M2-like tumour-associated macrophage-secreted IGF promotes thyroid cancer stemness and metastasis by activating the PI3K/AKT/mTOR pathway

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Abstract. M2-like tumour-associated macrophages (TAMs) have been demonstrated to promote the growth of anaplastic thyroid carcinoma (ATC). However, the underlying mechanism of M2-like TAMs in ATC remains unclear. Thus, in the present study, the role and mechanism of M2-like TAMs in ATC were investigated. M2-like TAMs were induced by treatment with PMA, plus IL-4 and IL-13, and identified by flow cytometry. Transwell and sphere formation assays were applied to assess the invasion and stemness of ATC cells. The expression levels of insulin-like growth factor (IGF)-1 and IGF-2 were examined by ELISA and reverse transcription-quantitative PCR. Proteins related to the epithelial-mesenchymal transition (EMT), stemness and the PI3K/AKT/mTOR pathway were examined via western blotting. Immunohistochemistry (IHC) was used to detect the expression of the M2-like TAM markers CD68 and CD206 in ATC tissues and thyroid adenoma tissues. It was found that treatment with PMA plus IL-4 and IL-13 successfully induced M2-like TAMs. Following co-culture with M2-like TAMs, the invasive ability and stemness of ATC cells were significantly increased. The expression levels of the EMT-related markers N-cadherin and Vimentin, the stemness-related markers Oct4, Sox2 and CD133, and the insulin receptor (IR)-A/IGF1 receptor (IGF1R) were markedly upregulated, whereas E-cadherin expression was significantly decreased. In addition, the production of IGF-1 and IGF-2 was significantly increased. Of note, exogenous IGF-1/IGF-2 promoted the invasion and stemness of C643 cells, whereas blocking IGF-1 and IGF-2 inhibited metastasis and stemness by repressing IR-A/IGF-1R-mediated PI3K/AKT/mTOR signalling in the co-culture system. IHC results showed that the expression of CD68 and CD206 was obviously increased in ATC tissues. To conclude, M2-like TAMs accelerated the metastasis and increased the stemness of ATC cells, and the underlying mechanism may be related to the section of IGF by M2-like TAMs, which activates the IR-A/IGF1R-mediated PI3K/AKT/mTOR signalling pathway.

Introduction

Thyroid cancer is the most common malignancy of the endocrine system worldwide, and its incidence has shown a significant increase over the past 30 years (1,2). Thyroid carcinomas are classified as differentiated or undifferentiated according to their histological type. Anaplastic thyroid carcinoma (ATC) is an undifferentiated subtype of thyroid cancer with a high risk of invasion, recurrence and metastasis (3). Generally, 90% of patients with ATC pass away within 6 months of diagnosis, accounting for 14-39% of all thyroid cancer-related deaths annually worldwide (4). Despite therapeutic advances in diagnosis and clinical treatment, multiple studies have shown no obvious improvement in the survival rate of patients with ATC (3,5), perhaps due to the insufficient understanding of the underlying mechanism of cancer metastasis and the lack of effective therapeutic targets for ATC.

Cancer stem cells (CSCs), also known as tumour-initiating cells, are a small subpopulation of cancer cells with the properties of multidirectional differentiation and metastasis, unlimited proliferation and self-renewal (6). An increasing number of studies have indicated that CSCs are closely related to chemoradiotherapy tolerance, tumour metastasis and recurrence (7-9). The clinical implication of the CSC model is that it may be possible to eradicate the tumour by removing all CSCs or other factors that promote the characteristics of CSCs (10),

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Abbreviations: TAMs, tumour-associated macrophages; ATC, anaplastic thyroid carcinoma; CSCs, cancer stem cells; IGF, insulin-like growth factor; IR, insulin receptor; EMT, epithelial-mesenchymal transition

Key words: M2-like TAMs, IGF, PI3K/AKT/mTOR pathway, thyroid cancer, cancer stemness

which provides a largely novel idea for targeted treatment of thyroid cancer. The tumour microenvironment is crucial in regulating the plasticity of CSCs (11). However, the effect of the tumour microenvironment on the stemness of thyroid cancer cells is unclear. Tumour-associated macrophages (TAMs) are vital immune cells in the tumour microenvironment that have been shown to be associated with poor prognosis in a variety of malignant tumours (12,13), especially M2-like TAMs, which directly communicate with CSCs to promote their stemness and/or subsequent oncogenic properties, thereby triggering tumour invasion and metastasis (14,15). Therefore, the present study focused on investigating the role of M2-like TAMs in cancer stemness and thyroid cancer metastasis.

The insulin-like growth factor (IGF) system plays an important role in regulating the development and growth of mammals (16). Notably, the two main receptors of this system, IGF-1 receptor (IGF-1R) and insulin receptor (IR), which occurs in two isoforms (IR-A and IR-B), are usually overexpressed in tumour cells, supporting their biological significance in cancer development (17,18). There is extensive crosstalk between IR-A and IGF-1R, and the pleiotropy of IR-A/IGF-1R signals is mediated by a variety of downstream pathways, including the PI3K/AKT and ERK pathways (19). IGF-1 and IGF-2 activate downstream signalling pathways and participate in the regulation of stemness, epithelial-mesenchymal transition (EMT), proliferation and metastasis of tumour cells by binding to receptors IGF-1R and IR-A (20). Studies have shown that upregulation of IGF-1 by TAMs increases the proliferation and migration of cancer cells (21,22). In addition, high expression of IRs and IGF was observed in thyroid cancer cells (23). Therefore, the present study hypothesized that upregulation of IGF by M2-like TAMs promotes stemness and metastasis by activating IR-A/IGF-1R-mediated PI3K/AKT/mTOR signalling in human ATC.

In the present study, the data showed that M2-like TAMs were enriched in human ATC tissues and that M2-like TAM-secreted IGF promoted the metastasis and stemness of ATC cells by activating the IR-A/IGF1R-mediated PI3K/AKT/mTOR signalling pathway. These data could improve the understanding of ATC progression and provide promising therapeutic targets for the treatment of ATC.

Materials and methods

Tissue samples. Tissues from 12 patients with thyroid adenoma (seven women and five men; mean age, 48.08±15.43 years old, range 22-68 years old), and tissues from 12 patients with ATC (six women and six men; mean age, 52.25±16.99 years old, range 19-73 years old) were acquired from the Third Affiliated Hospital of Kunming Medical University, also known as Yunnan Cancer Hospital (Kunming, China), collected from February 2019 to January 2020. Informed consent was obtained from all participants and the Research Ethics Committee of the Yunnan Cancer Hospital approved this study (approval no. KY2020220). The inclusion criteria were undifferentiated thyroid carcinoma patients with complete data. Patients with severe chronic diseases (such as renal insufficiency) and severe liver disease were excluded. Tissue samples were collected during surgery and immediately stored in liquid nitrogen and 4% paraformaldehyde.

