HLA-DR regulates macrophage phenotypic transformation and affects malignant behavior in esophageal squamous cell carcinoma

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Research

Keywords: esophageal squamous cell carcinoma, tumor microenvironment, tumor-associated macrophages, phenotypic transformation, HLA-DR

Posted Date: August 3rd, 2020

DOI: https://doi.org/10.21203/rs.3.rs-49282/v1

Abstract

Background Tumor-associated macrophages (TAMs) are an important immune cell component of the tumor microenvironment. This study aimed to explore the molecular mechanism of TAMs phenotype transformation and the role in the development of esophageal squamous cell carcinoma (ESCC).

Methods Co-culture conditions were employed to determine the phenotypic effects of TAMs on ESCC cell biological behavior. Tumor metastasis related molecules VEGF-C and MMP-9 produced by TAMs was evaluated by qRT-PCR and western blot. Expression of HLA-DR was knocked down in TAMs in vitro to determine the effects on macrophage polarization and the biological behavior of ESCC. We determined whether co-injection with M2 TAMs and macrophages depletion affected tumor growth in vivo tumor challenge model. Associations between HLA-DR, TAM density, and clinical outcomes were evaluated in patients with ESCC.

Results TAMs in ESCC samples were found to closely reflect the M2 phenotype of TAMs, and exhibited low expression of HLA-DR. Which was involved in ESCC tumor invasion and metastasis. Low expression of HLA-DR positively correlated with high-density of M2 TAMs, indicating high invasiveness and poor prognosis in patients with ESCC. Downregulation of HLA-DR in TAMs led to additional M2-type TAM polarization and more VEGF-C and MMP-9 secretion, promoted the malignant transformation of ESCC.

Conclusions These results demonstrate that downregulation of HLA-DR promote the transformation of M2 TAMs, and participate in the invasion and metastasis of ESCC.

Background

The aggressive invasion and high metastatic capacity of esophageal squamous cell carcinoma (ESCC) are important factors contributing to the poor prognosis of this disease [1]. The occurrence and development of tumor from the participation of a series of complex factors, and the role of the surrounding tumor microenvironment is crucial [2].The tumor microenvironment of ESCC contains various inflammatory and immune cells, and tumor associated macrophages (TAMs) comprise the main component of the immune cell compartment [3]. TAMs can induce epithelial–mesenchymal transition (EMT) in ESCC cells to promote tumor invasion and metastasis [4–6]. Increased TAMs density in tumor tissue is a poor prognostic factor in patients with ESCC [7]. Macrophages can differentiate into two polarized states according to their function and phenotype: tumor-supportive (M2 type) macrophages and tumor suppressive (M1 type) macrophages [8]. Macrophages have functional plasticity and can alter their functional profiles in response to environmental stimuli. For example, when macrophages are exposed to lipopolysaccharide (LPS) or gamma interferon (IFN-γ), they are polarized to the M1 phenotype and exhibit antitumor activities. However, exposure of macrophages to Th2 cytokines, such as interleukin-4 (IL-4) and IL-13, results in polarization to an M2 phenotype [9]. Anti-CD47 treatment alone can shift the phenotype of macrophages toward an M1 subtype *in vivo*[10]. The regulation of macrophage phenotypes is the key to the role of macrophages in promoting or suppressing tumor progression. However, the molecular signals that regulate these phenotypes and the corresponding regulatory mechanisms remain to be elucidated.

The expression of MHC class II proteins (human leukocyte antigen class II, HLA-II) on antigen-presenting cells (APCs) promotes CD4 + T-helper-mediated activation of B cells and cytotoxic CD8 + T cells [11]. Macrophages are a classic type of APC cells. Studies have shown that there is a significant negative correlation between the expression of HLA class II molecules (HLA-DR, DP, DQ) and CD68 + CD163 + TAMs, and low expression of HLA-II and high-density TAMs are associated with poor prognosis of cancer patients[12, 13]. HLA-DR as an important antigen of HLA-II gene, is associated with the immune response to tumor cells, which can lead to poor outcome of tumor patients [14]. Suggested that HLA-DR is involved in the regulation of tumor progression, which may be mediated by TAMs, but the regulatory effect and mechanism have not been reported. In the present study, we evaluated the effects of HLA-DR in TAMs polarization and the impact of it on malignant behavior of ESCC.

Results

TAMs promoted migration, and invasion of ESCC

To understand the role of TAMs in the progression of ESCC, we co-cultured macrophages (PMA-treated THP-1) with ESCC cells (EC109 and EC9706). Compared with ESCC cells cultured alone, co-culture of ESCC with macrophages for 36 h altered cell morphology from the original round, oval-shaped morphology with epithelial characteristics to an elongated circle-shaped or spindle-shaped mesenchymal morphology, with enlarged gaps between cells, diffuse growth, and decreased adhesion (Fig. 1a). CCK8 and plate cloning experiments showed that TAMs did not significantly increase the proliferative capacity of ESCC cells (Fig. 1b–c). However, transwell assay with migration and invasion experiments demonstrated that TAMs significantly promote the migration and invasion of ESCC cells (Fig. 1d–e).

To interrogate the role of TAMs infiltration in ESCCs *in vivo*, we applied nude mice tumor formation experiment and human tissue for analysis. Tumor formation experiments in nude mice showed that the volume and weight of tumors from ESCC cells with chlorophosphate-depleted macrophages were significantly smaller than untreated group (Fig. 1f–h). We used human ESCC tissues for further verification, and found that increased number of tumor-infiltrating CD68 + TAMs was closely related to lymphatic invasion, lymph node metastasis, and clinical progression of ESCC ($P < 0.05$, Fig. 1i). It suggested that there is a significant correlation between TAMs and tumor invasion and metastasis. In our previous research, we found that the VEGF-C in the VEGF family and MMP-9 in the tumor microenvironment have been proved possess a certain effect on ESCC metastasis[15].To further explore macrophage specific regulatory role on ESCC invasion, we evaluated expression of VEGF-C and MMP9 (considered to be key genes that promote tumor invasion and metastasis) in tumor tissue. We found that the expression of *VEGF-C* and *MMP9* in ESCCs was significantly higher than that in normal tissues (Fig. 2a–-b), and there was a significant higher expression of VEGF-C and MMP-9 in TAMs compare with no-co-culture group (Fig. 2c). Consistently, it was confirmed by qRT-PCR (Fig. 2d), suggesting that TAMs may promote the invasion and metastasis of ESCC, which is closely related to VEGF-C and MMP9 secreted by TAMs.

