Histol Histopathol (2019) 34: 563-572

http://www.hh.um.es

Histology and Histopathology

From Cell Biology to Tissue Engineering

Infiltrated M2 tumour-associated macrophages in the stroma promote metastasis and poor survival in oesophageal squamous cell carcinoma

Jian Zhou[1,3,4]*, Shutao Zheng[2,3]*, Tao Liu[2,3], Qing Liu[2,3], Yumei Chen[1,2], Rong Ma[1,2], Doudou Tan[1,3] and Xiaomei Lu[1,3]

[1]Tumor Hospital Affiliated to Xinjiang Medical University, [2]Clinical Medical Research Institute, First Affiliated Hospital of Xinjiang Medical University, [3]State Key Laboratory of Pathogenesis, Prevention, Treatment of Central Asian High Incidence Diseases, Xinjiang Uygur Autonomous Region, Urumqi and [4]Department of Pathology, the Affiliated Hospital of Southwest Medical University, Luzhou, PR China

*Contributed equally

Summary. Although M2 tumour-associated macrophages (M2 TAMs) have been shown to be associated with the progression and metastasis of breast cancer, their role in oesophageal squamous cell carcinoma (ESCC) remains less well understood. Therefore, to understand the clinicopathological significance of infiltrated M2 TAMs in ESCC, statistical analysis was performed after immunohistochemical evaluation of CD163 expression, a well-accepted surface marker of M2 TAMs in ESCC. To gain insight into the effect of M2 TAMs, ESCC cell lines Eca109 and KYSE150 cells were co-cultured with M2 TAMs artificially induced from THP-1 cells. The variations in the proliferation, migration and invasion were assessed using the MTT, wound-healing and Transwell assays, respectively. The variation in the typical biomarkers of the epithelial-mesenchymal transition (EMT) was evaluated using western blotting. Infiltrated M2 TAMs were confirmed to predominate in the stroma of ESCC relative to normal controls. Moreover, it turned out that M2 TAMs were shown to promote the migration and invasion of ESCC cells but not proliferation. Furthermore, M2 TAMs were observed to induce EMT in ESCC cells. Together, our results showed that infiltrated M2 TAMs in the stroma is a feature accompanying ESCC metastasis and that M2 TAMs can promote the migration and invasion, but not proliferation, of ESCC cells, thereby inducing EMT. Thus, M2 TAMs could be an alternative therapeutic target in ESCC.

Key words: Oesophageal squamous cell carcinoma (ESCC), M2 macrophage, Invasion, Migration, Epithelial-mesenchymal transition (EMT)

Offprint requests to: Dr. Xiaomei Lu, Tumor Hospital Affiliated to Xinjiang Medical University, State Key Laboratory of Pathogenesis, Prevention, Treatment of Central Asian High Inicidence Dieases, Urumqi 830000, Xinjiang Uygur Autonomous Region, PR China. e-mail: luxiaomei88@163.com

DOI:10.14670/HH-18-061

Introduction

Oesophageal cancer (EC) is one of the most common cancers in the world and the fourth leading cause of cancer-related death in China (Chen et al., 2016). Oesophageal squamous cell carcinoma (ESCC) is the main histopathologic subtype (Rustgi and El-Serag, 2014). Despite the advancement in surgical techniques and neoadjuvant therapy of ESCC, the 5-year prognosis of patients with ESCC remains poor, and most patients die of metastasis (Niwa et al., 2016). Consequently, elucidation of the molecular mechanism underlying the metastasis of ESCC will be crucial to improve the clinical outcome of ESCC patients.

The tumour microenvironment is the local pathological environment that has been reported to play critical roles in tumour invasion and metastasis and has become a focus in cancer research (Lin et al., 2016). Tumour-associated macrophages (TAMs), the major

component of the immune cell reaction (Hatogai et al., 2016) reside in the tumour microenvironment, have been found to be involved in tumour initiation, progression and prognosis (Zhu et al., 2017). Considering the heterogeneity of macrophages, TAMs are classically divided into two different subtypes (Hu et al., 2016; Rhee, 2016)-M1 and M2. M1 macrophages are usually regarded as playing a tumour-suppressor role, whereas M2 macrophages are reported to promote tumour-enhancing migration and invasion. Several lines of evidence have revealed that the increased density of infiltrated M2 TAMs in tumours is correlated with a poor prognosis in many cancers, including ESCC (Hu et al., 2017) and that M2 TAMs could increase tumour growth and metastasis (Shigeoka et al., 2013). However, the role of M2 TAMs in ESCC remains less well unknown.

In our present study, to better understand the clinicopathological significance of the infiltrated of M2 TAMs in ESCC, immunohistochemistry was performed with the surface marker CD163, a well-accepted specific marker of M2 TAMs in our own cases. Infiltrated M2 TAMs were demonstrated to be predominantly present in the stroma of ESCC compared with those in matched normal controls. Clinicopathologically, infiltrated M2 macrophages in the stroma were significantly correlated with metastasis and a poor survival of ESCC. Moreover, M2 TAMs were shown to promote the migration and invasion of ESCC cells, but not proliferation, inducing EMT in ESCC cells.

Materials and methods

Clinical samples

One hundred twenty cases of ESCC and paired normal control were enrolled including one tissue microarray comprising 90 paired cases of ESCC and matched normal adjacent tissues from Shanghai Outdo Biotech Co. Ltd. (Shanghai, China). Another additional independent 30 cases of ESCC who underwent operation were retrieved from the archives at the Department of Pathology of the First Affiliated Hospital of Xinjiang Medical University. Staging and grading were performed in accordance with the World Health Organization classification and grading system. None of the patients had received treatment before surgery. Both the carcinoma and its adjacent normal tissues were confirmed by two experienced pathologists who were blind to our study. Informed consent was obtained from each patient involved, and the study was approved by the Medical Ethics Committee of the Tumor Hospital Affiliated to Xinjiang Medical University.

Cell lines and cell culture

The ESCC cell line Eca109 with high differentiation potential was purchased from WuHan University (WuHan, China). KYSE150 with low differentiation potential was provided by the State Key Laboratory of Chinese Academy of Medical Sciences (Beijing, China). THP-1 cells (human acute monocytic leukaemia cell line) were purchased from Shanghai Cell Bank. Phorbol 12-myristate13-acetate (PMA), IL-4 and IL-13 were purchased from Sigma Chemical Company (St. Louis, MO, USA). The cells were cultured in RPMI-1640 (Thermo Fisher Scientific, Waltham, MA, USA) medium supplemented with 10% foetal bovine serum (FBS; HyClone, Logan, UT, USA), 100 units/ml of penicillin, and 100 μg/ml of streptomycin (Invitrogen, Carlsbad, CA, USA) and were maintained at 37°C in a humidified 5% CO_2 atmosphere.

