultured with TAMs cultural medium; TAMs (Curcumin 20 μM), SKOV3 cells cultured with TAMs cultural medium stimulated with 20 μM curcumin for 48 h in advance; TAMs + Curcumin 20 μM, SKOV3 cells cultured with TAMs cultural medium in combination of 20 μM curcumin. **B** OVCAR-3 cells were treated for 48 h. Qualitative analysis of cell viability values via CCK-8 (n = 3 per group). OVCAR-3, OVCAR-3 cells cultured with normal medium (10% FBS and 1.5% penicillin/steptomycin solution in 1640 medium); TAMs, OVCAR-3 cells cultured with TAMs cultural medium; TAMs (Curcumin 20 μM), OVCAR-3 cells cultured with TAMs cultural medium stimulated with 20 μM curcumin for 48 h in advance; TAMs + Curcumin 20 μM, OVCAR-3 cells cultured with TAMs cultural medium in combination of 20 μM curcumin. *P < 0.05, **P < 0.01, ***P < 0.001. Note: In all figures, the terms "SKOV3" and "OVCAR-3" refer to untreated (vehicle) conditions for the respective cell lines unless otherwise specified



Curcumin attenuates the increase of cell viability in SKOV3 and OVCAR-3 in the cultural medium of TAMs

Since TAMs contributed to the regulation of tumor cell activities, we then cultivated TAMs with or without the stimulation of curcumin for 48 h. After cultivation, the TAM cultural medium was collected for the cultivation of SKOV3 or OVCAR-3 cells in certain groups. Compared with the normal group, SKOV3 cells cultured with TAM cultural medium showed a significant increase in cell viability, while the administration of curcumin in TAM cultural medium in advance largely attenuated the increasing effect. No obvious attenuative effect was observed in those in TAMs cultural

◂**Fig. 4** Effects of curcumin on migration of single-layer SKOV3 and OVCAR-3 in the culture medium of TAMs. **A**, **B** SKOV3 cells were treated for 48 h. Representative images and qualitative analysis of migration of single-layer cells via Cell scratch test (n = 3 per group; Scale bar: 200 μm). SKOV3, SKOV3 cells cultured with normal medium (10% FBS and 1.5% penicillin/steptomycin solution in 1640 medium); Curcumin 20 μM, SKOV3 cells cultured with normal medium in combination of 20 μM curcumin; TAMs, SKOV3 cells cultured with TAMs cultural medium; TAMs (Curcumin 20 μM), SKOV3 cells cultured with TAMs cultural medium stimulated with 20 μM curcumin for 48 h in advance; TAMs + Curcumin 20 μM, SKOV3 cells cultured with TAMs cultural medium in combination of 20 μM curcumin. **C** and **D** OVCAR-3 cells were treated for 48 h. Representative images and qualitative analysis of migration of single-layer cells via Cell scratch test (n = 3 per group; Scale bar: 200 μm). OVCAR-3, OVCAR-3 cells cultured with normal medium (10% FBS and 1.5% penicillin/steptomycin solution in 1640 medium); Curcumin 20 μM, OVCAR-3 cells cultured with normal medium in combination of 20 μM curcumin; TAMs, OVCAR-3 cells cultured with TAMs cultural medium; TAMs (Curcumin 20 μM), OVCAR-3 cells cultured with TAMs cultural medium stimulated with 20 μM curcumin for 48 h in advance; TAMs + Curcumin 20 μM, OVCAR-3 cells cultured with TAMs cultural medium in combination of 20 μM curcumin. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ Note: In all figures, the terms "SKOV3" and "OVCAR-3" refer to untreated (vehicle) conditions for the respective cell lines unless otherwise specified

medium alongside curcumin stimulation (Fig. 3A). Similar results were detected in OVCAR-3 cells (Fig. 3B).

