Tumor-selective blockade of CD47 signaling with CD47 antibody for enhanced anti-tumor activity in malignant meningioma

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Article

Keywords: meningioma, CD47, immune escape, EMT, targeted therapy

Posted Date: September 9th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-2002161/v1

Abstract

Patients with WHO grade III meningioma have a poor prognosis with a median survival of less than two years and a high risk of recurrence. However, traditional treatment options have failed to improve prognosis. Therefore, development of novel immunotherapy targets is urgently needed. CD47 acting as a “don't eat me” signal to macrophages of the immune system can trigger tumor immune escape. However, the role of CD47 in malignant meningioma is not well understood. We collected 190 clinical meningioma samples and detected the expression of CD47 and immune infiltrate in WHO grade I-III by immunohistochemistry, western blot, Q-PCR and analyzed the correlation with pathological classification using statistical software. We also examined the functional effects of anti-CD47 antibody (B6H12) on cell proliferation, migration and invasion, macrophage-mediated phagocytosis and tumorigenicity both in vitro and vivo. The expression of tumor relating pathways were also detected by immunohistochemistry. We found that CD47 expression was increased in malignant meningioma along with a decreased number of T cells and an increase in CD68 expressing macrophages. Blocking CD47 with anti-CD47 antibody suppressed cancer cell growth, motility and promoted macrophage-mediated phagocytosis in the malignant meningioma cell line (IOMM-Lee). Additionally, p-ERK and EGFR showed higher expression than other signaling pathways in malignant meningioma with high expression of CD47 and showed decreased expression in CD47 downregulation tumor tissues. These results demonstrate that CD47 may be involved in the disease progression and prognosis and offer a novel therapeutic option by targeting CD47 in malignant meningioma.

Introduction

Meningiomas account for up to 30% of all primary intracranial tumors. The prognosis of meningioma is closely related to the pathological classification. They are histologically classified into three types according to the World Health Organization (WHO) classification of tumors [1]. Approximately 3% variants correspond with WHO grades III [2], which presents a worse prognosis with a higher risk of recurrence revealing a 5-year mortality rate of up to 50–80% and shorter median survival times of less than two years [3].

However, traditional treatments such as surgical resection, chemotherapy have difficulty in treating malignant meningioma effectively [4]. Recently, immune therapies have showed impressive effects in various tumors [5]. Programmed death-ligand 1 (PD-L1), was shown to have trigger immune escape in malignant meningioma [6]. Meanwhile, CD47 as an immunoglobulin family member protein overexpressed in various cancers [7]. CD47 acts as a “don’t eat me” signal by binding to signal regulatory protein α (SIRPα) on macrophages and thereby inhibiting phagocytosis of target cells. Previous studies have proved this ability of CD47 [8–10]. Meanwhile, anti-CD47 antibodies are confirmed as an inhibitor to cancer cell proliferation through the EGFR/PI3K/AKT signaling pathway [11–13]. And it has proven that CD47 blockade can also enhance tumor cell phagocytosis by tumor-associated macrophages (TAMs) [14]. However, the role of CD47 in tumor immunity has not been studied in malignant meningioma.

In light of the findings above, we collected 190 meningioma samples to assess CD47 and TAMs expression. Additionally, we conducted vitro and vivo experiments to find whether anti-CD47 antibody can suppress the proliferation of malignant meningioma and promoted the TAMs.

Materials And Methods

Patients and Tissue Samples

We collected a total of 190 cases of formalin-fixed and paraffin-embedded (FFPE) tissues from Nan Fang Hospital. The cohort included 190 meningioma patients with a median age of 51 years (range, 13–82 years) including 68 male and 122 female patients with 128 WHO grade , 37 WHO grade and 25 WHO grade . They were all cases of meningioma diagnosed by the Department of Pathology of Southern Hospital from 2008 to 2019. Fresh samples for primary cells culture was obtained from surgery and diagnosis of meningioma with written informed consent from patients. The above specimens were examined and identified by three board certified surgical pathologists specializing in meningioma pathology, and were reclassified according to the 2007 WHO classification of meningioma. Details of patient information were presented in Table 1.