M2 macrophages were the primary TAM phenotype that promoted malignant behavior of ESCC cells

Macrophages are generally classified as M1 or M2 macrophages [16, 17]. Immunofluorescence (IF) imaging was used to evaluate changes in macrophage phenotypes in a co-culture system, and found that the number of CD163-positive M2-type TAMs was significantly increased following co-culture with ESCC cells, while the number of M1-type macrophages (indicated by co-staining of HLA-DR and CD68) was significantly reduced (Fig. 3a–b). We further evaluated macrophage phenotypes *in vitro* by qRT-PCR, and found that macrophages co-cultured with ESCC cells exhibited molecular characteristics of M2 phenotype macrophages, including significantly increased IL-10 expression and decreased TNF-α and HLA-DR expression (Fig. 3c). These data suggest that there is phenotypic transformation of macrophages in the ESCC microenvironment, and M2 TAMs may be the main component of macrophages in ESCC microenvironment.

Nude mice in Tumor formation experiments, we induced macrophages to an M2 phenotype *in vitro*, then co-injected these M2 macrophages along with ESCC cells as subcutaneous tumors. Found that tumors were larger and heavier in the M2 TAMs co-culture ESCC cell group compared with the ESCC cell-alone injection group (Fig. 3d–f). Further verification in human ESCC tissues, we found that the increase in the number of infiltrating CD163-positive M2 TAMs was closely related to lymphatic invasion, lymph node metastasis, and clinical progression in patients with ESCCs (Fig. 3g). It is suggests that M2 macrophages are the primary TAMs phenotype that promotes malignant behavior of ESCC cells

Hla-dr Mediated Macrophage Phenotypic Transformation

To screen for molecular mechanisms that may be related to the M2 polarization of macrophages, we analyzed microarray data (dataset GSE95405 from the GEO database) to compare gene expression between THP-1 cells that were induced into M1 macrophages by treatment with phorbol ester (PMA), lipopolysaccharide (LPS) and IFN-γ, and THP-1 cells that were induced into M2 macrophages by treatment with IL-4 and IL-13 (Fig. 4a). Significant differences in the expression of multiple members of the HLA-DR family between M1 and M2 type macrophages were identified. Compared with M1-type macrophages, M2-type macrophages expressed less HLA-DR (Fig. 4b). To address whether HLA-DR downregulation is a key molecular event involved in the transformation of macrophages into a cancer-promoting M2 phenotype, we transfected macrophages with HLA-DR lentiviral shRNA. HLA-DR expression in macrophages was significantly lower after shRNA transfection (Fig. 4c–e). Silencing HLA-DR significantly increased expression of the macrophage molecular marker IL-10, and significantly reduced expression of TNF-α (Fig. 4f). These data demonstrate that downregulation of HLA-DR promoting the macrophages polarization to M2 phenotype.

Effects of HLA-DR knockdown on TAM-mediated malignant behavior of ESCC

To investigate the effects of HLA-DR knockdown in macrophages on the biological behavior of ESCC tumor cells, we co-cultured HLA-DR knockdown macrophages and ESCC cells *in vitro*, and found that HLA-DR knockdown macrophages significantly increased the proliferation of ESCC cells compared with control macrophages ($P < 0.05$, Fig. 5a). Transwell migration and invasion assays showed that HLA-DR knockdown macrophages significantly promoted the migration and invasion of ESCC cells ($P < 0.05$, Fig. 5b–c). In addition, we found that knockdown of HLA-DR significantly increased the expression of VEGF-C and MMP-9

in TAMs ($P < 0.05$, Fig. 5d–e), suggesting that downregulation of HLA-DR in macrophages induces TAM M2 phenotypic transformation, promotes secretion of VEGF-C and MMP-9, and participates in the malignant transformation of ESCC.

Low expression of HLA-DR positively correlated with high-density of M2 TAMs, indicating high invasiveness and poor prognosis in ESCC

Our experiments *in vitro* demonstrated that downregulation of HLA-DR contributes to macrophage M2 polarization and promotes malignant behavior of ESCC cells. To further verify the pro-cancer effect of HLA-DR downregulation, the expression of HLA-DR in clinical ESCC samples was evaluated, and found that HLA-DR is mainly expressed in stromal cells in ESCC, and that macrophages constitute an important population of HLA-DR expressing cells (Fig. 6a). Moreover, we observed that some CD68-positive TAMs did not express HLA-DR, suggesting that some TAMs may have lost HLA-DR, and represent a group of M2 phenotype TAMs (Fig. 6a). Identification of M2 TAMs with CD163 staining revealed that ESCC areas with low levels of HLA-DR expression contained high densities of M2 TAMs. Furthermore, in areas with high expression of HLA-DR, there were fewer M2 TAMs (Fig. 6b). Comparing the distribution of M2 TAMs with clinicopathological information, we found that the lower expression of HLA-DR was positively correlated with an increase in the number of M2 TAMs, and positively correlated with invasion depth, lymph node metastasis, and clinical stage of ESCC (Table 1). Analysis of gene expression from the TCGA database and the prognostic data from patients in this study, revealed that lower expression of HLA-DR was closely related to poor prognosis (Fig. 6c–d). These data suggest that lower expression of HLA-DR may be related to macrophage transformation to an M2 phenotype and associated with increased invasiveness and poor prognosis of ESCC.

Table 1
Correlation between HLA-DR expression and clinicopathological parameters in esophageal squamous cell carcinoma (ESCC) tissues.

Variable	Cases (N)	HLA-DR	HLA-DR	*P*-value
		Negative (0,1)	Positive (2,3)	
Age (y)				
≤ Median (58y)	81	23 (28.4%)	58 (71.6%)	0.868
Median	59	16 (27.1%)	43 (72.9%)	
Gender				
Male	96	30 (31.3%)	66 (68.8%)	0.226
Female	44	9 (20.5%)	35 (79.5%)	
Tumor location				
Upper	2	0 (0.00%)	2 (100.0%)	0.100
Middle	109	35 (32.1%)	74 (67.9%)	
Lower	96	4 (13.8%)	25 (86.2%)	
Histologic grade				
Well	42	12 (28.6%)	30 (71.4%)	0.931
Moderate	68	18 (26.5%)	50 (73.5%)	
Poor	30	9 (30.0%)	21 (70.0%)	
Depth of invasion				
T1-T2	47	7 (14.9%)	40 (85.1%)	0.026*
T3-T4	93	32 (34.4%)	61 (65.6%)	
Venous invasion				
Negative	44	9 (20.5%)	35 (79.5%)	0.263
Positive	96	30 (31.3%)	66 (68.8%)	
Nodal status				

*P < 0.05, **P < 0.01, ***P < 0.001

Variable	Cases (N)	HLA-DR	HLA-DR	*P*-value
		Negative (0,1)	Positive (2,3)	
pN −	75	13 (17.3%)	62 (82.7%)	0.005***
pN +	65	26 (40.0%)	39 (60.8%)	
TNM stage				
I–II	91	15 (16.5%)	76 (83.5%)	*P* < 0.001***
III–IV	49	24 (49.0%)	25 (51.0%)	
*P < 0.05, **P < 0.01, ***P < 0.001				

Discussion

ESCC contains a large number of TAMs, which constitute an important immune cell population within the tumor microenvironment. TAMs play a key role in tumor progression by secreting a variety of cytokines, chemokines, and angiogenic factors [18]. TAMs have been reported to be related to the malignant behavior of tumors and to be important factors leading to poor prognosis in a variety of tumors, such as pancreatic ductal carcinoma and breast cancer [19–21]. It is crucial to further define the mechanisms of TAM phenotypic transformation and their cancer-promoting mechanisms.