Curcumin attenuates the increases in tumor cell migration and invasion of SKOV3 and OVCAR-3 in the cultural medium of TAMs

We then detected the effects of curcumin on tumor cell activities, including cell migration and invasion of SKOV3 and OVCAR-3 in the cultural medium of TAMs for 48 h. Cell migration was analyzed using a cell scratch test. Compared with the normal group, SKOV3 cells cultured with TAMs cultural medium showed a significant increase in cell viability. In contrast, the administration of curcumin in TAM cultural medium in advance and those in TAM cultural medium alongside curcumin stimulation largely attenuated the increasing effect. No obvious change was observed in those cultured with normal medium in combination with curcumin (Fig. 4A, B). Similar results were detected in OVCAR-3 cells (Fig. 4C, D). We then detected invasive ability via Cellular immunofluorescence. Compared with the normal group, SKOV3 cells cultured with TAMs cultural medium showed a significant increase in invasive ability. In contrast, the administration of curcumin in the TAM cultural medium in advance and alongside curcumin stimulation largely attenuated the increasing effect. No obvious change was observed in those cultured with normal medium in combination with curcumin (Fig. 5A, B). Similar results were detected in OVCAR-3 cells (Fig. 5C, D).

Curcumin attenuates the decrease of EMT-related E-cadherin and increases of N-cadherin and vimentin in SKOV3 and OVCAR-3 under the cultural medium of TAMs

EMT is widely recognized as closely linked to the pathogenesis and progression of tumors. To investigate the effect of curcumin on EMT, we analyzed the levels of EMT-related proteins, including E-cadherin, N-cadherin, and vimentin, in SKOV3 and OVCAR-3 cells cultured in TAMs-conditioned medium using RT-PCR (Fig. 6) and Western blot (Fig. 7). Compared with the normal group, SKOV3 cells cultured in a TAM-conditioned medium exhibited a significant increase in the mRNA level of E-cadherin and a decrease in the mRNA levels of N-cadherin and vimentin. Pre-treatment with curcumin in a TAM-conditioned medium largely attenuated these effects. In contrast, in SKOV3 cells cultured with normal medium plus curcumin, the mRNA level of E-cadherin was significantly increased, with no significant changes observed in N-cadherin or vimentin (Fig. 6A–C). Similar results were observed in OVCAR-3 cells (Fig. 6D–F), along with corresponding changes in protein levels of E-cadherin, N-cadherin, and vimentin in SKOV3 (Fig. 7A) and OVCAR-3 (Fig. 7B).

Discussion

Malignant ascites are one of the most common complications of ovarian cancer, forming the special tumor microenvironment for ovarian cancer [24, 25]. In this study, we obtained primary TAMs from abdominal dropsy of ovarian cancer patients via immunomagnetic bead sorting to mimic the micro-environment of ovarian cancer. All of the samples of this study were detected by biochemical and pathological examination for abdominal dropsy to ensure the assembly of cancer cells in abdominal dropsy. Six clinical samples were included in this study. Recently, many studies have been conducted to demonstrate the connection between the number/density of TAMs and poor prognosis of ovarian cancer [26, 27]. For instance, it was reported by Zhang et al. that the density of TAMs was significantly increased, and the ratio of M1/M2 was decreased in advanced ovarian cancer, showing the positive connection between M1/M2 ratio and survival period [27]. Consistent with those previous findings, in our study, we found that the percentage of the M2 subtype was $54.89 \pm 2.92(\%)$ in TAMs, which was much higher than that of the M1 subtype (21.44 ± 7.16) ($P < 0.001$). Those data indicated that there were a large number of immune cells in the abdominal dropsy of ovarian cancer, with the M2 subtype as the majority cell type. Isolating TAMs from ascites and establishing a co-culture model of primary TAMs with