Cell culture and treatment. The human monocyte cell line THP-1 was purchased from the American Type Culture Collection. The human anaplastic thyroid cancer cell line C643 was purchased from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. Both cell lines were maintained in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) containing 10% foetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin (Beijing Solarbio Science & Technology Co., Ltd.) in a humidified atmosphere at 37˚C with 5% CO_2.

C643 cells were incubated with serum-free medium containing 100 ng/ml IGF-1 or IGF-2 (Sigma-Aldrich; Merck KGaA) for 24 h at 37˚C. To block IGF-1 and IGF-2 signals, cells were treated with serum-free containing anti-IGF-1 (cat. no. ab40657, Abcam) and anti-IGF-2 (cat. no. ab63984, Abcam) antibodies for 2 h at 37˚C, and then co-cultured with M2-like TAMs. To block the PI3K/AKT pathway, cells were pre-incubated with serum-free medium containing PI3K/AKT pathway inhibitor LY294002 (20 μM; Sigma-Aldrich; Merck KGaA) for 2 h, and then co-cultured with M2-like TAMs for 24 h at 37˚C.

Preparation of M2-like macrophages. THP-1 cells ($1x10^6$/well) were cultured in 6-well plates. To generate M2-like TAMs, 320 nM PMA (Sigma-Aldrich; Merck KGaA) was used to treat THP-1 cells for 6 h, and then the cells were treated with PMA plus 20 ng/ml IL-4 (Sigma-Aldrich; Merck KGaA) and 20 ng/ml IL-13 (Sigma-Aldrich; Merck KGaA) for another 18 h (24).

Flow cytometry analysis. Following washing, trypsin digestion and centrifugation (1,000 x g, 4˚C, 5 min), the M2-like TAMs were resuspended in 100 μl PBS ($1x10^6$ cells) and stained with 5 μl mouse anti-human CD14-FITC, CD68-FITC, CD206-PE and CD163-FITC antibodies for 30 min at 4˚C. The stained cells were then analysed using a FACSCalibur flow cytometer (BD Biosciences) and Cell Quest 3.3 software (BD Biosciences). Antibodies against CD14-FITC (cat. no.367115; 1:500), CD68-FITC (cat. no. 333805; 1:500), CD206-PE (cat. no. 321105; 1:500) and CD163-FITC (cat. no. 333617; 1:500) were purchased from BioLegend, Inc.

M2-like TAMs and C643 cell co-culture. A 0.4-μm Transwell chamber (Corning, Inc.) was used in the co-culture assay. Briefly, M2-like TAMs ($2x10^5$) were seeded into the upper chamber and co-cultured with C643 cells ($2x10^5$/well) in 6-well plates. After 24 h of co-culture at 37 ˚C, the upper chamber was discarded, and C643 cells in the lower chamber were collected and used for subsequent experiments.

Transwell invasion assay. A 24-well Transwell cell culture chamber (Corning, Inc.) coated with Matrigel (BD Biosciences) for 30 min at 37˚C was used to investigate cell invasion ability. To assess invasion, C643 cells were harvested after co-culture with M2-like TAMs for 24 h at 37˚C. Then, the C643 cells were diluted to $1x10^5$/ml in 200 μl serum-free RPMI-1640 medium and added to the upper chamber. RPMI-1640 medium (600 μl) containing 10% FBS was placed in the lower chamber. After incubation for 24 h at 37˚C, the invaded cells in the lower chamber were fixed with 4% paraformaldehyde at 25˚C for 30 min and stained with 0.1% crystal violet at 25˚C for 15 min. The invaded

cells were imaged and counted at x200 magnification using a light microscope in five different fields for each chamber.

Sphere formation assay. C643 cells were harvested after co-culture with M2-like TAMs for 24 h. Then, C643 cells ($4x10^4$/well) were plated in ultra-low-attachment 24-well plates (Corning, Inc.) and maintained in serum-free DMEM-F12 (Sigma-Aldrich; Merck KGaA) containing B27 supplement minus vitamin A (Gibco; Thermo Fisher Scientific, Inc.), 20 ng/ml epidermal growth factor (R&D Systems) and 20 ng/ml basic fibroblast growth factor (R&D Systems). After 2 weeks, cell spheroids with a diameter >75 μm were counted at x100 magnification using a light microscope.

Western blot analysis. Cellular lysates were prepared using radioimmunoprecipitation assay lysis buffer (Wuhan Boster Biological Technology, Ltd.) supplemented with protease inhibitors (Roche Diagnostics) according to the manufacturer's protocols. A bicinchoninic acid protein assay kit (Wuhan Boster Biological Technology, Ltd.) was used to determine the protein concentration. Equal amounts of protein (30 μg of lysates) were separated via 10% SDS-PAGE, and then separated proteins were transferred onto PVDF membranes (EMD Millipore). The membranes were then blocked with 5% non-fat milk at room temperature for 1 h, followed by incubation at 4˚C overnight with primary antibodies against E-cadherin (cat. no. ab15148; 1:500), N-cadherin (cat. no. ab18203; 1:500), Vimentin (cat. no. ab137321; 1:1,000), CD133 (cat. no. ab19898; 1:1,000), Oct4 (cat. no. ab18976; 1:500), Sox2 (cat. no. ab97959; 1:500), IGF1R (cat. no. ab131476; 1:500), phosphorylated (p)-IGF1R (cat. no. ab39398; 1:1,000), IR (cat. no. ab137747; 1:1,000), p-IR (cat. no. ab60946; 1:1,000), AKT (cat. no. ab18785; 1:500), p-AKT (cat. no. ab38449; 1:500), mTOR (cat. no. ab2732; 1:2,000), p-mTOR (cat. no. ab84400; 1:1,000) and GAPDH (cat. no. ab9485; 1:2,000). All antibodies were purchased from Abcam. After the membrane was incubated with HRP-conjugated goat anti-rabbit immunoglobulin G secondary antibody (cat. no. BA1054; 1:2,000; Wuhan Boster Biological Technology, Ltd.) at room temperature for 1 h, the signals were developed using an enhanced chemiluminescence reagent (EMD Millipore) according to the manufacturer's instructions. Image-Pro Plus 6.0 software (Media Cybernetics, Inc.) was used to semi-quantify the protein bands, and GAPDH was used as a loading control.