In our study, co-culture of macrophages and tumor cells did not significantly change the proliferation of ESCC cells, but did significantly enhance the invasion and migration of ESCC cells. *In vivo* tumorigenesis experiments in nude mice further confirmed that depletion of TAMs by chlorophosphite treatment significantly inhibited tumor growth. These results are similar to a study by Fan et al, in which it was reported that TAMs can promotes tumorigenicity, tumor invasion and metastasis [22]. Our results further support the pro-tumor role for TAMs, as our analysis of human ESCC tissue specimens, revealed that the density of CD68 + TAMs in ESCC was positively related to vascular infiltration and lymph node metastasis. This phenomenon has also been reported in variety of other tumors[23]. But specific mechanisms by which TAMs promote the development of ESCC still need to be further explored. MMP-9 and VEGF have been investigated as important factors related to invasion and metastasis in tumors[24]. In this study, VEGF-C and MMP-9 were found to be highly expressed in ESCC, and TAM was an important source of them compared to normal macrophages. These indicated that TAM is an important factor in promoting the invasion and metastasis of ESCC, mechanistically due to its production of VEGF-C and MMP-9. It is similar to Li et al report that, the induction of VEGF and MMP9 expression by macrophages from the tumor microenvironment is also one of the important sources [25].

Previous studies have found that the TAMs in ESCCs are mainly M2 type. M2 TAMs are considered to have low antigen delivery capacity, which may facilitate immunosuppression and promote tumor vascular growth and lymph node metastasis [26]. In the present study, immunofluorescence and qRT-PCR were used to evaluate changes in TAM phenotypes in a co-culture system with ESCC cells. This study demonstrated

that the number of CD163-positive M2-type TAMs was significantly increased following co-culture with ESCC cells, while the number of M1-type TAMs was significantly reduced, suggesting that M2 phenotype macrophages play an important role in ESCC. In order to more fully explore the effects of M2 TAM on ESCCs, we induced macrophages into M2 TAMs by co-culture with ESCC cells. We then injected the M2 macrophages subcutaneously in nude mice, either alone or co-innoculated with ESCC cells. In this co-innoculation experiment, we found M2 TAMs significantly promoted the growth of ESCC tumors. Moreover, our results further suggest that M2 TAMs have a significant correlation with tumor metastasis and clinical progress in human ESCCs.

The regulation of macrophage phenotypes is key to the role of macrophages in promoting or suppressing tumor progression. To screen for molecular mechanisms that may be related to the M2 polarization of macrophages, we analyzed microarray data (dataset GSE95405 from the GEO database), and found that there are significant differences in the expression of multiple members of the HLA-DR family between M1 and M2 macrophages. And HLA-DR as a key marker has been used to distinguish M1 macrophages from M2 macrophages [27, 28]. Suggest HLA-DR deletion may be a molecular mechanism that mediates M2 TAM-mediated immune escape, but a causal relationship between loss of HLA-DR on M2 TAMs and immune escape of ESCC remains to be shown. So next HLA-DR lentiviral shRNA was used to downregulate macrophage HLA-DR expression, the results demonstrate that Macrophage was co-cultured with EC109 and EC9706 and exhibited a mixed M1 / M2 phenotype, while down-regulation of HLA-DR can further polarize macrophages to the M2 phenotype. Recent reports also showed the role of HLA in regulating macrophage phenotype [29, 30]. Suggesting the loss of macrophage HLA-DR mediates immune escape and is an upstream molecular event that leads to M2 macrophage polarization.

In the co-culture system, we found that HLA-DR knockdown macrophages significantly increased the proliferation, migration and invasion of ESCC cells. In addition, we found that knockdown of HLA-DR significantly increased the expression of VEGF-C and MMP-9 in TAMs, suggesting that downregulation of HLA-DR in macrophages induces TAM M2 phenotypic transformation, promotes secretion of VEGF-C and MMP-9, and participates in the malignant transformation of ESCC.

Human ESCC tissue samples showed that HLA-DR was negatively correlated with the number of M2 type TAMs. This supports the findings from our *in vitro* experiments. We also demonstrated that low expression of HLA-DR in ESCC tumor tissues was associated with low survival rates of ESCC patients. This is consistent with previous studies, which report that low HLA-DR expression is a risk factor for poor prognosis in various tumors [31, 32]. Therefore, we believe that the low expression of HLA-DR has predictive value for the recurrence and metastasis of ESCC. The expression of HLA-DR could be used as an important biomarker for the recurrence and metastasis of ESCC.

Conclusion

In summary, TAMs is critical factor that influence ESCC tumor formation, and the migration and invasion of ESCC cells. M2 type TAMs are a critical tumor microenvironment component that promote the malignant growth of ESCC. Moreover, downregulation of HLA-DR induces a transformation of macrophages into M2-

type TAMs, resulting in enhanced production of VEGF-C and MMP-9, which may represent an important molecular mechanism by which M2 TAMs promote ESCC invasion and metastasis.

Materials And Methods

Patients and immunohistochemistry

All participants were patients at the Xinjiang Yili Friendship Hospital, China. Prior to participating in this study, each participant provided written informed consent. The Institutional Ethics Committee of the Erie Friendship Hospital ratified these protocols in accordance with the Declaration of Helsinki. From 2008 to 2014, tissue samples were collected from 140 cases of Xinjiang Kazakh (Kazakh) ESCC from Xinjiang Yili Kazakh Autonomous Prefecture Friendship Hospital by surgical resection for formalin fixation and paraffin embedding. According to NCCN guidelines, pathologists determined the tumor stage. The case information is shown in Supplementary Table 1

The survival status of all participants was followed by telephone contact until December 2015. The median follow-up time for living patients was 30 months (range 1–84 months). Overall survival (OS) was defined as the interval between surgery and death, or between surgery and the last patient follow-up. Of the 140 patients recruited, 73 (52.14%) remained alive and 57 (47.86%) died during the follow-up interval.