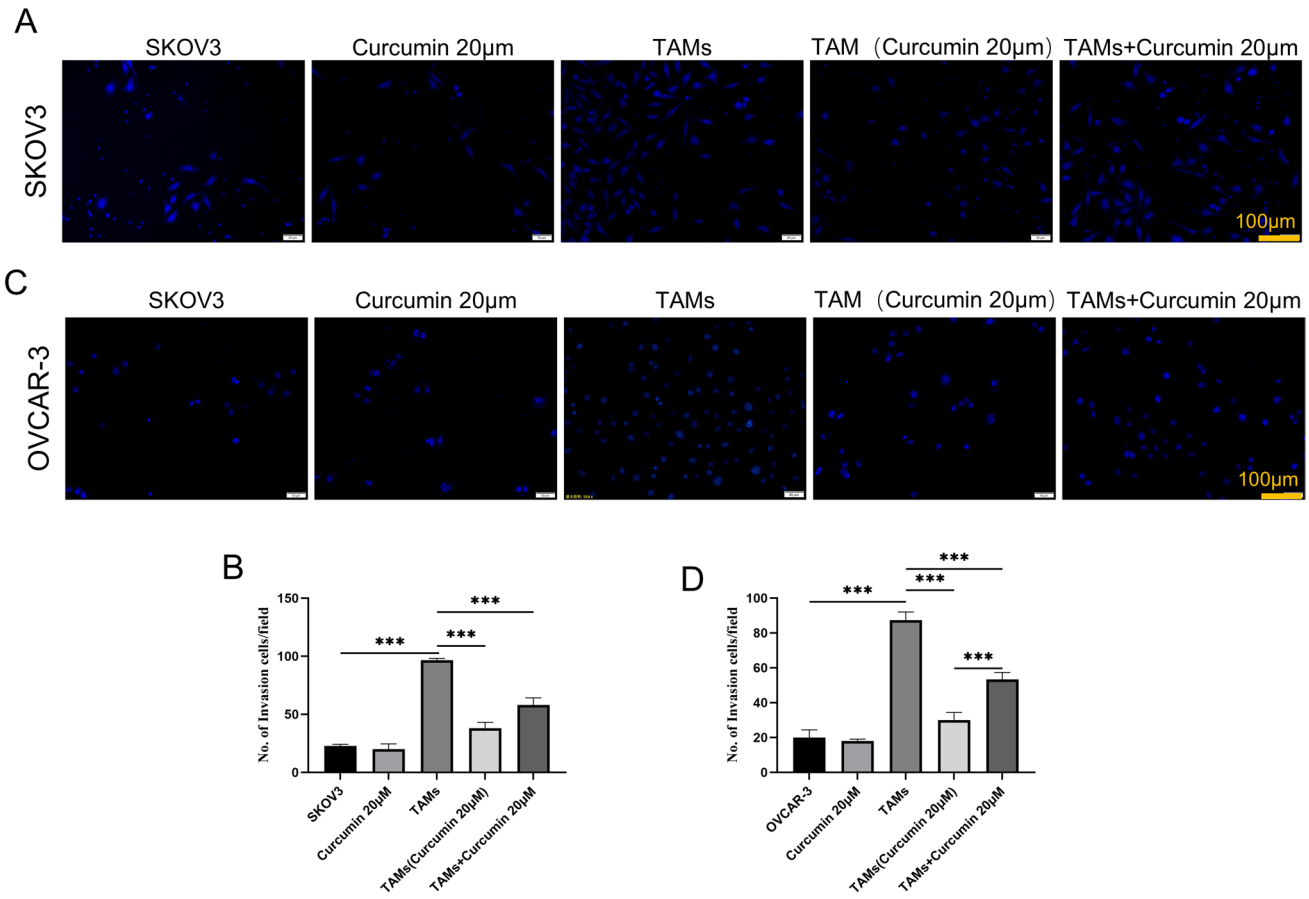


Fig. 5 Effects of curcumin on invasive ability of SKOV3 and OVCAR-3 in the cultural medium of TAMs. **A**, **B** SKOV3 cells were treated for 48 h. Representative images and qualitative analysis of invasive ability of SKOV3 cells via Cellular immunofluorescence (n = 3 per group; Scale bar: 100 μm). SKOV3, SKOV3 cells cultured with normal medium (10% FBS and 1.5% penicillin/steptomycin solution in 1640 medium); Curcumin 20 μM, SKOV3 cells cultured with normal medium in combination of 20 μM curcumin; TAMs, SKOV3 cells cultured with TAMs cultural medium; TAMs (Curcumin 20 μM), SKOV3 cells cultured with TAMs cultural medium stimulated with 20 μM curcumin for 48 h in advance; TAMs + Curcumin 20 μM, SKOV3 cells cultured with TAMs cultural medium in combination of 20 μM curcumin. **C**, **D** OVCAR-3 cells were treated for 48 h. Representative images and qualitative analysis of invasive ability of OVCAR-3 cells via Cellular immunofluorescence (n = 3 per group; Scale bar: 100 μm). OVCAR-3, OVCAR-3 cells cultured with normal medium (10% FBS and 1.5% penicillin/steptomycin solution in 1640 medium); Curcumin 20 μM, OVCAR-3 cells cultured with normal medium in combination of 20 μM curcumin; TAMs, OVCAR-3 cells cultured with TAMs cultural medium; TAMs (Curcumin 20 μM), OVCAR-3 cells cultured with TAMs cultural medium stimulated with 20 μM curcumin for 48 h in advance; TAMs + Curcumin 20 μM, OVCAR-3 cells cultured with TAMs cultural medium in combination of 20 μM curcumin. ***P < 0.001. Note: In all figures, the terms "SKOV3" and "OVCAR-3" refer to untreated (vehicle) conditions for the respective cell lines unless otherwise specified