Reverse transcription-quantitative (RT-q) PCR analysis. Isolation of total RNA from cultured cells was performed with TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Briefly, RT was performed according to the manufacturer's protocol and cDNA was generated using a PrimeScript™ RT reagent kit (Takara Biotechnology Co., Ltd.). qPCR was conducted using SYBR Green P0.remix Ex Taq II (Takara Biotechnology Co., Ltd.) in an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). qPCR was performed under the following conditions: 95˚C for 30 sec, followed by 40 cycles of 95˚C for 3 sec and 60˚C for 30 sec. The $2^{-\Delta\Delta Cq}$ method was used for comparative quantitation (25). *GAPDH* was used as an internal normalization control. The primer sequences used were as follows: *IGF1* forward, 5'-CAGCAGTCTTCCAACCCAAT-3' and reverse, 5'-CCACACACGAACTGAAGAGC-3'; *IGF2* forward, 5'-CGGACAACTTCCCCAGATAC-3' and reverse, 5'-GTCTTGGGTGGGTAGAGCAA-3'; and *GAPDH* forward, 5'-CCAGGTGGTCTCCTCTGA-3' and reverse, 5'-GCTGTAG CCAAATCGTTGT-3'.

ELISA for IGF-1 and IGF-2. C643 cells were co-cultured with M2-like TAMs and C643 cells alone were used as a control group. After 24 h of co-culture at 37˚C, the supernatant was collected and centrifuged at 200 x g for 5 min at 4˚C. Then, following the instructions of the Human IGF Signaling Antibody Array (cat. no. ab197446; Abcam), the optical density of the collected supernatant was detected at 450 nm using a microplate reader, and the concentration of IGF-1 or IGF-2 was calculated.

Immunohistochemistry (IHC) staining. The tissues were fixed in 4% paraformaldehyde for 24 h at 4˚C and dehydrated with a gradient of ethanol (100, 95, 80 and 70%). The tissues were embedded in paraffin and sectioned at a thickness of 3 μm. The sections were soaked in 3% H_2O_2 for 10 min and blocked with 5% non-immune goat serum (cat. no. 5425; Cell Signaling Technology, Inc.) at room temperature for 10 min, followed by incubation with anti-CD68 (cat. no. ab213363; 1:2,000; Abcam) or anti-CD206 (cat. no. 91992; 1:400; Cell Signaling Technology, Inc.) antibody at 4˚C for ~12 h. Subsequently, the sections were washed with PBS buffer three times and incubated with secondary antibodies (cat. no. 8114; 1:2,000; Cell Signaling Technology, Inc.) at 25˚C for 30 min. Then, the sections were stained with DAB reagent (Sigma-Aldrich; Merck KGaA) for 5 min at room temperature and stained with haematoxylin for 2 min at room temperature. The histomorphological changes were observed and imaged under a light microscope (Leica DM LB2; Leica Microsystems, Inc.).

Statistical analysis. Statistical evaluation was performed using SPSS 20.0 software (IBM Corp.). The values are presented as the mean ± standard deviation. Differences among multiple groups were compared by one-way analysis of variance followed by Tukey's post hoc test and differences between two groups were compared by unpaired Student's t-test (for parametric data). $P<0.05$ was considered to indicate a statistically significant difference.

Results

M2-like TAMs promote invasion and stemness of human ATC cells. Human THP-1 cells are widely used as a model for macrophage differentiation (26). To investigate the role of M2-like TAMs in ATC, the THP-1 cells were first treated with PMA, and then IL-4 and IL-13 were continuously used. As shown in Fig. 1A, M2-like macrophages were larger and more stacked in morphology. The THP-1 macrophages treated with PMA plus IL-4 and IL-13 exhibited notable expression of M2-like TAM surface markers, including CD68, CD206 and CD163, whereas the expression of CD14 (a monocyte marker) was markedly decreased (Fig. 1B). These results indicated that the M2-like TAM model was successful. M2-like TAMs were then used in co-culture with C643 cells. The results revealed that after co-culture with M2-like TAMs, the invasion capacity of ATC cells was significantly increased (Fig. 1C and D). Furthermore, a