Induction of M2 TAMs and co-culture with ESCC cells

The ESCC cell lines Eca109 and KYSE150 and THP-1-derived M2 TAMs were co-cultured using a cell culture insert (Corning, NY, USA) with a 0.4-μm porous membrane to separate the upper and lower chambers. The THP-1 monocytes (3×10^5 cells/ml), which were seeded into the upper chamber of the Transwell apparatus, were stimulated to differentiate into inactivated macrophages by the addition of 3.2×10^7 mol PMA (320 nmol/L; Sigma Chemical) for 24 h. To induce M2 TAMs, the inactivated macrophages were treated with IL-4 (20 ng/ml; Sigma Chemical) and IL-13 (20 ng/ml; Sigma Chemical) for 48 h. The Eca109 and KYSE150 cells were placed in the lower chamber at a density of 3×10^5 cells/ml for 24 h to allow their adherence to the walls. The chambers with the THP-1-derived M2 TAMs were then placed directly on top of the six-well plates containing the Eca109 and KYSE150 cells, and the co-culture systems were incubated for 24 h in serum-free RPMI 1640.

Immunohistochemistry (IHC)

Briefly, the tissue microarray was incubated at 60°C for 12 hours and then was de-paraffinized and dehydrated in gradient ethanol. The activity of endogenous peroxide was blocked in 3% H_2O_2 for 10 minutes. The antigen was retrieved in 0.01 M citrate buffer heated in a microwave oven at 98°C for 15 minutes and then was cooled at room temperature for 20 minutes. After washing in phosphate-buffered saline (PBS), the tissue microarray was incubated with normal serum to block nonspecific staining for 30 minutes. Monoclonal mouse anti-CD163 (1:200 dilution; Beijing Zhongshan Golden Bridge Biotechnology Co, Ltd) was incubated with the tissue section overnight at 4°C in a humidified chamber. After washing with PBS, the tissue section was treated with biotinylated anti-mouse secondary antibody, followed by further incubation with streptavidin-horseradish peroxidase complex and staining using the diaminobenzidine kit (Beijing Zhongshan Golden Bridge Biotechnology Co, Ltd). IHC staining was assessed in a series of randomly selected four fields at ×400 magnification.

Immunoscoring of infiltrated M2 TAMs

Four fields per section were randomly selected, and the infiltration of M2 TAMs was estimated by immunoscoring of CD163 expression by two separate clinical pathologists. The infiltration of M2 TAMs was classified into the following four categories: 0, absent (no positive cells); 1, weak infiltration (between 20 and 40 macrophages per mm^2); 2, moderate infiltration (between 40 and 60 macrophages per mm^2); 3, strong infiltration (>60 macrophages per mm^2). For statistical analysis, these categories were categorized into two groups, sparse (0-1) and dense (2-3) infiltration.

Immunofluorescence

The THP-1 monocytes were plated onto glass-bottomed cell culture dishes (NEST 801001). UaM incubated with anti-CD68 antibodies (1:200; Beijing Zhongshan Golden Bridge Biotechnology Co, Ltd) and M2 TAMs incubated with anti-CD163 antibodies (1:200; Beijing Zhongshan Golden Bridge Biotechnology Co, Ltd), anti-E-cadherin antibody (dilution at 1:1000; catalogue number: #3195, Cell Signaling Technology), anti-Vimentin antibody (dilution at 1:1000; catalogue number: #5741, Cell Signaling Technology) for 12 h at 4°C. After washing with PBS 3 times, the cells were incubated with Anti-Mouse IgG Fab2 Alexa Fluor 488 (Mouse)-conjugated secondary antibody (Cell Signaling Technology) for 2 h at room temperature in the dark. The nucleus was stained with 4', 6-diamidino-2-phenylindole (DAPI; Sigma, St. Louis, USA). After washing with PBS, the cell expression was observed and counted by fluorescent confocal microscope (Leica TCS SP8).

MTT

Eca109 and KYSE150 cells were co-cultured with THP-1-derived M2 TAMs were placed in a 96-well plate at a density of 3×10^3/well and were incubated overnight. At 0, 24, 48, 72, and 96 h, 20 μL (5 g/L) of MTT (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2-H-tetrazolium bromide) labelling reagent was added to the designated wells. After a 4 h incubation, the MTT formazan precipitate was dissolved in dimethylsulphoxide (DMSO) (150 μL/well, Sigma-Aldrich, St. Louis, MO, USA) in a shaker before reading the absorbance at 490 nm using a 96-well plate reader (Bio-Rad, Winooski, VT USA). The results were the average of the four wells. Non-co-cultured Eca109 and KYSE150 cells were used as the controls.

Wound healing assay

Co-cultured Eca109 and KYSE150 were seeded in 6-well plates. After overnight incubation, the cells were scraped with a 0.01-mL pipette tip to create a straight-line cell-free scratch. Migrating cells were photographed every 24 h. Each well was washed with PBS to remove the remaining unattached cells before imaging. Cell motility was quantified by measuring the distance between the migrating cell boundaries. Non-co-cultured Eca109 and KYSE150 cells were used as the controls.

Transwell assay

The invasion assays were conducted using a 24-well Transwell chamber with a polycarbonate membrane with a pore size of 8 μm (Corning, NY, USA). The membrane was coated with 60 μl of a 1:5 mixture of Matrigel (BD Sciences, San Jose, CA, USA) and serum-free RPMI 1640 medium to form a matrix barrier. After the Matrigel was allowed to solidify at 37°C for 2 h, Eca109 and KYSE150 cells (1×10^5 cells/ml) that had been co-cultured with THP-1-derived M2 TAMs in 200 μl of serum-free RPMI-1640 medium were added to the upper compartment of the chamber; the lower chamber was filled with 0.6 ml of medium supplemented with 10% FBS as a chemo-attractant. Untreated Eca109 and KYSE150 cells were added to the lower compartment of the chamber as control cells. After incubation at 37°C for 24 h, co-cultured and non-co-cultured cells were then rinsed with PBS and fixed in 100% methanol. The cells remaining at the top of the polycarbonate membrane were removed using a moist cotton-tipped swab. The cells that had migrated through pores to the lower surface were stained with haematoxylin solution and distilled water several times, and the cell numbers in six random fields at a magnification of ×200 were counted and averaged.