ovarian cancer is a viable approach that better reflects the disease state.

Curcumin is a kind of liposoluble polyphenol with multiple biological activities [7–9]. A large number of studies have shown that curcumin has extensive anti-cancer effects, including inhibiting tumor cell proliferation, inducing apoptosis, and preventing metastasis. [28, 29]. It has been previously reported that curcumin in high concentrations could effectively suppress the proliferation of ovarian cancer cells. Nazli, Güllü, et al. found that curcumin at 50 μM can significantly inhibit the proliferation of colorectal cancer cells by suppressing the expression of the *MACC1* gene [30]. Also, a study found that 50 μM curcumin significantly inhibits the proliferation of head and neck tumor cells by downregulating the PI3K-AKT-mTOR pathway [31]. In this study, we also found that curcumin had a significant inhibitory effect on the proliferation of SKOV3 and OVCAR-3 ovarian cancer cells at doses greater than 40 μ M ($P < 0.001$). The inhibitory effect of curcumin on tumor cells shows distinct time- and dose-dependent characteristics, especially when the treatment duration exceeds 48 h [32, 33]. Here in our current study, we focused on the exploration of the regulatory effects of curcumin on the immune microenvironment in ovarian cancer, aiming to investigate whether curcumin could further alleviate the malignant behaviors of ovarian cancer cells through the regulation of TAM polarization. To minimize