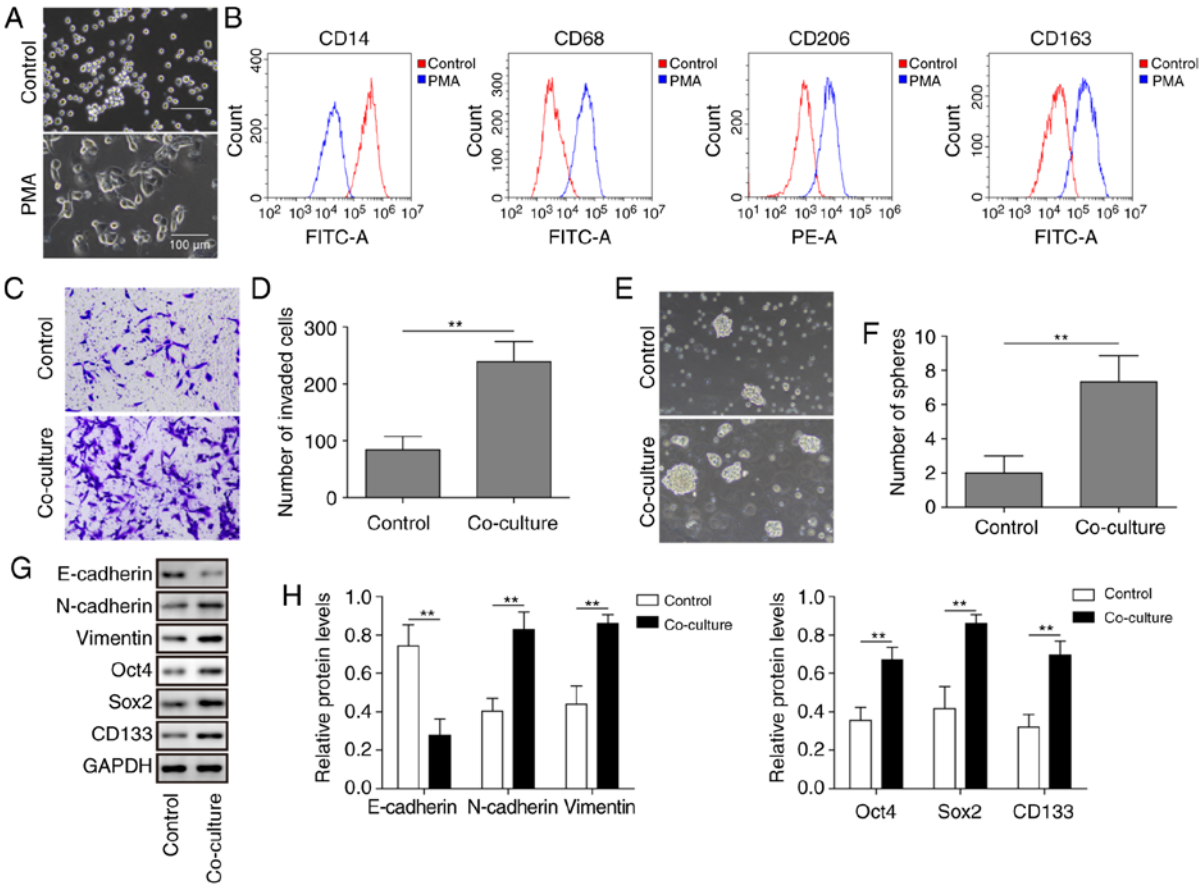

Figure 1. Effects of M2-like TAMs on the invasion, stemness and EMT of anaplastic thyroid carcinoma cells. (A) THP-1 cell morphology was observed via microscopy after treatment with PMA plus IL-4 and IL-13. Scale bar, 100 μm. (B) M2-like TAM surface markers, including CD14 (monocytes), CD68 (M2 TAMs), CD206 (M2 TAMs) and CD163 (M2 TAMs), were identified by flow cytometry after treatment with PMA plus IL-4 and IL-13. (C and D) The invasive ability of C643 cells was determined by Transwell assay after co-culture with M2-like TAMs for 24 h. (E and F) The cancer stemness of C643 cells was determined by a tumour sphere-formation assay after co-culture with M2-like TAMs for 24 h. (G and H) EMT and cancer stemness markers of C643 cells were examined by western blotting after co-culture with M2-like TAMs for 24 h. C643 cells were cultured alone as the control groups. The experiment was repeated three times, and the results are shown as the mean ± standard deviation. **P<0.01. TAM, tumour-associated macrophage; EMT, epithelial-mesenchymal transition.

sphere formation assay and western blotting were performed to assess cancer stemness. The results revealed that sphere formation and the expression of stemness-related markers Oct4, Sox2 and CD133 in ATC cells were significantly increased following co-culture with M2-like TAMs (Fig. 1E-H). EMT is considered to be an essential part of the process of tumour cell metastatic dissemination (27). Thus, the present study investigated whether M2-like TAMs were involved in EMT. As shown in Fig. 1G and H, co-culture with M2-like TAMs significantly decreased the expression of the epithelial marker E-cadherin and upregulated the expression of the mesenchymal markers N-cadherin and Vimentin in ATC cells. Taken together, these results indicated that M2-like TAMs accelerated the invasion and increased the stemness of ATC cells.

M2-like TAMs activate IR-A/IGF-1R signalling in human ATC cells by secreting IGF-1 and IGF-2. To further investigate the underlying mechanisms by which M2-like TAMs regulate cancer stemness and invasion of human ATC, RT-qPCR, ELISA and western blot assays were performed to discover the potential molecular mechanism. Significant induction of IGF-1 and IGF-2 mRNA expression was found in the supernatants of the co-culture group (Fig. 2A and B). ELISA results also confirmed that the secretion of IGF-1 and IGF-2 was significantly increased in the supernatant of the co-cultured cells compared with that of C643 cells alone (as a control group) (Fig. 2C and D). In addition, the western blotting results showed that ATC cells exhibited higher expression of p-IR-A and p-IGF1R after co-culture with M2-like TAMs (Fig. 2E and F), indicating that the M2-like TAMs activated IR-A/IGF-1R signalling in the ATC cells.

Exogenous IGF-1/IGF-2 promotes invasion and stemness of C643 cells. Next, exogenous IGF-1 or IGF-2 were used to treat C643 cells. As shown in Fig. 3A-D, it was found that exogenous IGF-1 or IGF-2 promoted the invasion and stemness in C643 cells. Furthermore, exogenous IGF-1 or IGF-2 increased the protein expression levels of N-cadherin, Vimentin, Oct4, Sox2 and CD133, and decreased the protein expression level of E-cadherin in C643 cells (Fig. 3E and F). Taken together, these data suggested that exogenous IGF-1/IGF-2 promoted the invasion and stemness of C643 cells.

Blockade of IGF1/IGF2 inhibits the effect of M2-like TAMs on the invasion and stemness of human ATC cells. After observing that M2-like TAMs accelerated the invasion and increased the stemness of C643 cells and simultaneously promoted

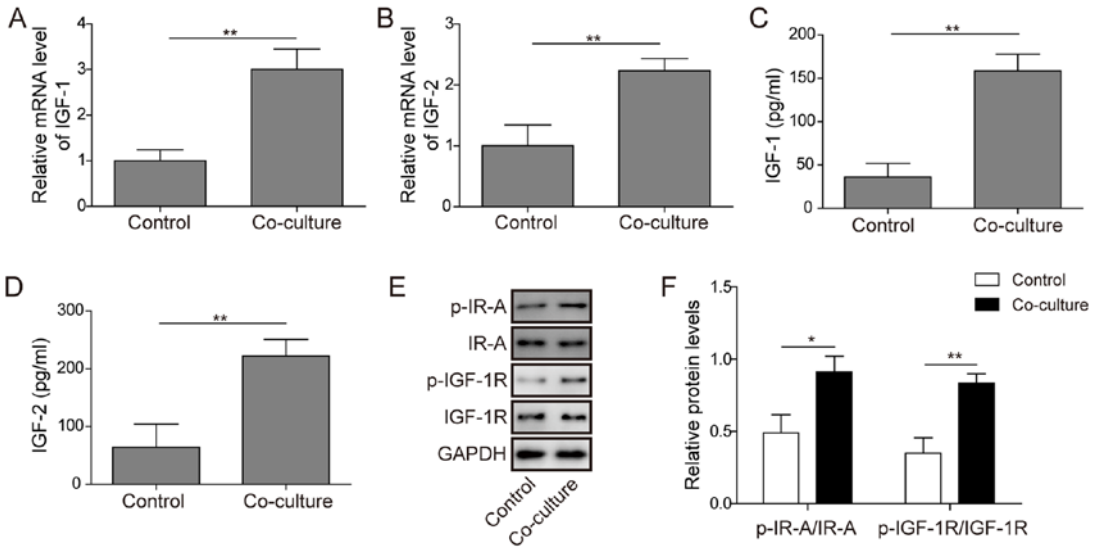

Figure 2. Effects of M2-like TAM-secreted IGF-1 and IGF-2 on the activation of IR-A/IGF-1R signalling in anaplastic thyroid carcinoma cells. (A and B) The IGF-1 and IGF-2 mRNA levels in C643 cells, M2-like TAMs and co-cultured cells were measured by reverse transcription-quantitative PCR. (C and D) The concentrations of IGF-1 and IGF-2 in supernatants obtained from C643 cells, M2-like TAMs and co-cultured cells were measured by ELISA. (E and F) The protein expression levels of IR-A, IGF-1R, p-IR-A and p-IGF-1R in C643 cells were measured by western blotting after co-culture with M2-like TAMs for 24 h. C643 cells were cultured alone as the control groups. The experiment was repeated three times, and the results are shown as the mean ± standard deviation. *P<0.05 and **P<0.01. TAM, tumour-associated macrophage; IGF, insulin-like growth factor; IR, insulin receptor; IGF-1R, IGF1 receptor; p-, phosphorylated.