Western blotting

Western blotting was performed as described previously. Cells were lysed in RIPA lysis buffer. The protein concentrations were determined using the BCA protein quantitation assay (Bioteke, Beijing, China). Equal amounts of protein (80 μg) were separated on 10% SDS-PAGE gels and were transferred to a PVDF membrane (Millipore, Bedford, MA, USA). The membranes were blocked for 1 h using blocking buffer and then were probed overnight with an anti-E-cadherin antibody (1:1000; #3195; Cell Signaling Technology), anti-Vimentin antibody (1:1000; #5741; Cell Signaling Technology); anti-Snail antibody (1:1000; #3879; Cell Signaling Technology) and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (1:1000; Cell Signaling Technology) at 4°C. The membrane was washed three times for 5 min each time with washing buffer and was incubated with secondary antibody anti-rabbit IgG, HRP-linked antibody (1:10,000; Cell Signaling Technology) for 1.5 h. Proteins were detected using an ECL detection reagent.

Statistical analysis

The data were expressed as the means±standard

error of mean (SEM). Statistical analysis was carried out using SPSS 19.0 software (SPSS, Chicago, IL, USA). Independent sample T-test and one-way ANOVA were used for statistical analyses between groups of continuous variables that followed the normal distribution. Cross-table analysis was conducted for categorical variables. Kaplan-Meier survival analysis was performed for overall prognostic analyses. A value of $P<0.05$ was considered statistically significant.

Results

M2 TAMs are predominantly infiltrated in the stroma of ESCC relative to the normal control

To evaluate the specificity of the primary antibody, we carried out antigen pre-adsorption trials as suggested previously (Holmseth et al., 2012). The antigen pre-adsorption result showed that the specificity of the primary antibody to CD163 that was commercially available was adequate (data not shown). To investigate the infiltration of M2 TAMs in ESCC, IHC was performed using CD163, a specific marker of M2 TAMs. As shown in Fig. 1A, CD163-positive M2 TAMs were overwhelmingly infiltrated in the stroma of cancerous tissues. By contrast, only a few normal control cases occurred to infiltrate in the stroma for M2 TAMs (Fig. 1B). Regarding subcellular localization, immunostaining of infiltrated M2 TAMs was evident in both the membrane and cytoplasm.

Infiltrated M2 TAMs in the stroma is significantly correlated with lymph node metastasis, T classification and overall survival

Next, we sought to analyse the association between the extent to which M2 TAMs infiltrated and clinicopathological variables of ESCC. It was found that a high density of infiltrated M2 TAMs was shown to significantly correlate with lymph node metastasis and T classification. Nonetheless, no significant correlation

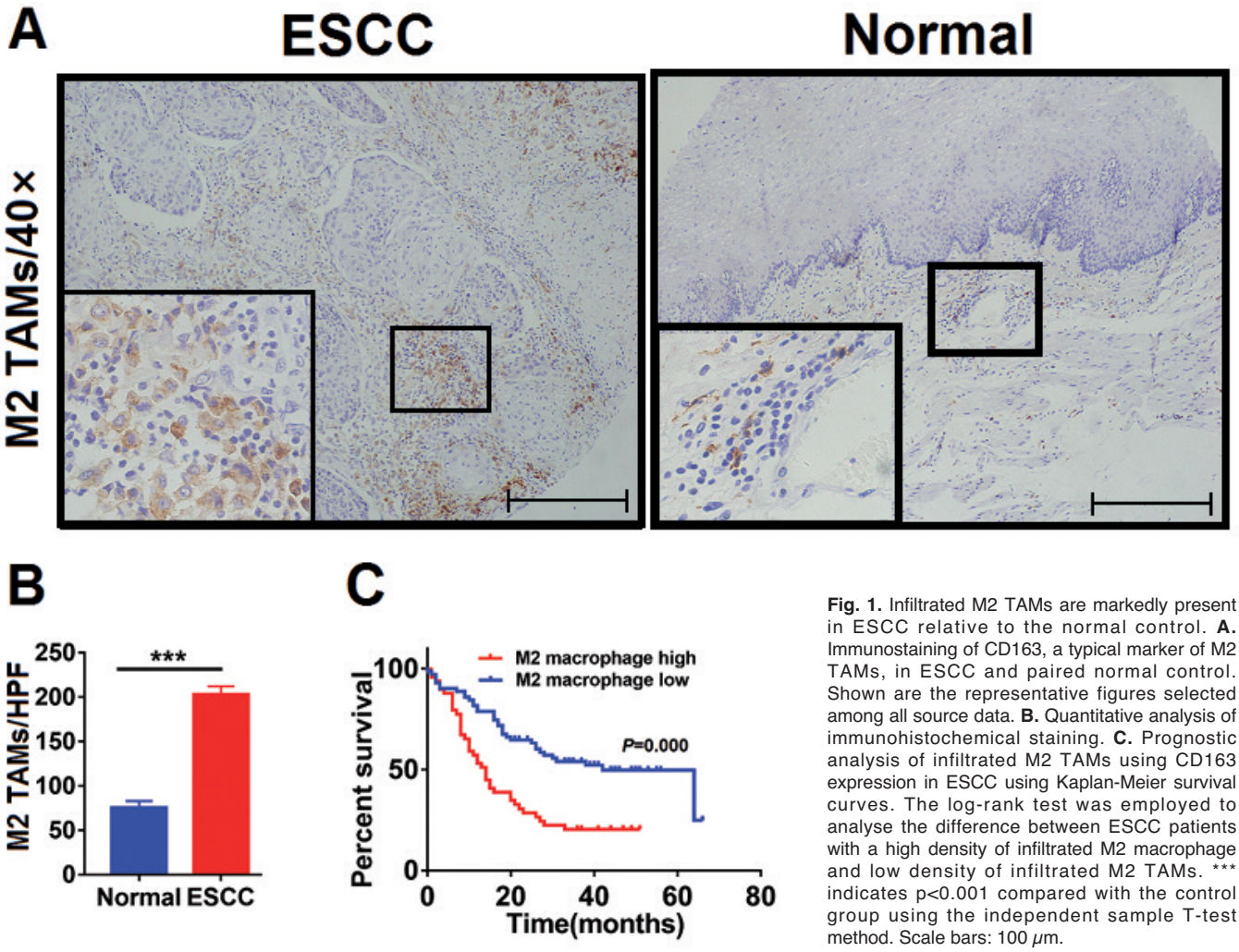

Fig. 1. Infiltrated M2 TAMs are markedly present in ESCC relative to the normal control. **A.** Immunostaining of CD163, a typical marker of M2 TAMs, in ESCC and paired normal control. Shown are the representative figures selected among all source data. **B.** Quantitative analysis of immunohistochemical staining. **C.** Prognostic analysis of infiltrated M2 TAMs using CD163 expression in ESCC using Kaplan-Meier survival curves. The log-rank test was employed to analyse the difference between ESCC patients with a high density of infiltrated M2 macrophage and low density of infiltrated M2 TAMs. *** indicates $p<0.001$ compared with the control group using the independent sample T-test method. Scale bars: 100 μm.

was observed between the density of infiltrated M2 TAMs and other clinicopathological variables, including gender, age, clinical stage, tumour diameter and gross classification (Table 1). The association of infiltrated M2 TAMs with overall survival (OS) for ESCC patients was analysed using the Kaplan-Meier survival curve with the log-rank test. As shown in Fig. 1C, patients with high infiltration of M2 TAMs had a shorter OS, whereas those with a low infiltration of M2 TAMs had a longer OS, with a statistically significant difference (log-rank, P<0.01).