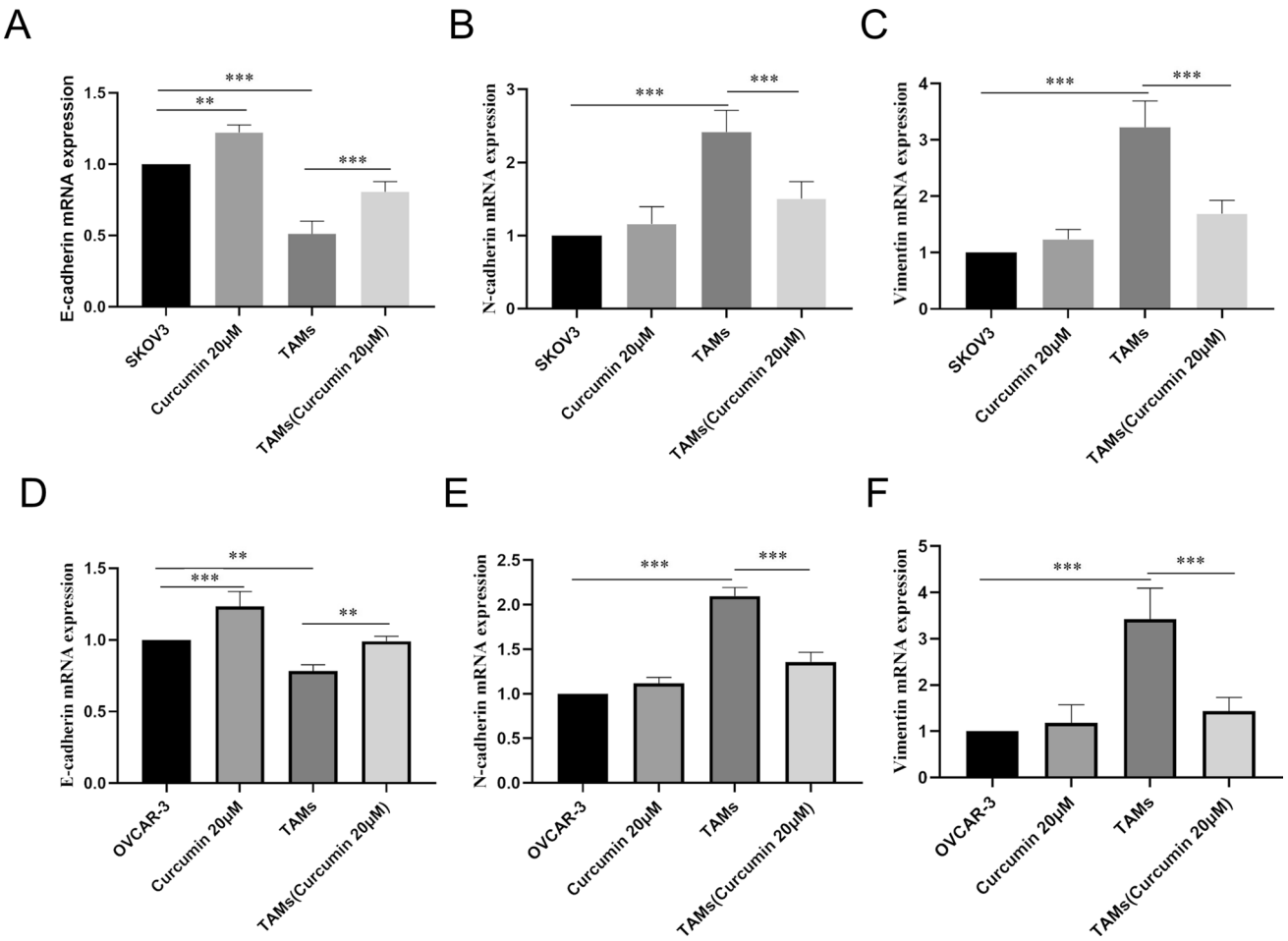


Fig. 6 Effects of curcumin on mRNA levels of EMT related proteins in SKOV3 and OVCAR-3 under TAMs cultural medium. **A**–**C** SKOV3 cells were treated for 48 h. Qualitative Analysis of EMT related proteins including E-cadherin, N-cadherin and vimentin in SKOV3 cells via RT-PCR (n = 3 per group). SKOV3, SKOV3 cells cultured with normal medium (10% FBS and 1.5% penicillin/steptomycin solution in 1640 medium); Curcumin 20 μM, SKOV3 cells cultured with normal medium in combination of 20 μM curcumin; TAMs, SKOV3 cells cultured with TAMs cultural medium; TAMs (Curcumin 20 μM), SKOV3 cells cultured with TAMs cultural medium stimulated with 20 μM curcumin for 48 h in advance. **D**–**F** OVCAR-3 cells were treated for 48 h. Qualitative Analysis of EMT related proteins including E-cadherin, N-cadherin and vimentin in OVCAR-3 cells via RT-PCR (n = 3 per group). OVCAR-3, OVCAR-3 cells cultured with normal medium (10% FBS and 1.5% penicillin/steptomycin solution in 1640 medium); Curcumin 20 μM, OVCAR-3 cells cultured with normal medium in combination of 20 μM curcumin; TAMs, OVCAR-3 cells cultured with TAMs cultural medium; TAMs (Curcumin 20 μM), OVCAR-3 cells cultured with TAMs cultural medium stimulated with 20 μM curcumin for 48 h in advance. **P < 0.01, ***P < 0.001. Note: In all figures, the terms "SKOV3" and "OVCAR-3" refer to untreated (vehicle) conditions for the respective cell lines unless otherwise specified

the direct inhibitory effects of long-term, high-dose curcumin on ovarian cancer cells, the observation time in this experiment was set to 48 h. At the same time, we found that curcumin had no significant inhibitory effect on the proliferation of these three types of cells within the dose range of 0 to 20 μ M. As a result, curcumin in the doses of 0–20 μM would be applied in subsequent experiments.