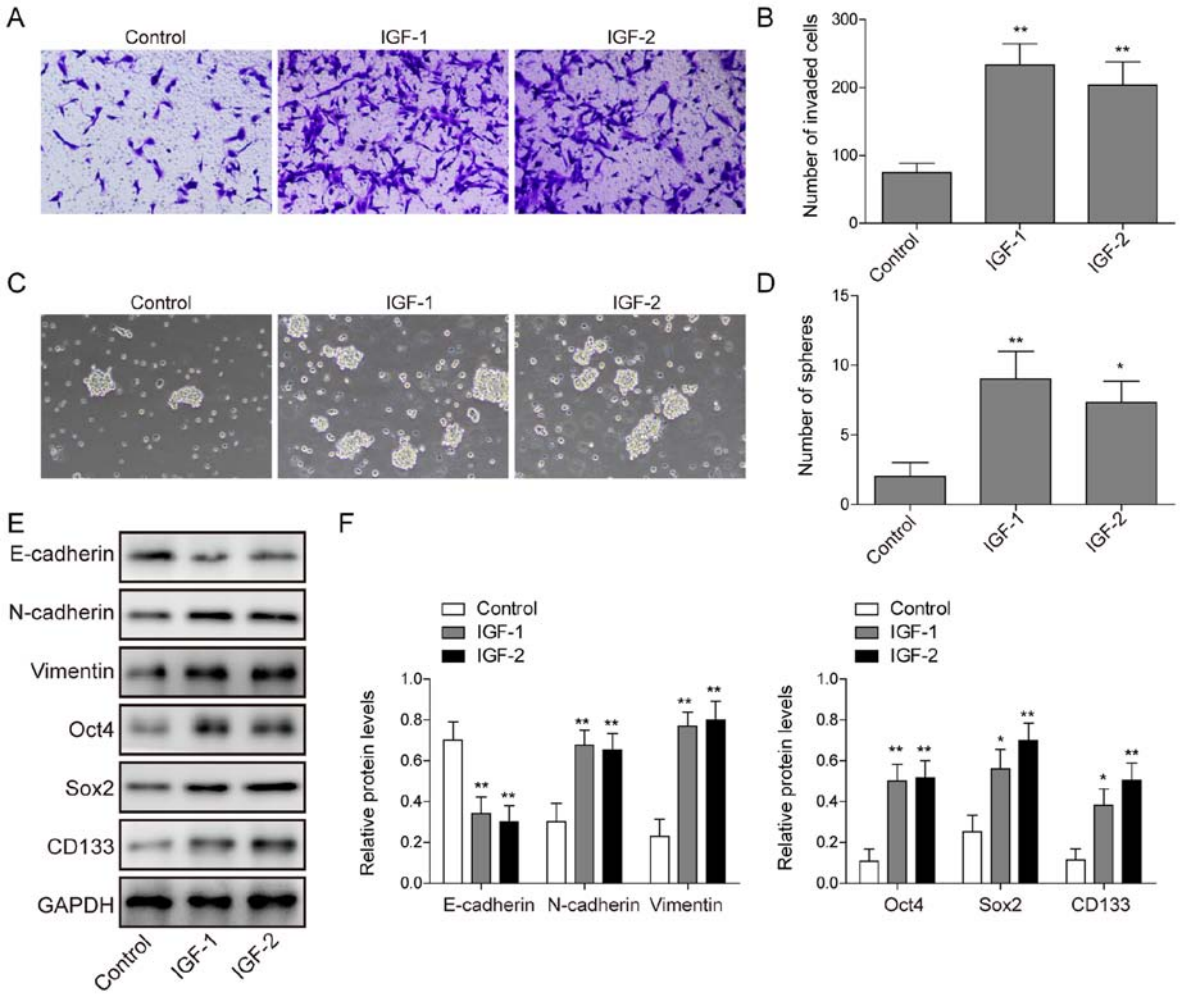

Figure 3. Effects of exogenous IGF-1/IGF-2 on the invasion and stemness of C643 cells. (A and B) Invasive ability was determined using a Transwell assay in C643 cells treated with IGF-1 or IGF-2. (C and D) The cancer stemness of C643 cells was determined by a tumour sphere-formation assay. (E and F) epithelial-mesenchymal transition and cancer stemness markers of C643 cells were examined by western blotting. The experiment was repeated three times, and the results are shown as the mean ± standard deviation. *P<0.05 and **P<0.01 vs. control. TAM, tumour-associated macrophage; IGF, insulin-like growth factor.