Monocytes immobilized with formalin can be induced to differentiate into macrophages after 36 h, and can be co-cultured with cancer cells after 36 h. After 36 h, cells were incubated with diluted primary antibody (see below) overnight at 4°C. Cells were then incubated with secondary antibody (1:50 dilution) for 30 min. Cell nuclei were counterstained with 4,6-diamino-2-phenylindole for 15 min. Images were obtained using a Leica SP5 spectral confocal microscope or an AMG EVOS FL microscope. The primary antibodies used were: for IF single staining: anti HLA-DR (1:50), anti-cd163 (1:50), for IF double staining: anti CD68 (1:200) (Abcam, ab5076, 1:50). Staining was observed and images were recorded using a fluorescence microscope.

Cell culture

The human ESCC cell lines EC109 and EC9706 were purchased from the American Type Culture Collection (ATCC) and cultured in RPIM-1640 (Gibco) containing 10% fetal bovine serum (FBS, Gibco). Human THP-1 cells were purchased from ATCC and cultured in RPMI-1640 medium containing 10% FBS. THP-1 cells were polarized into macrophages using PMA treatment (Sigma, 5 nM). Briefly, THP-1 cells were inoculated into a 6-well plate Transwell chamber at 2×10^6 cells/ml, and cultured with RPMI-1640 medium (10% serum) containing 200 ng/ml PMA for 24 h. The ESCC cell lines EC109 and EC9706 were inoculated into the upper chamber of a 6-well plate chamber at 4×10^5 cells/ml, and 2 ml/well of complete DMEM were added to the upper and lower chambers. The cells were allowed to adhere for 12 h. Macrophages and ESCC cell lines co-cultivation was established bythe non-contact co-culture transwell system (Corning, USA). Inserts containing THP-1 induced macrophages were transferred to 6-well plate seeded with ESCC cells (1×10^5

cells per well) in advance and co-cultured , and the medium was replaced with fresh RPMI 1640 complete medium. Cells were incubated in a 5% CO_2 cell incubator at 37°C for 36 h, and then used for experiments.

Conditioned medium

THP-1 cells were transformed into macrophages by induction with RPMI-1640 medium (10% serum) containing 200 ng/ml PMA for 24 h. ESCC cells were placed in a Transwell cell and cultured with the macrophages for 36 h. The cell culture medium of the upper and lower chambers was drawn into centrifuge tubes, centrifuged at 800 rpm for 5 min, and the supernatant was collected as conditioned medium and preserved at −20°C.

Morphological observation of ESCC cells

Co-cultured and monocultured ESCC cells were observed under a microscope. Images were taken with an Olympus imaging system in a 100× and 200× field.

Cell proliferation experiment

Cell proliferation was evaluated using the CCK8 assay. Briefly, 10 μl CCK8 working buffer were added to each well of a 96 well plate, and absorbance was measured at 450 nm using a TECAN infinite m200 Pro enzyme scale.

RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was isolated using Trizol reagent. Total RNA was reverse transcribed with a Takara PrimeScript™ RT-PCR kit and amplified using a SYBR Premier ex Taq™ II Kit (Takara), according to the manufacturer's instructions. All primers used are listed in Table S1. PCR was carried out using the eco (Illumina) system with parameters: initiation at 95°C for 30 s, followed by 40 thermal cycles at 95°C for 3 s, 55°C for 30 s and 72°C for 30 s, then 95°C for 15 s, 65°C for 60 s, and 95°C for 15 s. All data were analyzed by CT value comparison.

Animal models

Six-week-old BALB/c nude mice were purchased from the Shanghai Experimental Animal Center of the Chinese Academy of Sciences, and were maintained in a pathogen free environment. Thirty mice were randomly divided into three groups. Mice received subcutaneous injection of cell suspension of 1×10^6 EC109 cells into the left armpit; one group was also injected with 1×10^6 M2 TAM cells. When the diameter of the transplanted tumor reached 0.2–0.4 cm, chlorophosphate liposomes and phosphate-buffered saline (PBS) control liposomes (Fu Sheng Biotechnology Co, Ltd, China) were injected into the nude mice in the corresponding groups every 4 days until the end of the experiment. The mice were euthanized by cervical dislocation 4 weeks after injection, and the tumors were isolated for analysis.

Plate clone formation assay

EC109 and EC9706 cells (about 1×10^3 cells) co-cultured with macrophages were inoculated into a six-well culture dish. After incubation at 37°C for 2 weeks, the cells were washed twice with PBS and stained with 0.1% crystal violet solution. The colonies containing ~50 cells were counted under a microscope.

Cell migration and cell invasion assays

About 5×10^4 EC109 and EC9706 cells from the different co-culture groups were resuspended in 200 μl serum-free medium and inoculated into the upper chamber of a Transwell invasion insert, and 500 μl medium supplemented with 10% FBS were added to the lower compartment. After 24 h of culture, the cells that had migrated to the lower surface of the membrane were fixed with 4% paraformaldehyde, stained with 0.1% crystal violet, and counted under a microscope. These experiments were performed three times.

Immunofluorescence assay

Cells grown in 6-well plates were fixed for 20 min in 4% (w / v) paraformaldehyde at room temperature, washed 3 times with PBS, and treated with 0.5% (v / v) Triton X-100 (Solarbio, T8200) for 20 min and then blocked for 30 min with 5% (v / v) normal serum in PBS. After that, cells were induced with primary antibodies at 4 ° C overnight and secondary antibodies(1:50) for 1 h at room temperature, followed by washing and staining with DAPI (2 μg / ml, Solarbio, c0065).

Western blotting

Western blotting was performed as previously described [33]. Protein was extracted from TAM cells with RIPA lysis buffer (Beyotime, Haimen, China) containing protease inhibitor PMSF (CW Biotechnology, Beijing, China). Primary antibodies to VEGF-C (1:1000; Santa Cruz), MMP-9 (1:500; Santa Cruz), and HLA-DR (1:2000; Santa Cruz) were used. These antibodies were detected using goat polyclonal anti rabbit IgG antibody (1:10000; Abcam). Protein bands were detected using ECL.

Construction of the lentivirus shRNA vector

Human HLA-DR specific shRNA carrier and non-targeting (shnt) vector were purchased from Jikai Company of China .