Previous studies have reported that curcumin contributes to the alleviation of tumor progress via the regulation of TAMs and T cell activities [34–36]. Here in this study, we stimulated primary TAMs with curcumin in doses of 0, 5, 10, and 20 μM for 48 before Flow cytometry detection. We found that curcumin in the doses of 5,10, and 20 μM could reduce the percentage of the M2 subtype in total TAMs, with 20 μM curcumin showing the most obvious effect ($P < 0.01$). However, we did not detect the obvious change in the M1 subtype percentage via flow cytometry, as reported by previous studies. Despite this, we found an increase in the mRNA levels of M1 subtype-related cytokines, including IL-1, IL-12, and TNF-α and a decrease of those of M2 subtype-related *TGF-β, CCL23*, and *CXCR2* via RT-PCR analysis. Those data further indicated the regulatory effect of curcumin in the dose of 20 μM on TAM polarization. Similarly, Shiri et al. showed that 20 μM curcumin encapsulated in

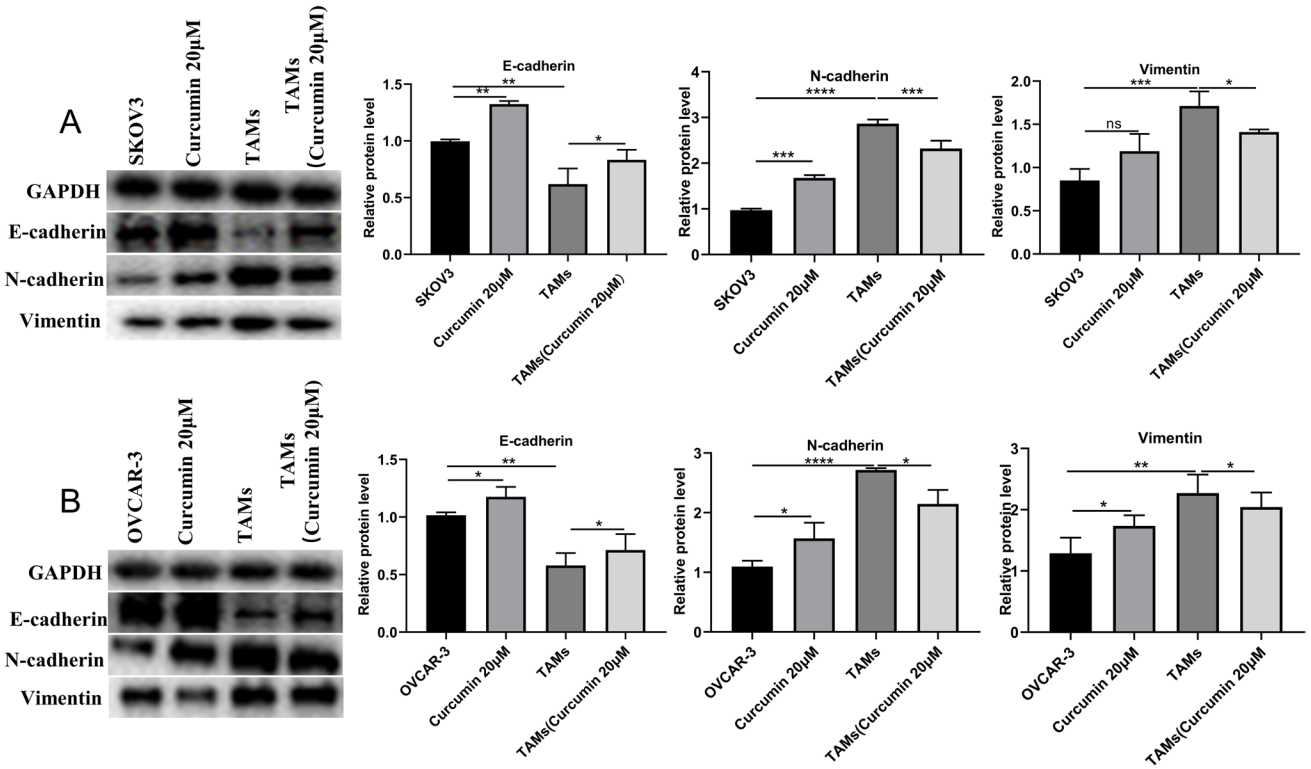


Fig. 7 Effects of curcumin on proteins levels of EMT related proteins in SKOV3 and OVCAR-3 under TAMs cultural medium. **A** SKOV3 cells were treated for 48 h. Qualitative Analysis of EMT related proteins including E-cadherin, N-cadherin and Vimentin in SKOV3 cells via Western blot (n = 3 per group). SKOV3, SKOV3 cells cultured with normal medium (10% FBS and 1.5% penicillin/steptomycin solution in 1640 medium); Curcumin 20 μM, SKOV3 cells cultured with normal medium in combination of 20 μM curcumin; TAMs, SKOV3 cells cultured with TAMs cultural medium; TAMs (Curcumin 20 μM), SKOV3 cells cultured with TAMs cultural medium stimulated with 20 μM curcumin for 48 h in advance. **B** OVCAR-3 cells were treated for 48 h. Qualitative analysis of EMT related proteins including E-cadherin, N-cadherin and Vimentin in OVCAR-3 cells via Western blot (n = 3 per group). OVCAR-3, OVCAR-3 cells cultured with normal medium (10% FBS and 1.5% penicillin/steptomycin solution in 1640 medium); Curcumin 20 μM, OVCAR-3 cells cultured with normal medium in combination of 20 μM curcumin; TAMs, OVCAR-3 cells cultured with TAMs cultural medium; TAMs (Curcumin 20 μM), OVCAR-3 cells cultured with TAMs cultural medium stimulated with 20 μM curcumin for 48 h in advance Note: In all figures, the terms "SKOV3" and "OVCAR-3" refer to untreated (vehicle) conditions for the respective cell lines unless otherwise specified

nanoparticles suppresses metastatic breast cancer by shifting the macrophage balance from M2 to M1 [37].