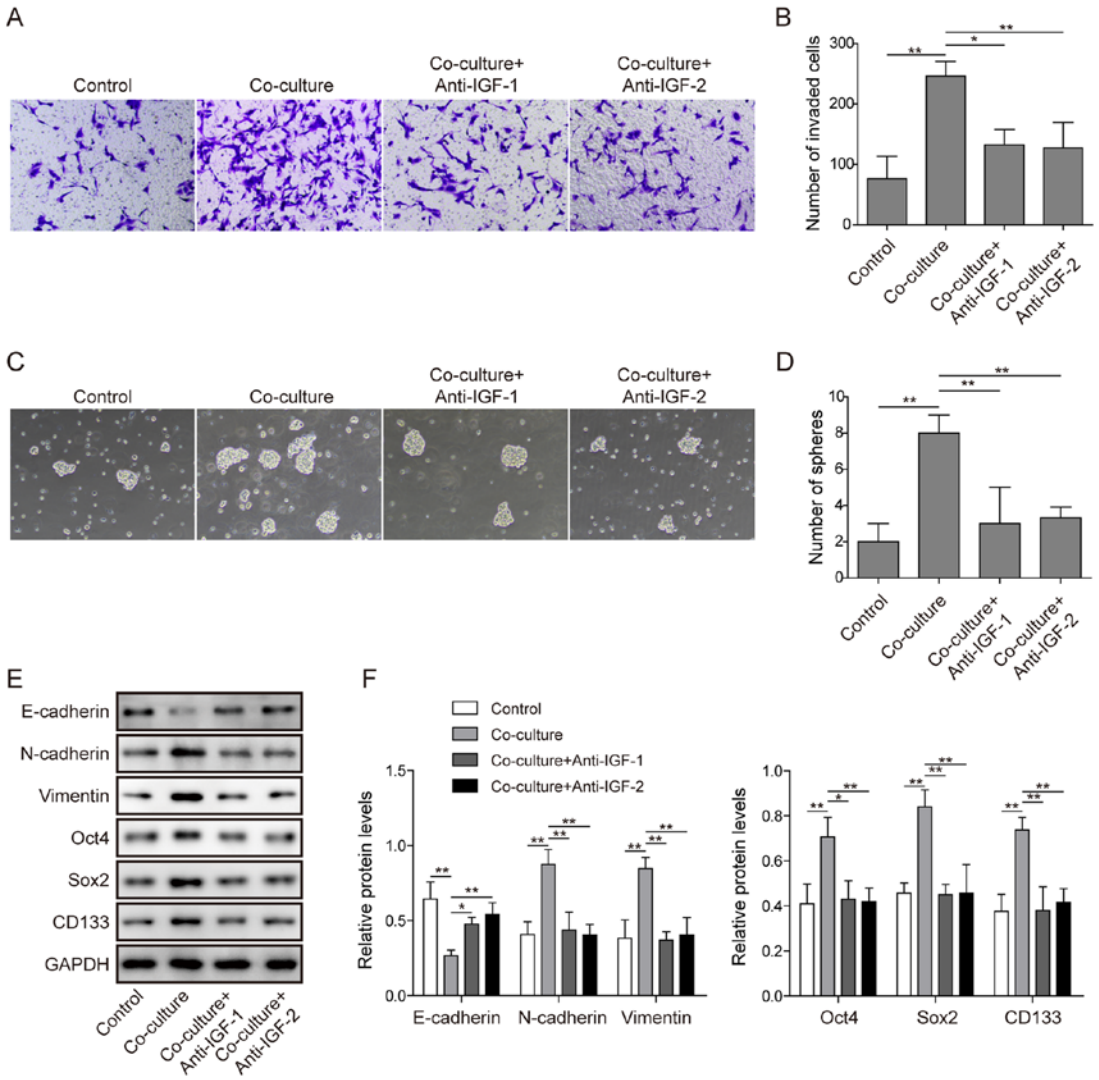

Figure 4. Effects of IGF-1-/IGF-2-neutralizing antibody on invasion and stemness of co-cultured cells. The co-culture system of C643 cells and M2-like TAMs was cultured in RPMI-1640 medium containing IGF-1-/IGF-2-neutralizing antibody (2 μg/ml) for 24 h. (A and B) The invasive ability of C643 cells was determined by a Transwell assay. (C and D) The cancer stemness of C643 cells was determined by a tumour sphere-formation assay. (E and F) Epithelial-mesenchymal transition and cancer stemness markers of C643 cells were examined by western blotting. The experiment was repeated three times, and the results are shown as the mean ± standard deviation. *P<0.05 and **P<0.01. TAM, tumour-associated macrophage; IGF, insulin-like growth factor.

the production of IGF-1 and IGF-2, the present study next assessed whether blockade of IGF-1/IGF-2 would suppress the M2-like TAM-induced alteration of invasion and stemness of C643 cells. An IGF-1-/IGF-2-neutralizing antibody was used to block the IGF pathway, and the blockade of IGF-1 and IGF-2 inhibited the invasion and stemness induced by M2-like TAMs in C643 cells (Fig. 4A-D). In addition, blockade of IGF-1 and IGF-2 reversed the increase in N-cadherin, Vimentin, Oct4, Sox2 and CD133 protein expression and the decrease in E-cadherin protein expression induced by M2-like TAMs in C643 cells (Fig. 4E and F). These data indicated that blocking IGF-1/IGF-2 suppressed the alteration of invasion and stemness of C643 cells induced by M2-like TAMs.

Blockade of IGF1/IGF2 inhibits IR-A/IGF-1R-mediated PI3K/AKT/mTOR signalling in the co-culture system. Next, the effects of blocking IGF1/IGF2 on the IR-A/IGF-1R-mediated PI3K/AKT signalling pathway in the co-culture system were examined. As shown in Fig. 5A and B, blockade of IGF1 and IGF2 significantly attenuated the protein expression of p-IR-A, p-IGF1R, p-AKT and p-mTOR. Although the expression of IR-A, IGF1R, AKT and mTOR showed no obvious change in any group, the ratios of p-IR-A/IR-A, p-IGF1R/IGF1R, p-AKT/AKT and p-mTOR/mTOR were markedly decreased following the blockade of IGF1/IGF2 in the co-culture system. These results indicated that IR-A/IGF-1R-mediated PI3K/AKT signalling was activated in the co-culture system and that blocking IGF1/IGF2 inhibited its activation.

PI3K/AKT/mTOR pathway is critical for the M2-like TAM-induced invasion and stemness of C643 cells. As the present study observed activation of the PI3K/AKT/mTOR signalling pathway in the co-culture system, it was next investigated whether the PI3K/AKT/mTOR pathway was involved in cell invasion and stemness in the co-culture system. The PI3K/AKT pathway inhibitor LY294002 was used, and inhibition of the PI3K/AKT pathway significantly inhibited the invasion and stemness of the co-culture system (Fig. 6A-D).