Oligonucleotide sequence information:

No.	5′	STEM	Loop	STEM	3′
HLA-DRA-RNAi(55452-1)-a	Ccgg	ccATCTTCATCATCAAGGGAT	CTCGAG	ATCCCTTGATGATGAAGATGG	TTTTTg
HLA-DRA-RNAi(55452-1)-b	aattcaaaaa	ccATCTTCATCATCAAGGGAT	CTCGAG	ATCCCTTGATGATGAAGATGG	
HLA-DRA-RNAi(55453-1)-a	Ccgg	gcTTGAAGAATTTGGACGATT	CTCGAG	AATCGTCCAAATTCTTCAAGC	TTTTTg
HLA-DRA-RNAi(55453-1)-b	aattcaaaaa	gcTTGAAGAATTTGGACGATT	CTCGAG	AATCGTCCAAATTCTTCAAGC	
HLA-DRA-RNAi(55454-1)-a	Ccgg	ctGACCAATCAGGCGAGTTTA	CTCGAG	TAAACTCGCCTGATTGGTCAG	TTTTTg
HLA-DRA-RNAi(55454-1)-b	aattcaaaaa	ctGACCAATCAGGCGAGTTTA	CTCGAG	TAAACTCGCCTGATTGGTCAG	

Microarray analysis from bioinformatics database

The Oncomine database (https://www.oncomine.org/resource/login.html) was used to analyze the expression of VEGF and MMP-9 in ESCC and normal tissues [34]. Microarray data were obtained from Gene Expression Omnibus (Geo, http://www.ncbi.nlm.nih.gov/geo) from the GSE95405 study. GSE95405 microarray data included gene expression data from M0 and M1 macrophages from six cases. The expression of HLA-DR-related molecules was analyzed from this dataset. Data Were $\log_2$ transformed and the resulting heatmap was constructed using hemi software [35].

To further explore the expression and prognostic value of genes of interest in ESCC, we used a newly developed interactive website, ualcan, (http://ualcan.path.uab.edu/analysis. HTML) to analyze ESCC gene expression data from the TCGA database [36].

Statistical analysis

Statistical analysis was performed using SPSS 20.0 software (Chicago, IL, USA).. Measurement data between two groups were compared using paired tests and/or independent sample t-tests. Measurement data between three groups were compared using single factor analysis of variance. A difference was considered to be statistically significant at $P < 0.05$.

Abbreviations

ESCC: Esophageal squamous cell carcinoma; TAMs: Tumor-associated macrophages; HLA-II : Human leukocyte antigen class II; APCs: Antigen-presenting cells; qRT-PCR: Quantitative real-time PCR; IF: Immunofluorescence; PMA: Phorbol ester; LPS: Lipopolysaccharide; IL-4: Interleukin-4; IL-13: Interleukin-13; IFN-γ: interferon; FBS: Fetal bovine serum; PBS: Phosphate-buffered saline; GEO: Gene expression omnibus; OS :Overall survival

Declarations

Ethics approval and consent to participate

All participants were patients at the Xinjiang Yili Friendship Hospital, China. Prior to participating in this study, each participant provided written informed consent. The Institutional Ethics Committee of the Erie Friendship Hospital ratified these protocols in accordance with the Declaration of Helsinki.All animal experiments were conducted according to protocols approved.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article .

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by grants from the National Natural Science Foundation of China (No. 81460363, No. 81760428 and No. 81960435), the Start-up Project of High-level Talents Scientific Research in Shihezi University (RCZK2018C19), the Science and Technology Development Project of Xinjiang Production and Construction Corps (No. 2018AB033), the National Early Detection and Treatment Project for Upper Digestive Tract in Rural Area in China (No. 2018), and The Youth Science and Technology Innovation Leading Talents Project of Corps (No. 2017CB004).

Authors' contributions

JF,YF performed the experiments, analyzed the data and wrote the manuscript.WH, HJ,XL,JH,LK,XL did the experiments and analyzed the data. CH, XY, AZ did some experiments and provided useful helps. LJ,XB,LY,CX,WY gave valuable suggestions. FL,JM designed and supervised the study, explained the data and wrote the manuscript. All authors read and approved the final manuscript.

Acknowledgements

Not applicable.