In our current study, we found that TAMs could promote the proliferation of two kinds of ovarian cancer cells ($P < 0.001$). Compared with the TAMs group, cells in TAMs (Curcumin 20 μM) were more suppressed in proliferation ($P < 0.05$). In addition, TAMs could significantly suppress the migration and invasion of ovarian cancer cells through Cell scratch tests. Our results demonstrated that although curcumin in low doses (20 μM) could directly suppress the malignant activity of ovarian cancer, its alleviative effect on ovarian cancer tended to rely on the regulation of TAM polarization. In the Cell scratch test, we found the change of cell form in TAMs, TAMs (Curcumin 20 μM), and TAMs + Curcumin 20 μM groups compared with the control group. Compared with changes in OVCAR-3 cells, those in SKOV3 cells showed more obvious changes.

We finally study the effects on tumor activities via the detection of EMT. EMT is referred to as a vital biological process of the transformation from polarized epithelial cells into mesenchymal cells. Through the process of EMT, epithelial cells partly or totally lose their epithelial phenotype and basement membrane connection and subsequently gain higher ability in migration, invasion, anti-apoptosis, and degrading extracellular matrix [38–40]. In this study, we detected the levels of EMT-related markers, including E-cadherin, N-cadherin, and Vimentin, through RT-PCR and Western blot. We found that compared with the control group, the level of E-cadherin was decreased, and those of N-cadherin and Vimentin were increased in both SKOV3 and OVCAR-3 cells, indicating the co-culture of SKOV3/OVCAR-3 and TAMs led to EMT process. In addition, compared with the TAMs group, the level of the EMT process

was largely suppressed in the TAMs (Curcumin 20 μM) group.

While our study provides valuable insights into curcumin's effects on TAMs and ovarian cancer cells, it has several limitations. The small sample size and reliance on in vitro models limit the generalizability of our findings. Future studies should include larger sample sizes, in vivo experiments, and clinical trials to validate our results.

Conclusion

In conclusion, in this study, we demonstrated that curcumin in low doses (20 μM) suppressed the malignant behaviors of ovarian cancer cells, including migration, invasion, and EMT process through the regulation of TAM polarization. We believe that our findings might provide a novel insight into the treatment of ovarian cancer and the development of novel therapies against ovarian cancer.

Author contributions Xi Li: Writing-original draft, Methodology; Lingzi Su: Writing-review & editing, Validation; Chen Qian: Formal analysis, Software; Wenchao Qiu: Formal analysis, Visualization; Lin Tao: Visualization; Zhaowei Guo: Investigation; Chaoqin Yu: Supervision; Jun Shi: Project administration. All authors have read and approved the published version of this manuscript.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Conflict of interest The authors declare no competing interests.

Ethical approval This study was approved by Shanghai Changhai Hospital Ethics Committee (approval number: CHEC2024-063).

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