Table 1
Clinicopathological characteristics of parents

Clinical Parameters	Entire population(n = 190)	
	n	%
Meningioma subtype		
WHO grade I	128	
Meningothelial	25	19.5
Fibrous	32	19.6
Transitional	61	47.7
Angiomatous	6	4.5
Psammomatous	3	2.3
Metaplastic	1	1.0
WHO grade II	37	
Atypical	29	78.4
Chordoid	8	21.6
WHO grade III	25	
Anaplastic	16	64.0
Rhabdoid	5	20.0
papillary	4	16.0
Age		
Median	51	
Range	13–82	
15–44	55	28.9
45–59	78	41.1
60–100	57	30.0
Gender		
Male	68	35.8
Female	122	64.2

Cell lines

The human malignant meningioma cell line IOMM-Lee (RRID: CVCL_5779) was cultured in complete medium, specifically Dulbecco's Modified Eagle Medium (DMEM, PAA) with 10% fetal bovine serum (FBS, Invitrogen) and 1% penicillin/streptomycin (Invitrogen) at 37°C with 5% CO2. All human cell lines have been authenticated using STR (or SNP) profiling within the last three years has been included. All experiments were performed with mycoplasma-free cells.

Antibodies

PD-L1 (1:400; CST; #13684), CD47 (1:200; Sigma; HPA044659), GAPDH (1:2000; CST; #5174), CD3 (1:200; Thermo Fisher; 14-0038-80), CD4 (1:150; Thermo Fisher; 14-0041-82), CD8 (1:100; Thermo Fisher; 14-0081-82), CD20 (1:100; Abcam; ab78237), FoxP3 (1:300; Abcam; ab20034), Ki-67 (1:200; Abcam; ab16667), EGFR (1:50; CST; #2085), p-ERK (1:400; CST; #3074), ERK (1:500; CST; #4695), β-catenin (1:200; Abcam; ab32572), CD68 (1:100; Abcam; ab213363), EMA (1:300; Abcam; ab109185), Anti-CD47 antibody (B6H12) (10 μg/mL; eBioscience), isotype control (mouse IgG1, Thermo Fisher).

Immunohistochemistry

HRP/DAB Detection IHC Kit (Abcam) was used for the following steps according to the manufacturer's protocol. All the samples were assessed by three pathologists in 10 different high-power fields (HPFs). The staining intensity of CD47 and PD-L1 was scored as 0 (no staining), 1 (weak staining), 2 (intermediate staining), or 3 (dark staining). The percentage of staining cells was scored as 0 (0–5%), 1 (6–25%), 2 (26–50%), 3 (51–75%), or 4 (76–100%). The product of the two scores was considered as the final score. A composite score greater than the median value for all cases was considered 'high expression' [15]. The histoscore of the membrane and nuclear staining quantification was assessed according to the formula (3 + per cent cells) ×3+(2 + per cent cells) ×2+(1 + per cent cells) ×1, and the formula total intensity/total cell number was used to assess the histoscore of pixel quantification. In this case, the normalized score is between 0 and 250 [16]. The χ^2 test was used to analyze the immune checkpoint expression rate in different grades of meningiomas.

Mice

A total of 18 nude mice (BALB/c) were provided and raised by the Experimental Animal Center of Nanfang Hospital of Southern Medical University. The mice were 4 weeks old and weighed 20-25g.

Instruments and Reagents

DMEM medium (Gibco, USA), 0.25% trypsin and 0.05% ethylenediamine tetraacetic acid (EDTA) (Gibco, USA), fetal bovine serum (FBS) (Gibco, USA), PBS buffer solution (Gibco, USA), CO_2 constant temperature incubator (Thermo company, USA), PMA (1:10000; Sigma), Transwell champer (Corning, 12 wells).