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Figures

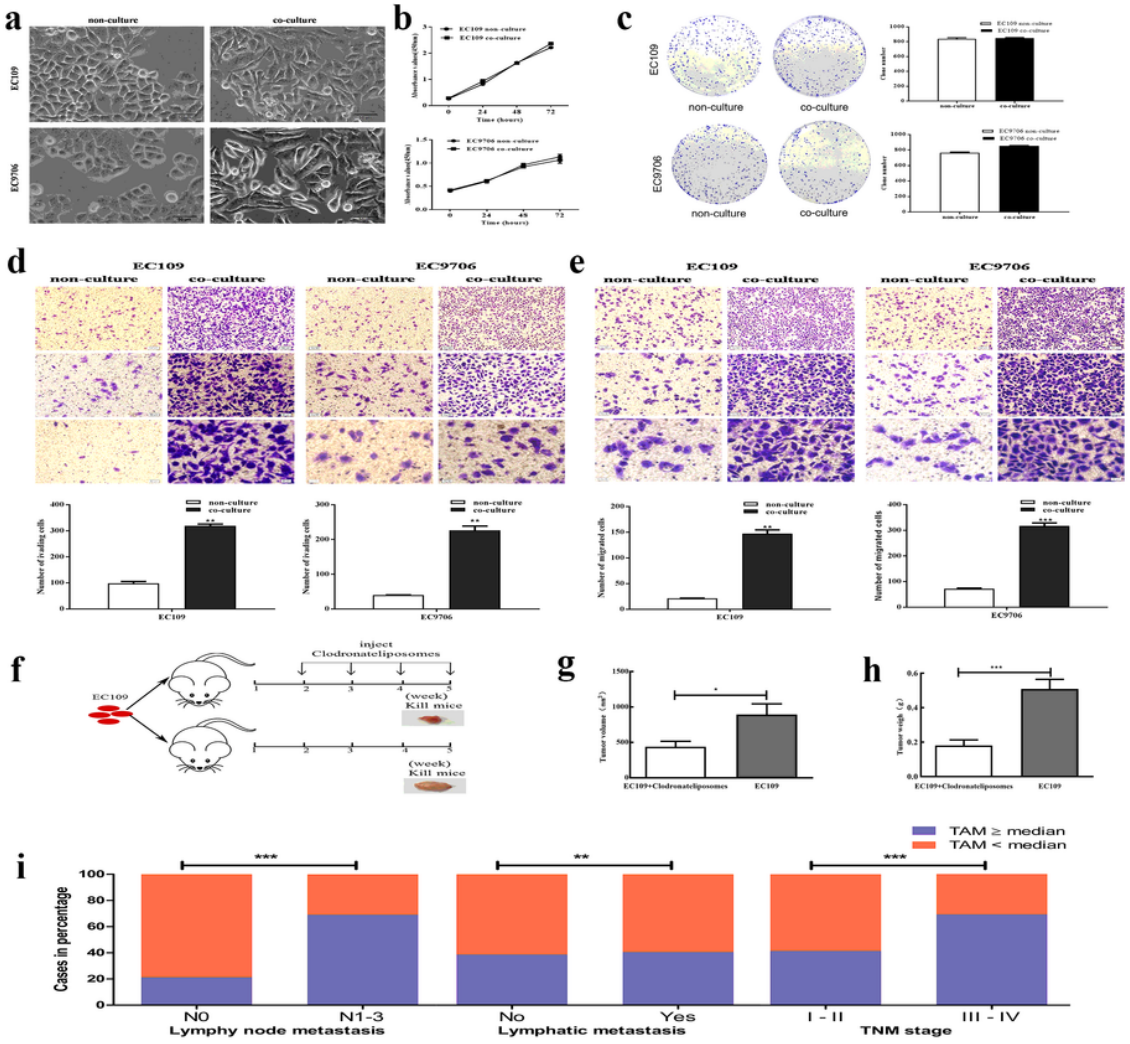

Figure 1

The effects of macrophages on the growth, invasion, and migration of esophageal squamous cell carcinoma (ESCC) cells. (a) The pattern of form EC109 and EC9706 were co-cultured with PMA-treated THP-1 macrophages for 48 h (magnification, ×200). (b-c) The effect of TAMs on the proliferation of EC109 and EC9706 cells via (b)CCK8 assay, (c) plate cloning experiment, TAM have no significantly effect on ESCC cells proliferation. (d-e) After EC109 and EC9706 co-culture with TAM or EC109 and EC9706 alone, (d) invasion experiment and (e) migration experiment were used to detect the migration and invasion capacity of EC109 and EC9706. (f) Schematic diagram of mice implanted with EC109 cells and injected with chlorate phosphate (depletion of TAMs). Differences in tumor volume (g) and weight (h) in BALB/c mice. (i) Relationship between CD68-positive TAMs and clinicopathological parameters in ESCC. TAM: tumor associated macrophage.

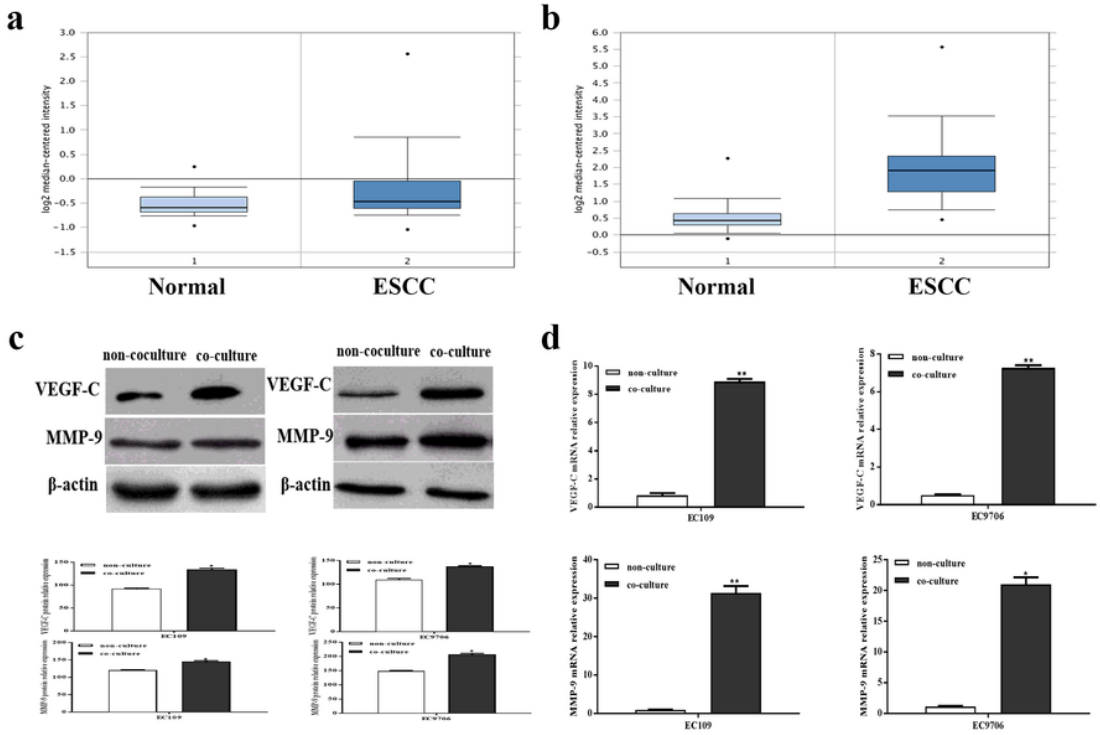

Figure 2

TAMs were an important source of VEGF-C and MMP-9 that promoted metastasis in ESCC tissue. (a-b) The Oncomine database was used to analyze the Su Esophagus dataset Compared with normal tissue, ESCC tissue exhibited (a) significantly higher expression of VEGF-C (P = 0.002; fold change = 1.264), and (b) significantly higher expression of MMP-9 (P = 1.02E−13; fold change = 2.787). Macrophages co-cultured with EC109 cells exhibited significantly higher expression of the metastasis-related factors VEGF-C and MMP-9 at both the protein expression level (c) and mRNA expression level (d).