Detection of the immunofluorescence of M2 TAMs induced from THP-1 cells

To gain insight into the role of M2 TAMs, we attempted to artificially induce M2 TAMs from THP-1 cells as previously reported (Yamaguchi et al., 2016, 2017). THP-1 cells presented a small and round morphology and grew in suspension as single cells or partly in clusters (Fig. 2A). The THP-1 cells were differentiated into inactivated macrophages after stimulation with PMA for 24 h. During the differentiation process, the cell morphology changed from round to almost spindle shaped (Fig. 2A). Simultaneously, the cells became attached to the bottom of the culture flask and were increased in size. Next, the inactivated macrophages were further induced into M2 TAMs after further stimulation with IL-4 and IL-13 for 48 h. The cell morphology became irregular or spindle shaped, and even pseudopods can be observed in some cells (Fig. 2A). To identify the M2 TAMs we induced, immunofluorescence was performed. Given that almost all M2 TAMs concomitantly expressed CD68 and CD163, two well-established specific markers of M2 TAMs, we stained the THP-1-derived M2 TAMs with an anti-CD68 (Fig. 2B)

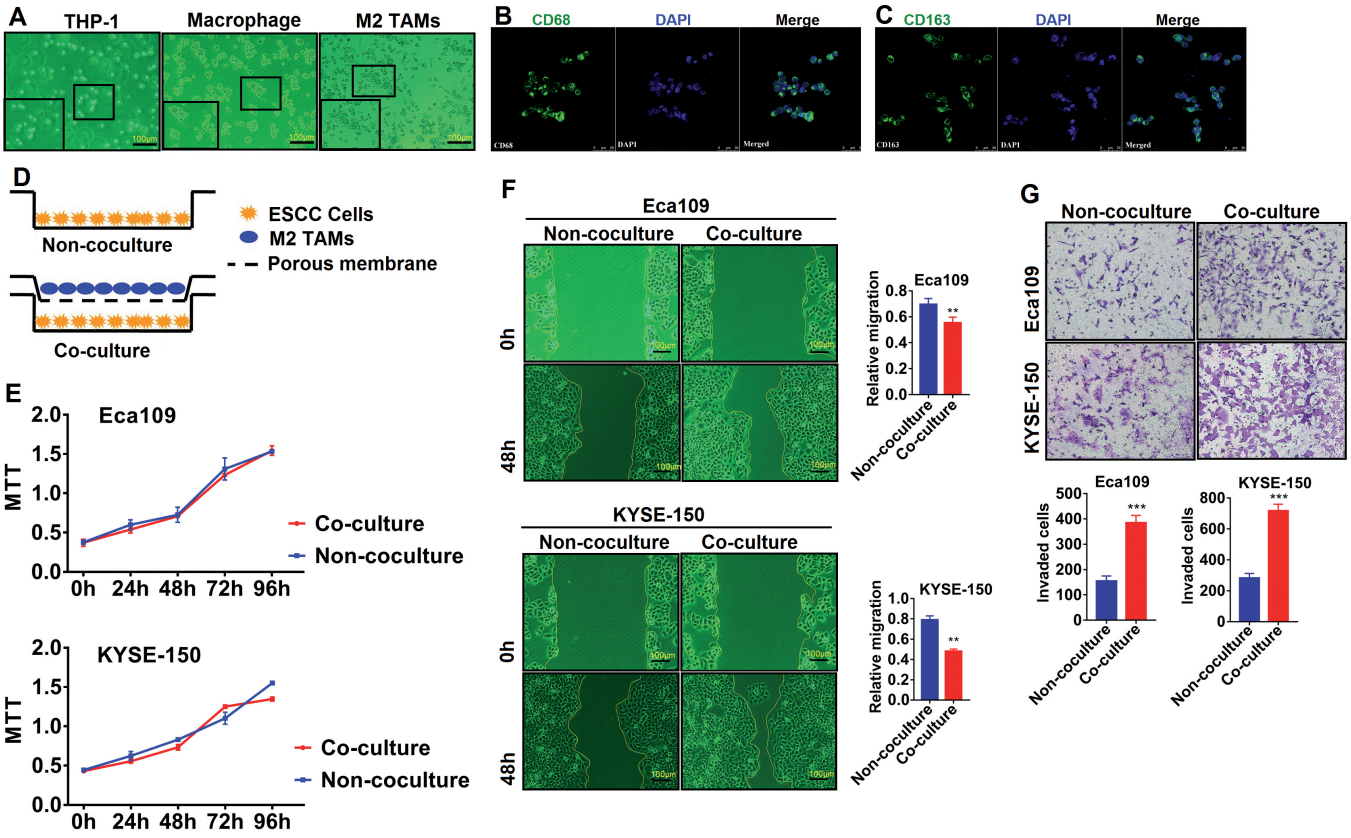

Fig. 2. M2 TAMs remarkably promote the migration and invasion, but not proliferation, of ESCC cells. **A.** Morphological variation of M2 TAMs induced from the human monocyte cell line THP-1 stimulated with PMA (macrophage), IL4 and IL13 (M2 TAMs) for 48 hours. **B.** Identification of the induced macrophages using immunofluorescence staining of CD68 (green colour), a well-accepted cell surface marker of pan-macrophages; the nucleus was stained blue with DAPI. **C.** Similarly, identification of the induced M2 TAMs using immunofluorescence staining of CD163, a well-accepted cell surface marker of M2 TAMs (the fold magnification was ×200, and the zoom factor was 10.50 when captured using laser confocal microscopy). **D.** Schema of the in vitro cell co-culture system. **E.** M2 TAMs showed little effect on the proliferation of ESCC cells, as evidenced by the MTT assay. **F.** M2 TAMs markedly promoted the migration of ESCC cells, as revealed by the wound-healing assay. **G.** M2 TAMs pronouncedly promoted the invasion of ESCC cells, as evidenced by the Transwell assay. All experiments were performed independently in triplicate, and representative figures selected among the candidates from three independent experiments are shown. ** p<0.01, *** p<0.001 compared with the control group using the independent sample T-test method.

and anti-CD163 antibodies (Fig. 2C). As expected, we obtained and identified successfully M2 TAMs that were induced from THP-1 cells.