Isolation, Culture and Purification of primary cells

The fresh meningioma tissues specimens obtained from surgery were washed with PBS. The tissues were cut into 1 mm^3 patches by ophthalmology. Then, Tissue blocks were digested with 0.25% trypsin and filtered with 70 μm filter for 10 minutes. The filtered solution was centrifuged for 3 min at 1000 r/min to remove the impurities. We added 3 ml red blood cell lysate into the filtered solution for 3 min. Then, we added 6 ml PBS to dilute. Centrifuge was used again to remove the supernatant. Cells were planted in 25 cm^2 culture bottles with DMEM medium containing 10% FBS and 1% penicillin/streptomycin in 37℃, 5% CO_2 cell incubator under aseptic conditions. Subculturing was performed when then density of cells was greater than 90% about every 2 to 3 d.

Western blot analysis

We extracted protein from malignant meningioma cells. The concentration of protein was determined using a BCA Protein Assay Reagent kit (Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Protein bands were detected by chemiluminescence technology using an ECL advanced western blot analysis detection reagent (EMD Millipore), quantified and normalized to GAPDH using Image J software (version 1.38, National Institutes of Health, Bethesda, MD, USA).

RNA extraction and Q-PCR

Quantitative PCR (Q-PCR) was performed using the SYBR Green Master Mix (Takara Bio Inc.) in a 20 μl reaction volume, according to the manufacturer's instructions. All reactions were run in triplicate on Step One™ PCR amplifier (Applied Biosystems), and GAPDH gene as endogenous control. The primer sequences for CD47 gene were 5'-AGAAGGTGAAACGATCATCGAGC-3' (sense primer) and 5'-CTCATCCATACCACCGGATCT-3' (antisense primer). The primer sequences for GAPDH gene were 5'-ACCACAGTCCATGCCATCAC-3' (sense primer) and 5'-TCCACCACCCTGTTGCTGTA-3' (antisense primer). A thermocycling program was set for 40 cycles of 3 s at 95°C, and 30 s at 60°C with an initial denaturation step at 95℃ for 20 s. Gene expression values were normalized to endogenous control GAPDH, and calibrated to sample with the lowest expression. The $2^{-\Delta\Delta Ct}$ method was used to calculate fold changes in the gene expression normalized to GAPDH [17]. Relative quantification (RQ) = $2^{-\Delta\Delta Ct}$, (ΔΔCt = ΔCt sample – ΔCt calibrater, where ΔCt = Ct target gene – Ct GAPDH) [18].

Cytotoxicity Assay

Human malignant meningioma cancer IOMM-Lee Cells at a density of 5×10^3 were seeded in a 96-well plate and cultured for 4 h with 100 μl complete medium per well at 37℃. IOMM-Lee cells were incubated with 200 μl culture media containing different concentrations of anti-CD47 antibodies, IgG antibodies and PBS for 24 h. The cell counting kit assay (KeyGEN BioTECH, CHINA) was used to measure cell proliferation [19]. According to the manufacturer's instructions, The Cell-Counting Kit (10 μl, CCK-8) reagent was added into each well, and the optical density (OD) at 450 nm was recorded using a microplate reader (BioTek, USA) after incubation for 3 h. The cell viability (%) was calculated using ((ODT- ODB)/(ODC-ODB)) × 100. Experiment was carried out three times independently.

Migration assay

Cell invasion assay was performed with self-coated Matrigel (BD Biosciences, San Jose, CA, USA) on the upper surface of a transwell chamber. Cells were planted in transwell chambers which were then put in a 12-well plate. The upper chambers were added with the cell suspension suspended with serum-free DMEM medium while the lower chambers were added the DMEM medium containing 10% FBS. Cells were incubated in a 5% CO_2 incubator at 37°C for 24 h. Anti-CD47 at the concentration of 10 μg/ml was added to the upper chamber of the experimental group. IgG at the concentration of 10 μg/ml was added to the upper chamber of the control group. PBS at the same concentration was added to the upper chamber of the blank control group. After 24 h, the medium was discarded. When cells had invaded through the extracellular matrix layer to the lower surface of the membrane, cells were fixed with 4% paraformaldehyde (PFA) for 30 min in PBS and stained with crystal violet for 15 min. We counted cells and captured photographs of four randomly selected fields of the fixed cells under the microscope. The experiment was repeated three times independently.