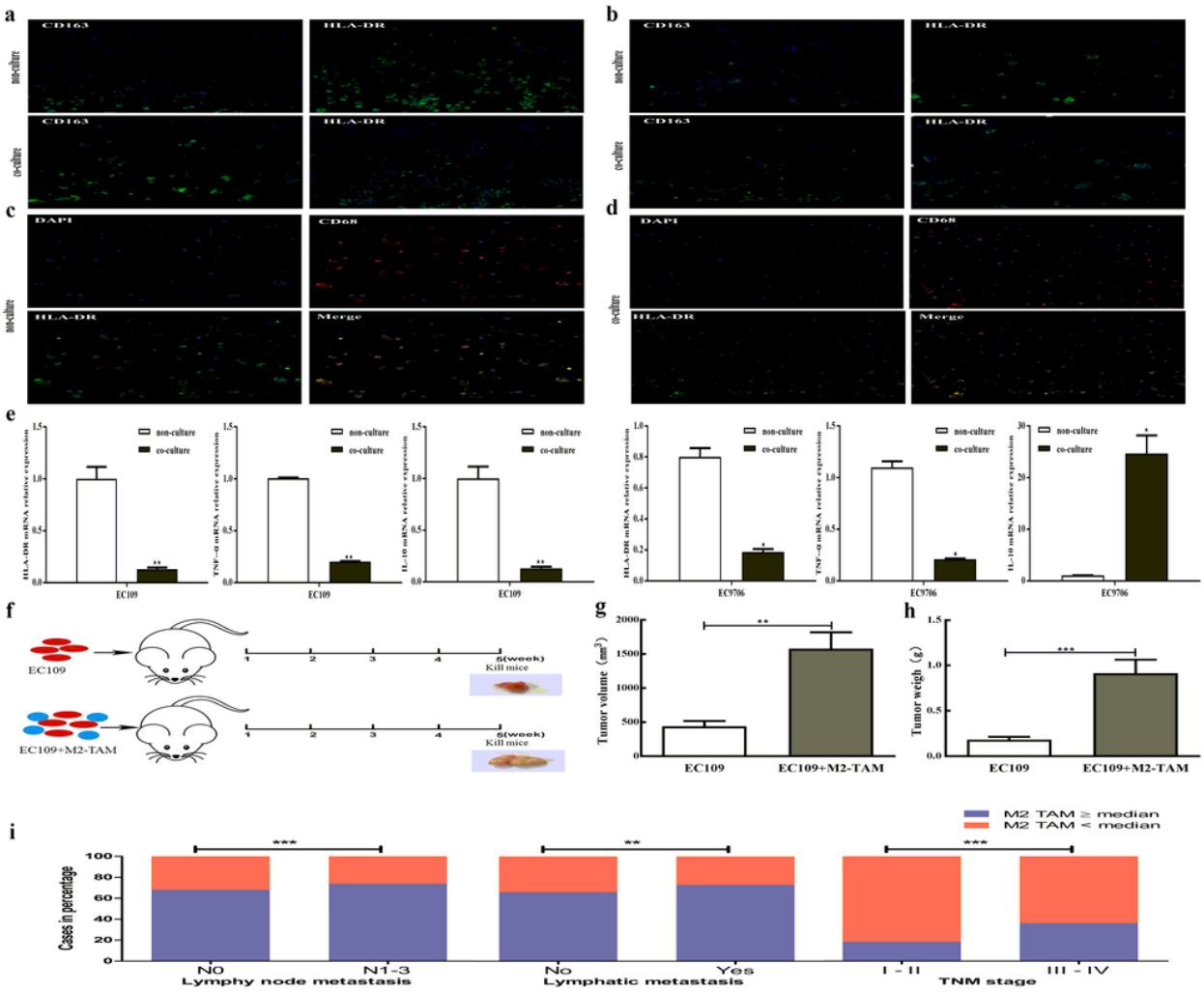

Figure 3

TAMs in ESCC were mainly M2 type, which promote metastasis and tumorigenicity of ESCC. (a) Immunofluorescence (IF) staining showed the expression of CD163 (M2 macrophages) and HLA-DR (M1 macrophages) in single-culture and co-culture with EC109/EC9706 cells and macrophages. Compared with single-culture, the number of CD163 macrophage-positive cells increased significantly in the co-culture setting, while the number of HLA-DR-expressing macrophages decreased significantly. (b) IF staining was used to detect the number of CD68 and HLA-DR double-expressing (M1 macrophage) cells before and after co-culture with EC109/EC9706 cells. Compared with single-culture, the number of CD68 and HLA-DR co-expressing cells was significantly reduced after co-culture. (c) qRT-PCR detection of gene expression indicative of macrophage phenotype changes after co-culture revealed a decrease in expression of the M1 type markers, HLA-DR and TNF-α, and an increase in expression of the M2 type marker IL-10. (d) Schematic diagram of mice co-implanted with EC109 cells and M2 macrophages, or with EC109 alone. Differences in tumor volume (e) and weight (f) in BALB/c mice. (g) Relationship between CD163-positive M2-TAMs and clinicopathological parameters of ESCC.

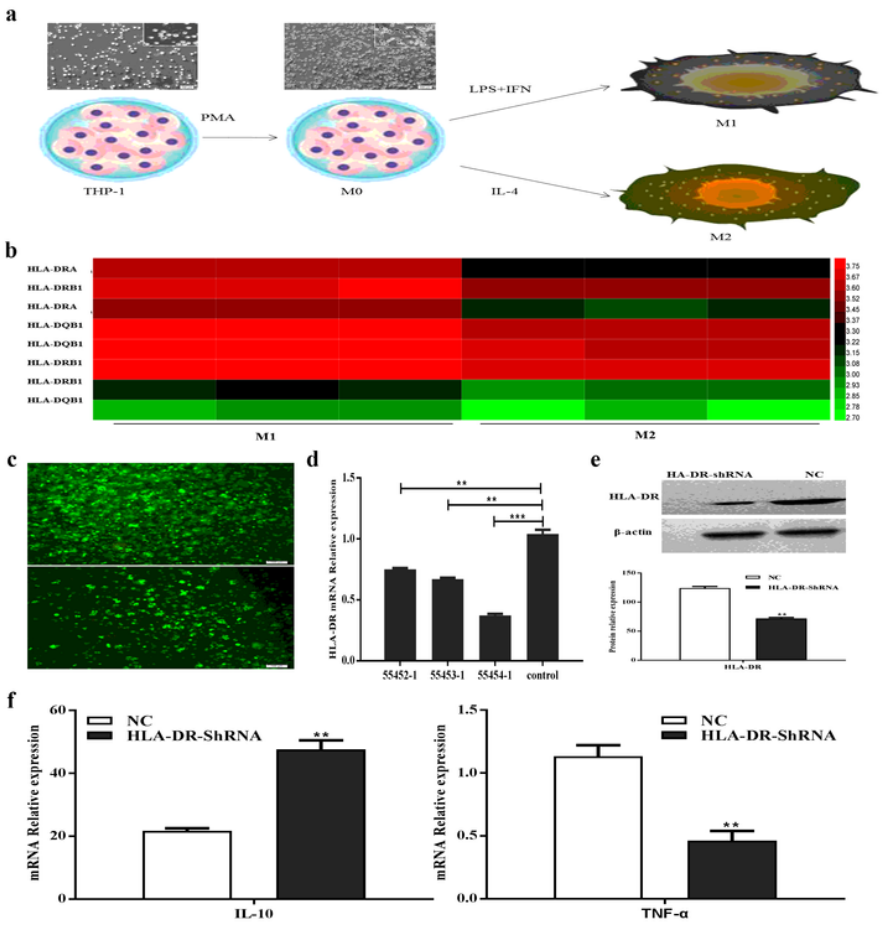

Figure 4

Knockdown of HLA-DR promoted macrophage transformation from the M1 to the M2 phenotype. (a) Schematic diagram of induction of THP-1 cells into M1 and M2 type macrophages, respectively. (b) Relative to M1 macrophages, expression of various members of the HLA-DR family was significantly downregulated in M2 macrophages (GEO: GSE95405), all data were standardized, and Hemap was used to visualize the heatmap. (c) Transfection efficiency of HLA-DR lentiviral shRNA-transfected macrophages. HLA-DRA-RNAi (55454-1)-transfected cells (green fluorescence) displayed by immunofluorescence after transfection of macrophages. qPCR (d) and Western blot (e) detection of three lentiviral shRNA and empty vector-treated control group indicated that macrophages transfected with HLA-DR shRNA exhibited reduced HLA-DR expression. (f) lentiviral shRNA transfection downregulated HLA-DR and influenced macrophage phenotype.