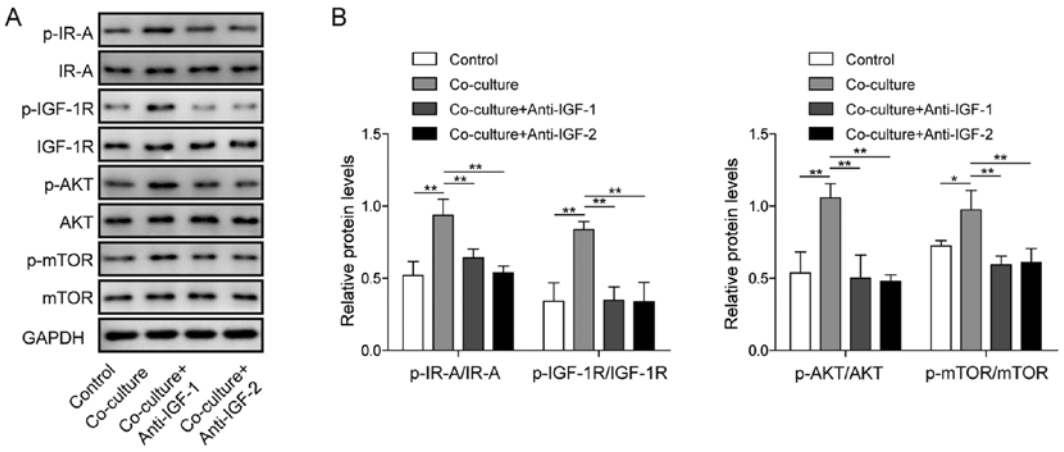

Figure 5. Effects of IGF-1-/IGF-2-neutralizing antibody on IR-A/IGF-1R-mediated PI3K/AKT/mTOR signalling in co-cultured cells. The co-culture system of C643 cells and M2-like TAMs was cultured in RPMI-1640 medium containing IGF-1-/IGF-2-neutralizing antibody (2 μg/ml) for 24 h. (A and B) The expression of IR-A/IGF-1R/PI3K/AKT signalling pathway proteins in C643 cells was examined by western blotting. C643 cells were cultured alone as the control groups. The experiment was repeated three times, and the results are shown as the mean ± standard deviation. *P<0.05 and **P<0.01. TAM, tumour-associated macrophage; IGF, insulin-like growth factor; IR, insulin receptor; IGF-1R, IGF1 receptor; p-, phosphorylated.

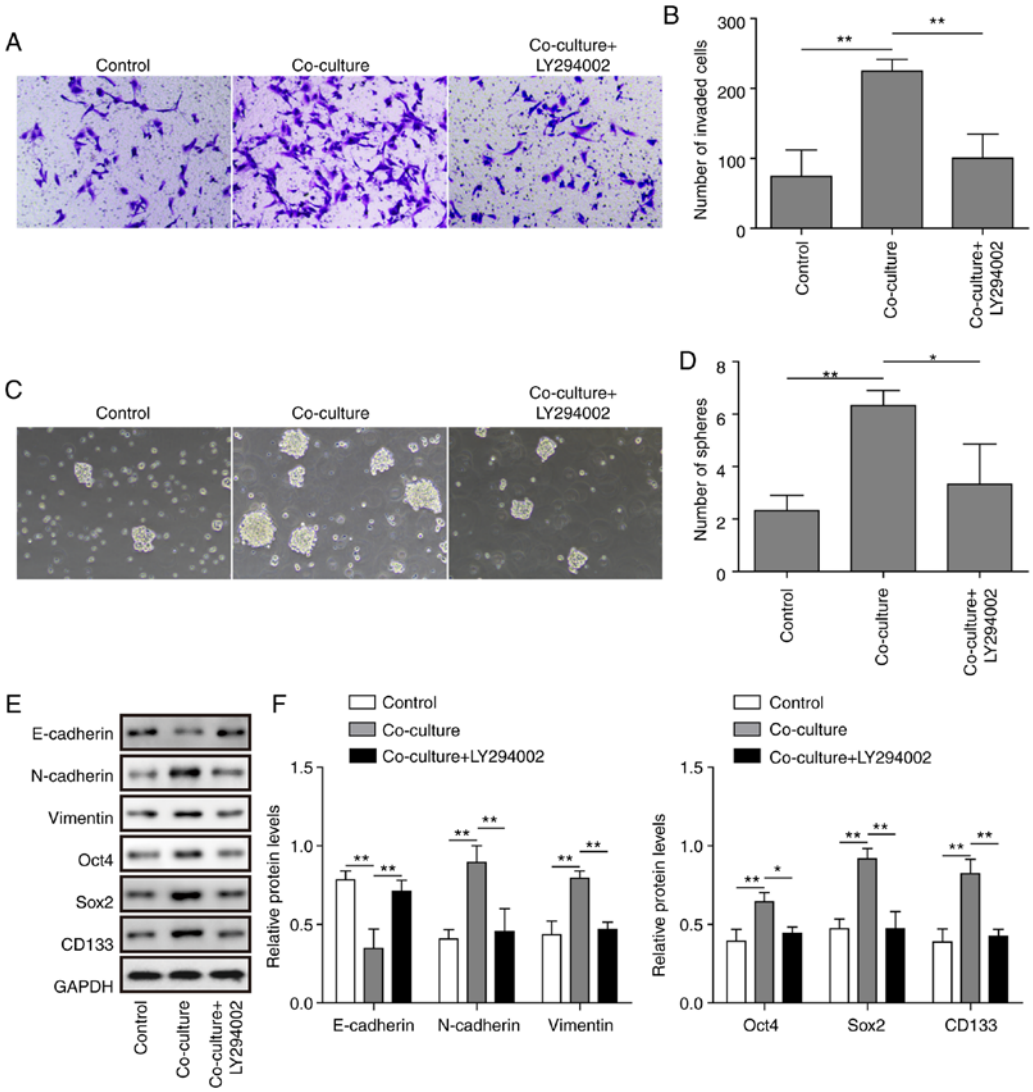

Figure 6. Effects of LY294002 on invasion, stemness and EMT in anaplastic thyroid carcinoma cells after co-culture with M2-like TAMs. C643 cells were pre-treated with LY294002 (20 μM) for 2 h and then co-cultured with M2-like TAMs for 24 h. C643 cells were cultured alone as the control groups. (A and B) The invasive ability of C643 cells was determined by a Transwell assay. (C and D) The cancer stemness of C643 cells was determined by tumour sphere-formation assay. (E and F) EMT and cancer stemness markers of C643 cells were examined by western blotting. The experiment was repeated three times, and the results are shown as the mean ± standard deviation. *P<0.05 and **P<0.01. EMT, epithelial-mesenchymal transition; TAM, tumour-associated macrophage.

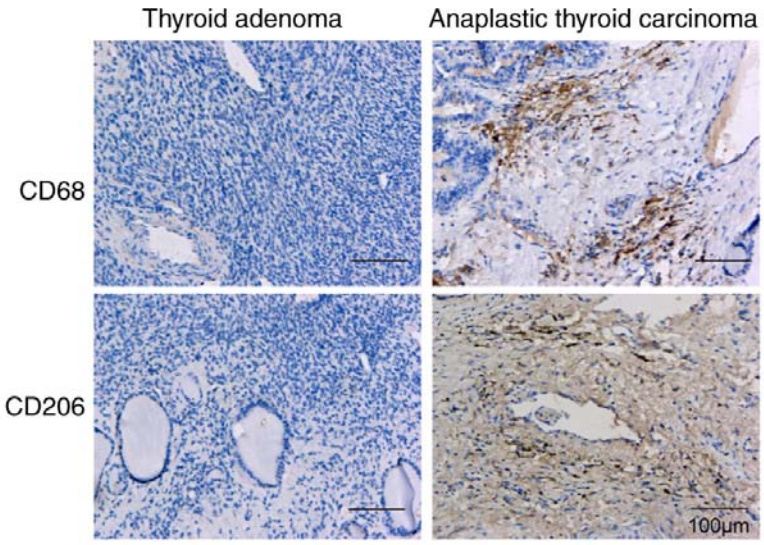

Figure 7. Expression of CD68 and CD206 in ATC tissues. CD68 and CD206 expression levels were notably increased in ATC tissues. The M2-like tumour-associated macrophage markers CD68 and CD206 in ATC tissues and thyroid adenoma tissues were analysed by immunohistochemistry staining. Scale bar, 100 μm. ATC, anaplastic thyroid carcinoma.