M2 TAMs promote the migration and invasion, but not proliferation, of ESCC cells in vitro

To understand the effect of M2 TAMs on cell migration, we established an *in vitro* cell co-culture system as reported previously (Fig. 2D). First, to observe the proliferative variation of Eca109 and KYSE150 cells, the MTT assay was performed. As shown in Fig. 2E, it turned out that co-culture with M2 TAMs had little effect on the proliferation of both Eca109 and KYSE150 cells. Next, to assess the migratory variation of co-culture with M2 TAMs, the wound-healing assay was carried out. After co-culture with M2 TAMs for 48 h, the scrape was significantly narrower than that in the non-coculture group in both Eca109 and KYSE150 cells (Fig. 2F), suggesting that M2 TAMs can enhance the migration of both Eca109 cell and KYSE150 cells. To observe whether co-culture with M2 TAMs could demonstrate a difference in the invasive ability of ESCC cells, the Transwell assay was carried out. As shown in Fig. 2G, the cell number of the group co-cultured with M2 TAMs cells that passed through was significantly greater than that of the non-cocultured group (Fig. 2G) in Eca109 and KYSE150 cells, indicating that M2 TAMs can promote the invasion of Eca109 and KYSE150 cells.

M2 TAMs can induce the EMT process

Based on previous studies that M2 TAMs can induce the EMT process in cancers (Liu et al., 2013; Dehai et

Table 1. Correlation between CD163 expression and clinicopathological characteristics of ESCC.

Characteristics	n	CD163 expression		X^2	P
		high (++,+++)	low (-,+)		
Type					
ESCC	120	69	51	30.625	0.000
Adjacent normal tissues	120	27	93		
Gender					
male	93	53	40	1.326	0.249
female	27	12	15		
Age (y)					
>60	79	43	36	0.03	0.862
≤60	41	23	18		
Clinical stage					
I	18	8	10	1.15	0.563
II	74	32	42		
III	28	9	19		
T classification					
T1-T2	43	18	25	5.293	0.021
T3-T4	72	46	26		
Lymph node metastasis					
N0	64	25	39	5.602	0.018
N1-N3	56	34	22		
Tumor diameter (cm)					
≤5	63	28	35	0.017	0.898
>5	57	26	31		
Gross classification					
Medullary type	33	15	18	0.883	0.829
Fungating type	22	8	14		
Ulcerative type	36	15	21		
Protrude type	26	9	17		

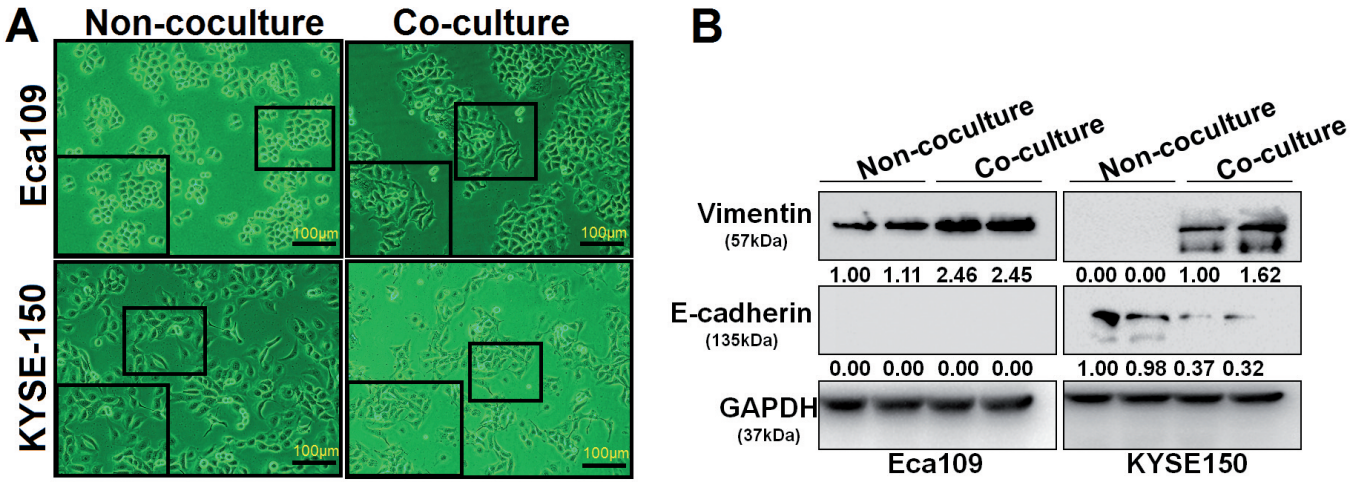

Fig. 3. M2 TAMs can induce the epithelial-mesenchymal transition (EMT) of ESCC cells *in vitro*. **A.** Morphological variation of ESCC cell lines Eca109 and KYSE-150 before and after co-culture with M2 TAMs. The fold magnification was 100 for the whole and inset. **B.** Variation in the protein expression of EMT biomarkers of ESCC cell lines Eca109 and KYSE-150 using immunoblotting before and after co-culture with M2 TAMs. All experiments involved were performed independently in triplicate, and representative figures selected from among candidate images from at least three independent experiments are shown.

al., 2014), we confirmed whether M2 TAMs could do so in ESCC. Initially, Eca109 and KYSE150 exhibited a typical cuboid epithelial morphology with tight cell-to-cell contacts. After co-culture with M2 TAMs for 48 h, the cell morphology of both Eca109 and KYSE150 was remarkably changed, with the morphology changing from pebble like or cuboid shaped to fibroblast like or spindle shaped, and the cell-to-cell contacts became less intense (Fig. 3A), suggesting that the Eca109 and KYSE150 cells could transit from the epithelial to mesenchymal state. To further confirm the phenomenon, the variation in the protein expression of typical epithelial (E-cadherin) and mesenchymal (Vimentin) biomarkers before and after co-culture with the induced M2 TAMs was detected using western blotting. The results showed that, because E-cadherin was missing in Eca109 cells, E-cadherin was seldom detectable. However, the typical mesenchymal marker Vimentin was markedly upregulated after co-culture compared with that in control Eca109 cells (Fig. 3B). Additionally, in KYSE150, the basal level of Vimentin was undetectable; thus, the variation in Vimentin expression was sharp from undetectable to appreciable similar to the trend observed with the E-cadherin level, which was remarkably reduced compared with that of the control (Fig. 3B), fundamentally corroborating the morphology differences shown in Fig. 3A. As further confirmation, immunofluorescence (IF) staining was employed and indicated that the expression variation of EMT markers, E-cadherin and Vimentin was consistent with that observed by western blotting (Fig. 4), demonstrating that M2 TAMs could induce EMT in ESCC cells.