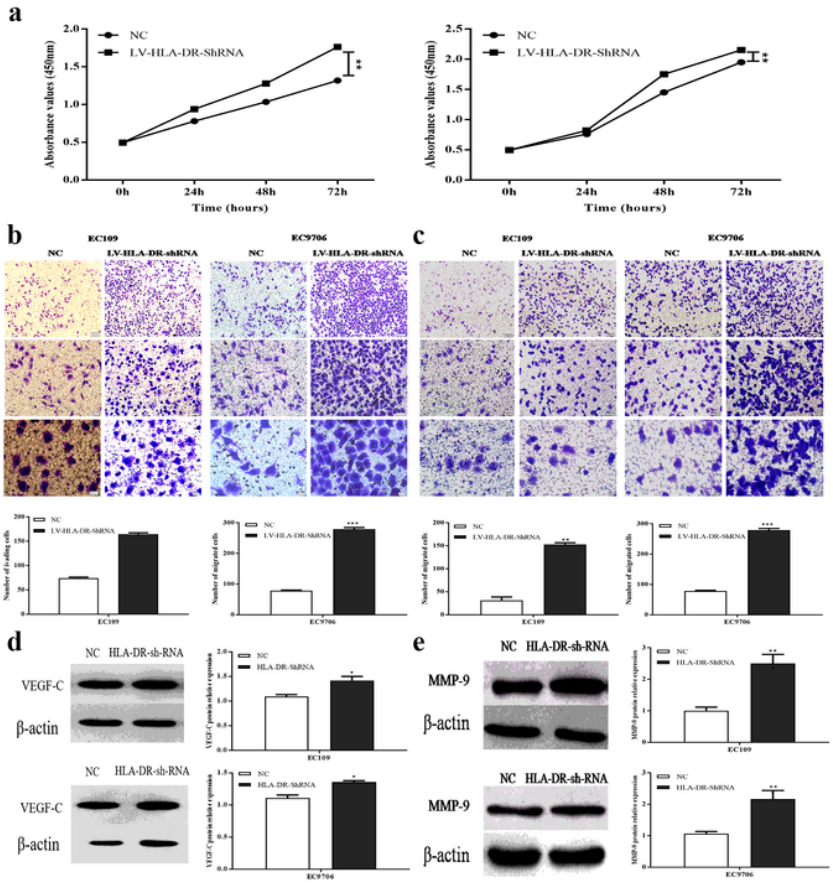

Figure 5

Knockdown of macrophage HLA-DR promoted proliferation, invasion and migration of ESCC. (a) Cell proliferation was higher in ESCC cells co-cultured with HLA-DR knockdown TAMs than in ESCC cells co-cultured with macrophages transduced with the empty vector negative control (EC109 cells, left panel; EC9706 cells, right panel). Co-culture with HLA-DR knockdown TAMs promoted migration (b) and invasion (c) of ESCC cells. Knockdown of HLA-DR in TAMs induced (d) VEGF-C overexpression and (e) MMP-9 overexpression.

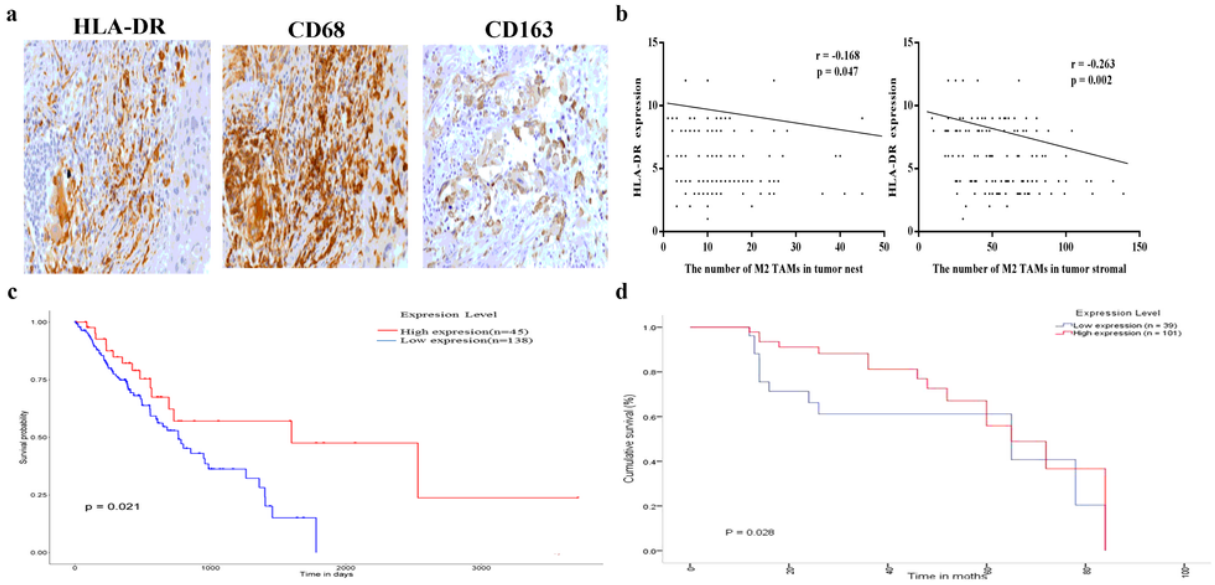

Figure 6

Low expression of HLA-DR is associated with low density M2 TAMs and poor prognosis in ESCC (a) HLA-DR is mainly expressed in stromal cells of ESCC, and macrophages are an important cellular component that express HLA-DR. (b) Lower expression of HLA-DR was positively correlated with an increase in the number of M2 TAMs. (c) Analysis of data from the TCGA database demonstrated that lower expression of HLA-DR was associated with poor outcomes in patients with ESCC. (d) Low HLA-DR expression is closely related to poor prognosis in patients with Kazakh ESCC.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTable1.docx
- SupplementaryTable1.docx