In addition, inhibition of the PI3K/AKT pathway reversed the increase in N-cadherin, Vimentin, Oct4, Sox2 and CD133 protein expression and the decrease in E-cadherin protein expression in the co-culture system (Fig. 6E and F). These results indicated that inhibition of the PI3K/AKT pathway inhibited the M2-like TAM-induced invasion and stemness of ATC cells.

Expression of CD68 and CD206 is notably increased in ATC tissues. Next, IHC was performed to detect the expression of the M2-like TAM markers CD68 and CD206 in ATC tissues and thyroid adenoma tissues. The results showed that the expression of CD68 and CD206 in ATC tissues was markedly increased compared with that in thyroid adenoma tissues (Fig. 7), indicating that the M2-like TAMs clustered with the ATC tissues.

Discussion

ATC remains one of the most fatal human malignancies, despite significant progress in diagnosis and treatment. Cancer stemness and distant metastasis are the main causes of poor survival in patients with ATC (4,28). Therefore, it is urgent to understand the underlying mechanism of the occurrence and metastasis of ATC to obtain effective treatments. Recent studies have shown that TAMs are one of the key immune cells in the tumour microenvironment that affect the degree of tumour malignancy (29,30). In the present study, it was found that the upregulation of IGF-1/IGF-2 induced by M2-like TAMs accelerated the metastasis and increased the stemness of ATC cells by activating the IR-A/IGF1R-mediated PI3K/AKT/mTOR signalling pathway.

Macrophages have functional plasticity and can be differentiated into differentially polarized TAMs under different microenvironmental stimuli (31). Macrophages differentiate into M1-like TAMs after exposure to lipopolysaccharides and IFN-γ, and have antitumour activities. When macrophages are exposed to Th2 cytokines, such as IL-4 and IL-13, they are polarized to M2 macrophages and promote tumour growth (32,33). M2-like TAMs have been reported to play a vital role in promoting tumour progression, and identification of M2-like TAMs during tumour progression is considered an appealing approach for cancer treatment (34). In thyroid cancers, an increased density of M2-like TAMs was associated with decreased survival (35). Caillou *et al* (36) confirmed the presence of a high number of TAMs in most ATC cases. In the present study, it was observed that when co-cultured with M2-like TAMs, ATC cells exhibited increased invasion, stemness and EMT. These findings indicated that M2-like TAMs may play a positive role in the promotion of ATC development. The TAM markers CD68 and CD206 were detected in ATC tissues and thyroid adenoma tissues. In addition, the IHC results showed that the expression of CD68 and CD206 in ATC tissues was notably increased compared with that in thyroid adenoma tissues, suggesting that the M2-like TAMs clustered with the ATC tissues.

IGFs are often present in large quantities in the tumour microenvironment and can be secreted by tumour stroma and/or malignant cells. Elevated circulating IGF levels are associated with a higher cancer risk, and experimental evidence has suggested that IGFs contribute to the growth, metastasis and chemoresistance of various cancers (37-39). In the present study, it was found that M2-like TAMs significantly increased the induction of IGF-1 and IGF-2 in ATC cells. More importantly, IGFs have been reported to be overexpressed in both thyroid cancer cell lines and tissues (40). Thus, in the present study it was demonstrated that M2-like TAMs promoted the progression of ATC by secreting IGFs. The biological effects of insulin and IGFs are mediated by the activation of their cell surface receptors, which possess tyrosine kinase activity (41). In the present study, high expression of p-IR-A and p-IGF1R in ATC cells were observed after co-culture with M2-like TAMs. Exogenous IGF-1/IGF-2 promoted the invasion and stemness of C643 cells, whereas an IGF1-/IGF2-neutralizing antibody reversed the alteration

of invasion, stemness and EMT. These results indicated that M2-like TAM-secreted IGFs may be key factors in the promotion of ATC progression via the IR-A/IGF-1R signalling pathway.

Activation of the PI3K/AKT pathway has been linked to cancer cell proliferation, survival and apoptosis by regulating downstream molecules (42). Fu *et al* (43) demonstrated that metallothionein 1G suppressed cell growth and invasiveness by regulating the PI3K/AKT signalling pathway in thyroid cancer. In the present study, it was found that M2-like TAMs increased ATC cell invasion, stemness and EMT by activating the PI3K/AKT pathway. Ligand binding to IGF-IR can trigger a variety of signalling pathways, such as the PI3K/AKT pathway, which is involved in the transmission of cell survival signals (44,45). Ma *et al* (46) reported that IGF-1 enhanced cell proliferation and invasion by activating the IGF-1/PI3K/AKT signalling pathway in pancreatic cancer cells. Targeting AKT or ERK signalling reversed IGF-1-induced EMT in gastric cancer (47). The present study revealed that blocking IGF1/IGF2 inhibited the activation of the IR-A/IGF-1R-mediated PI3K/AKT/mTOR signalling pathway in the co-culture system. Taken together, these results suggested that M2-like TAM-secreted IGFs promote ATC tumour progression by activating IR-A/IGF-1R-mediated PI3K/AKT/mTOR signalling.

In summary, the present study revealed that M2-like TAM-secreted IGF-1 and IGF-2 promoted cancer invasion, stemness and EMT of ATC via the IR-A/IGF-1R-mediated PI3K/AKT/mTOR signalling pathway. These data provided novel insights into the molecular mechanism underlying ATC progression, and currently available IGF1/IGF2 inhibitors may be a therapeutic strategy for ATC intervention.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ZYD was the guarantor of integrity for the entire study. ZYD and ZPF conceived the study, and ZYD and JL designed the study. PJL, LJ, ZXY and FH performed the experiments. ZXY acquired the data, CL performed data analysis and FKC conducted the statistical analysis. JL prepared and edited the manuscript. ZYD and ZPF reviewed the manuscript. ZYD and CL confirmed the authenticity of all the raw data. All authors read and reviewed the final manuscript.

Ethics approval and consent to participate

Informed consent was obtained from all participants and The Research Ethics Committee of the Yunnan Cancer Hospital approved this study (approval no. KY2020220; Kunming, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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