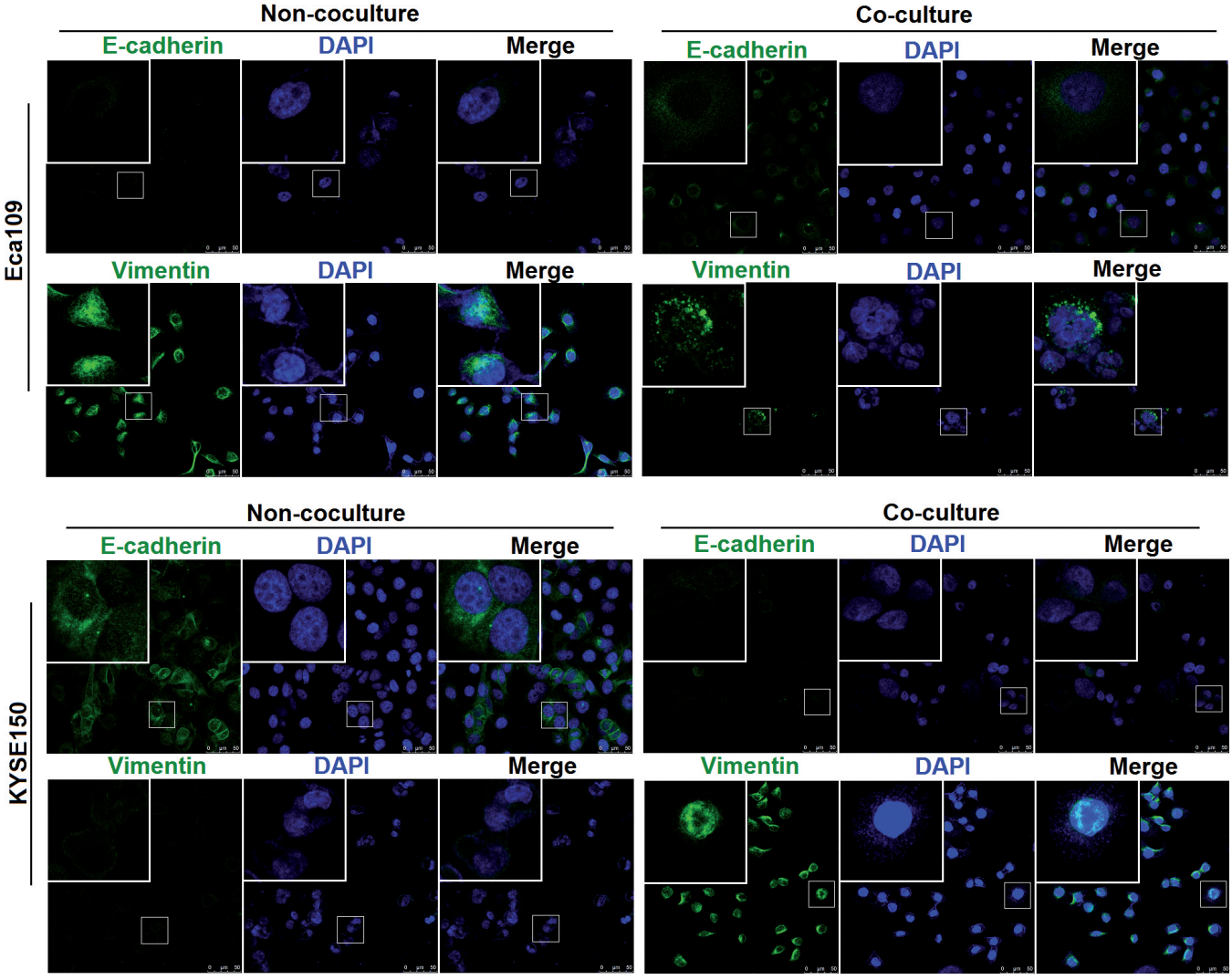

Fig. 4. Confirmation of the variation of EMT of ESCC cells using immunofluorescence staining. The variation of the expression of E-cadherin and Vimentin in Eca109 and KYSE150 co-cultured with or without M2 TAMs was confirmed using immunofluorescence staining. Representative figures selected from among candidate images are shown. Scale bars: 50 µm.

Discussion

In the present study, we showed that infiltrated M2 TAMs were overwhelmingly present in the stroma of ESCC relative to paired normal controls and that the density of the infiltrated M2 TAMs was significantly correlated with lymph node metastasis, T classification and inferior overall survival of ESCC. In the in vitro cell co-culture system, M2 TAMs could promote the invasion and migration, but not proliferation, of ESCC cells. Additionally, we confirmed that M2 TAMs can induce the EMT process in ESCC cells.

The role M2 TAMs has been extensively reported in different types of cancers, including breast cancer (Yang et al., 2016), pancreatic cancer (Liu et al., 2013), and gastric cancer (Yamaguchi et al., 2016). By contrast, limited literature exists regarding M2 TAMs in ESCC (Gao et al., 2014; Sugimura et al., 2015; Hu et al., 2017). Therefore, both the expression and its clinicopathological significance was relatively less well understood in ESCC than in other cancers that were extensively reported. Thus, we confirmed the role of M2 TAMs in ESCC in our own cases. Because the M2 TAMs displayed high expression of CD204 (Shigeoka et al., 2013) and CD163 (Hu et al., 2017), in our present study, we chose CD163 as the detection marker of M2 TAMs in ESCC. Previous studies regarding M2 TAMs in ESCC (Gao et al., 2014; Sugimura et al., 2015; Hu et al., 2017), although from different study groups, have consistently reported that the infiltrated M2 TAMs in the stroma were significantly associated with lymph node metastasis and poor overall prognosis, a finding that was in agreement with our findings that infiltrated M2 TAMs overwhelmingly presented in the stroma of ESCC compared with those in paired normal controls. Additionally, the density of the infiltrated M2 TAMs was significantly associated with lymph node metastasis, T classification and inferior overall survival of patients with ESCC, confirming that the extent to which M2 TAMs infiltrated into the stroma of ESCC can be a feature of the poor prognosis and metastasis of ESCC.

Having understood the clinicopathological significance of the infiltrated M2 TAMs in ESCC, we extended the investigation from in vivo to in vitro ESCC cells. Several lines of evidence have revealed that THP-1 cells can be artificially induced to M2-like macrophages (Yamaguchi et al., 2016, 2017) displaying concomitantly the positive expression of CD163 and CD68, general indicators of macrophages. In our present study, we reproduced successfully the induction of M2 TAMs from THP-1 cells following those previous reports (Yamaguchi et al., 2016, 2017). The results from wound-healing and Transwell assays revealed that M2 TAMs can promote the migratory and invasive abilities of ESCC cells in our co-culture system established as previously reported. Nevertheless, it turned out that M2 TAMs had little effect on the proliferation of ESCC cells, which greatly differed from earlier reports (Fu et al., 2015; Yamaguchi et al., 2016) that M2 TAMs could promote the growth of cancer cells after co-culture. The discrepancy remains unclear and might be due to the technical limitation in our experimental setting-that is, the effect exerted by M2 TAMs over proliferation was rather subtle and could not be significantly detected using the MTT approach. Additionally, the different cell co-culture system that we and others have established and used in vitro might have caused the different outcome. Accordingly, it was not surprising that our observation on proliferation differed from those of previous reports. However, the result that M2 TAMs had a negligible effect on proliferation could further rule out the potential artificial effects that probably existed in the migration and invasion of ESCC cells that could be caused due to their proliferative effects.

Considering the relationship between M2 TAMs and EMT established by relevant earlier studies, we tried to observe whether M2 TAMs could also induce EMT (Dehai et al., 2014) in ESCC cells. Initially, we provided morphological evidence that was strongly indicative of EMT occurring in ESCC cells. As further supporting biochemical evidence, we subsequently detected the variation in the protein expression of two typical markers associated with EMT, the epithelial marker E-cadherin and mesenchymal marker Vimentin, using western blotting. The date from western blotting corroborated what has been morphologically presented after co-culture with M2 TAMs, showing that the Vimentin level was markedly upregulated whereas the E-cadherin level was significantly reduced after co-culture with M2 TAMs relative to the control group. Our morphological and biochemical evidence was highly concordant and fully supported what has been previously reported for other cancers (Dehai et al., 2014) that is, M2 TAMs could induce EMT. Notably, in the western blotting analysis of EMT biomarkers E-cadherin and Vimentin in Eca109 and KYSE0-150 cells, we observed that in Eca109 cells (an extensively used ESCC cell lines in the study of ESCC), the endogenous level of E-cadherin was negligible before co-culture with M2 macrophages induced from THP-1 cells, thus enabling the detection of decreasing trend of E-cadherin of Eca109 after being co-cultured with M2 macrophages. Our results mentioned here were fully supported by the data (Fang et al., 2013; Zhang et al., 2015) from Li Enmin's studying group wherein they also found E-cadherin was almost undetectable in Ecal09 cells that they stored and used. However, our results strikingly contrasted those from Li Feng's serial reports (Pang et al., 2014, 2016) in which the basal level of E-cadherin was demonstrated to be normally detectable in Eca109 cells without undergoing any interference or treatment. The cause might be due to the same cell line used in different studying groups or laboratories showed variation (Korch et al., 2012; Capes-Davis et al., 2013a,b) to a certain extent, leading to the final labile results. Consequently, authentication of cell lines used or to be used is necessary in future studies.

Although we confirmed the phenomenon that M2

TAMs can promote migration and invasion, but not proliferation, in ESCC cells in our setting, the underlying molecular mechanism remains largely unknown and deserves to be further investigated. According to the suggestion given by the mechanistic studies available regarding M2 TAMs in cancer, the role exerted on cancer cells could be indirect-that is, it may be attributed to the cytokine/chemokine released from M2 TAMs (Liu et al., 2013; Mohamed et al., 2014). Consequently, additional studies are required to determine which cytokine/chemokine can be secreted by M2 TAMs. Taken together, our study confirmed that M2 TAMs can promote the migration and invasion, but not proliferation, of ESCC cells, to induce the EMT process, supporting that targeting M2 TAMs could be an alternative novel strategy in the cure of ESCC with metastasis.

Funding. This study was supported by the Natural Science Foundation of China (No. U1603284, 81260359, and U1303321), Science and Technology Aid Xinjiang Plan of the Xinjiang Uygur Autonomous Region (No.2018E02067), Major Science and Technology Projects of the Xinjiang Uygur Autonomous Region (No. 201430123-1), and State Key Lab Incubation Base of Xinjiang Major Diseases Research (SKLIB-XJMDR-2016).

Conflict of interest. All the authors declare that they have no conflict of interest.

Ethical approval. This study was approved by the Ethical Committee of the Tumor Hospital Affiliated to Xinjiang Medical University. All procedures in studies involving human participants were performed in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent. Informed consent was obtained from all individual participants included in the study.

References

Capes-Davis A., Alston-Roberts C., Kerrigan L., Reid Y.A., Barrett T., Burnett E.C., Cooper J.R., Freshney R.I., Healy L., Kohara A., Korch C., Masters J.R., Y. Nakamura Y., Nims R.W., Storts D.R., Dirks W.G., MacLeod R.A. and Drexler H.G. (2013a). Beware imposters: MA-1, a novel MALT lymphoma cell line, is misidentified and corresponds to Pfeiffer, a diffuse large B-cell lymphoma cell line. Genes Chromosomes Cancer 52, 986-988.

Capes-Davis A., Reid Y.A., Kline M.C., Storts D.R., Strauss E., Dirks W.G., Drexler H.G., MacLeod R.A., Sykes G., Kohara A., Nakamura Y., Elmore E., Nims R.W., Alston-Roberts C., Barallon R., Los G.V., Nardone R.M., Price P.J., Steuer A., Thomson J., Masters J.R. and Kerrigan L. (2013b). Match criteria for human cell line authentication: where do we draw the line? Int. J. Cancer 132, 2510-2519.

Chen W., Zheng R., Baade P.D., Zhang S., Zeng H., Bray F., Jemal A., Yu X.Q. and He J. (2016). Cancer statistics in China, 2015. CA Cancer J. Clin. 66, 115-132.

Dehai C., Bo P., Qiang T., Lihua S., Fang L., Shi J., Jingyan C., Yan Y., Guangbin W., and Zhenjun Y. (2014). Enhanced invasion of lung adenocarcinoma cells after co-culture with THP-1-derived macrophages via the induction of EMT by IL-6. Immunol. Lett. 160, 1-10.

Fang W.K., Liao L.D., Li L.Y., Xie Y.M., Xu X.E., Zhao W.J., Wu J.Y., Zhu M.X., Wu Z.Y., Du Z.P., Wu B.L., Xie D., Guo M.Z., Xu L.Y. and Li E.M. (2013). Down-regulated desmocollin-2 promotes cell aggressiveness through redistributing adherens junctions and activating beta-catenin signalling in oesophageal squamous cell carcinoma. J. Pathol. 231, 257-270.

Fu X., Shi H., Qi Y., Zhang W. and Dong P.. (2015). M2 polarized macrophages induced by CSE promote proliferation, migration, and invasion of alveolar basal epithelial cells. Int. Immunopharmacol. 28, 666-674.

Gao J., Wu Y., Su Z., Amoah Barnie P., Jiao Z., Bie Q., Lu L., Wang S. and Xu H. (2014). Infiltration of alternatively activated macrophages in cancer tissue is associated with MDSC and Th2 polarization in patients with esophageal cancer. PLoS One 9, e104453.

Hatogai K., Kitano S., Fujii S., Kojima T., Daiko H., Nomura S., Yoshino T., Ohtsu A., Takiguchi Y., Doi T. and Ochiai A. (2016). Comprehensive immunohistochemical analysis of tumor microenvironment immune status in esophageal squamous cell carcinoma. Oncotarget 7, 47252-47264.

Holmseth S., Zhou Y., Follin-Arbelet V.V., Lehre K.P., Bergles D.E. and Danbolt N.C. (2012). Specificity controls for immunocytochemistry: the antigen preadsorption test can lead to inaccurate assessment of antibody specificity. J. Histochem. Cytochem. 60, 174-187.

Hu W., Li X., Zhang C., Yang Y., Jiang J. and Wu C. (2016). Tumor-associated macrophages in cancers. Clin. Transl. Oncol. 18, 251-258.

Hu J.M., Liu K., Liu J.H., Jiang X.L., Wang X.L., Chen Y.Z., Li S.G., Zou H., Pang L.J., Liu C.X., Cui X.B., Yang L., Zhao J., Shen X.H., Jiang J.F., Liang W.H., Yuan X.L. and Li F. (2017). CD163 as a marker of M2 macrophage, contribute to predicte aggressiveness and prognosis of Kazakh esophageal squamous cell carcinoma. Oncotarget 8, 21526-21538.

Korch C., Spillman M.A., Jackson T.A., Jacobsen B.M., Murphy S.K., Lessey B.A., Jordan V.C. and Bradford A.P. (2012). DNA profiling analysis of endometrial and ovarian cell lines reveals misidentification, redundancy and contamination. Gynecol. Oncol. 127, 241-248.

Lin E.W., Karakasheva T.A., Hicks P.D., Bass A.J. and Rustgi A.K. (2016). The tumor microenvironment in esophageal cancer. Oncogene 35, 5337-5349.

Liu C.Y., Xu J.Y., Shi X.Y., Huang W., Ruan T.Y., Xie P. and Ding J.L. (2013). M2-polarized tumor-associated macrophages promoted epithelial-mesenchymal transition in pancreatic cancer cells, partially through TLR4/IL-10 signaling pathway. Lab. Invest. 93, 844-854.

Mohamed M.M., El-Ghonaimy E.A., Nouh M.A., Schneider R.J., Sloane B.F. and M. El-Shinawi M. (2014). Cytokines secreted by macrophages isolated from tumor microenvironment of inflammatory breast cancer patients possess chemotactic properties. Int. J. Biochem. Cell Biol. 46, 138-147.

Niwa Y., Koike M., Hattori M., Iwata N., Takami H., Hayashi M., Tanaka C., Kobayashi D., Kanda M., Yamada S., Fujii T., Nakayama G., Sugimoto H., Fujiwara M. and Kodera Y. (2016). The prognostic relevance of subcarinal lymph node dissection in esophageal squamous cell carcinoma. Ann. Surg. Oncol. 23, 611-618.

Pang L., Li Q., Li S., He J., Cao W., Lan J., Sun B., Zou H., Wang C., Liu R., Wei C., Wei Y., Qi Y., Hu J., Liang W., Zhang W.J., Wan M. and Li F. (2016). Membrane type 1-matrix metalloproteinase induces

epithelial-to-mesenchymal transition in esophageal squamous cell carcinoma: Observations from clinical and in vitro analyses. Sci. Rep. 6, 22179.

Pang L., Li Q., Wei C., Zou H., Li S., Cao W., He J., Zhou Y., Ju X., Lan J., Wei Y., Wang C., Zhao W., Hu J., Jia W., Qi Y., Liu F., Jiang J., Li L., Zhao J., Liang W., Xie J. and Li F. (2014). TGF-beta1/Smad signaling pathway regulates epithelial-to-mesenchymal transition in esophageal squamous cell carcinoma: in vitro and clinical analyses of cell lines and nomadic Kazakh patients from northwest Xinjiang, China. PLoS One 9, e112300.

Rhee I. (2016). Diverse macrophages polarization in tumor microenvironment. Arch. Pharm. Res. 39, 1588-1596.

Rustgi A.K. and El-Serag H.B. (2014). Esophageal carcinoma. N. Engl. J. Med. 371, 2499-2509.

Shigeoka M., Urakawa N., Nakamura T., Nishio M., Watajima T., Kuroda D., Komori T., Kakeji Y., Semba S. and Yokozaki H. (2013). Tumor associated macrophage expressing CD204 is associated with tumor aggressiveness of esophageal squamous cell carcinoma. Cancer Sci. 104, 1112-1119.

Sugimura K., Miyata H., Tanaka K., Takahashi T., Kurokawa Y., Yamasaki M., Nakajima K., Takiguchi S., Mori M. and Doki Y. (2015). High infiltration of tumor-associated macrophages is associated with a poor response to chemotherapy and poor prognosis of patients undergoing neoadjuvant chemotherapy for esophageal cancer. J. Surg. Oncol. 111, 752-759.

Yamaguchi T., Fushida S., Yamamoto Y., Tsukada T., Kinoshita J., Oyama K., Miyashita T., Tajima H., Ninomiya I., Munesue S., Harashima A., Harada S., Yamamoto H. and Ohta T. (2016). Tumor-associated macrophages of the M2 phenotype contribute to progression in gastric cancer with peritoneal dissemination. Gastric Cancer 19, 1052-1065.

Yamaguchi T., Fushida S., Yamamoto Y., Tsukada T., Kinoshita J., Oyama K., Miyashita T., Tajima H., Ninomiya I., Munesue S., Harashima A., Harada S., Yamamoto H. and Ohta T. (2017). Low-dose paclitaxel suppresses the induction of M2 macrophages in gastric cancer. Oncol. Rep. 37, 3341-3350.

Yang M., Ma B., Shao H., Clark A.M. and Wells A. (2016). Macrophage phenotypic subtypes diametrically regulate epithelial-mesenchymal plasticity in breast cancer cells. BMC Cancer 16, 419.

Zhang H.F., Alshareef A., Wu C., Li S., Jiao J.W., Cao H.H., Lai R., Xu L.Y. and Li E.M. (2015). Loss of miR-200b promotes invasion via activating the Kindlin-2/integrin beta1/AKT pathway in esophageal squamous cell carcinoma: An E-cadherin-independent mechanism. Oncotarget 6, 28949-28960.

Zhu Y., Li M., Bo C., Liu X., Zhang J., Li Z., Zhao F., Kong L. and Yu J. (2017). Prognostic significance of the lymphocyte-to-monocyte ratio and the tumor-infiltrating lymphocyte to tumor-associated macrophage ratio in patients with stage T3N0M0 esophageal squamous cell carcinoma. Cancer Immunol. Immunother. 66, 343-354.

Accepted November 12, 2018.