Scratch assay

Approximately 5×10^5 cells were seeded per well in a six-well plate. After the density of cells were about 100%, we used a 200 μl pipette tip to make a scratch vertically at the center of each well, perpendicular to the horizontal line. Afterwards, the cells were washed three times with PBS to washed away the streaked cells. Then, anti-CD47 antibodies at a concentration of 10 μg/ml prepared in 2ml DMEM medium were added in each well of the experimental group while IgG antibodies at the same concentration were added in the control group. Blank control group only contained 2 ml DMEM medium. Cells were incubated in a 0.5% CO_2 incubator at 37°C. We observed and captured photographs of cells every 6 h until 48 h after the treatment under the microscope [20].

In vivo test in mice and tumor measurement

The cells in the logarithmic phase of the third generation were digested with 0.25% trypsin and the supernatant was removed by centrifugation. 0.1 ml of the cell suspension was slowly injected into the flanks of 4 weeks ago nude mice with 5×10^6 cells per mouse using 1.0 ml syringe to form a subcutaneous mound [21]. All mice were subcutaneously injected with meningioma cells, and all tumor-forming mice were equally divided into 3 groups with 6 mice in each group. Anti-CD47 mAb was injected into the tumor-forming site of mice in the experimental group, and IgG1 isotype antibody was injected into mice in the control group. The same amount of PBS was injected into the NC group. Starting 3 days after tumor cell injection, mice were treated with 100 μg of anti-human CD47 mAb or mouse IgG1 isotype control mAb and PBS. Earrings were labeled to mark the mice [22].

The life status of nude mice was checked regularly every day. The body mass of nude mice in subcutaneous tumor group was weighed every week. We drawed the curve of the volume of tumor by the formula V = (π × width × length × height) / 6. After 5–6 weeks, mice were sacrificed followed by cervical dislocation and tumors were excised, measured and weighed.

Immunofluorescence assay

Co-cultured cells were fixed with 4% PFA at 4°C for 15min. After clearing the cells with PBS, 0.3% Triton was added into the cells for 10 min. Co-cultured cells were then blocked with 10% normal goat serum (Vector Laboratories, Inc, Burlingame, CA, USA) for 1 h at room temperature and incubated with primary antibodies overnight at 4°C. The primary antibody was CD68 antibody. The cells were then incubated with fluorescein-conjugated secondary antibodies. Anti-rabbit IgG antibody (1:200; Life Technologies) were used as secondary antibodies. The secondary antibody solution was then added and incubated at room temperature for 1 h. followed by nuclear counterstaining with DAPI (Abcam). The samples were detected by confocal microscopy. In all of the slices, five fields per sample and three to six tissues were quantified in each group. Expression was analyzed using Image-Pro Plus 6.0 software (Media Cybernetics Inc, Buckinghamshire, UK).

In vitro phagocytosis assay

Meningioma cells were labeled with 1 μM CFDA-SE (CFSE) using the Cell Trace CFSE Cell Proliferation Kit (Invitrogen). Macrophages were incubated with PMA for 48 h. Then macrophages were incubated with 1×10^6 CFSE-labeled tumor cells in serum-free medium in the presence of anti-CD47 antibodies (10 μg/ml, eBioscience) or IgG control (10 μg/ml, eBioscience) or PBS for 2 h. The macrophages were incubated with CD68 antibody to make macrophages RFP + before observation. Immunofluorescence assay was performed to observed the phagocytosis. The phagocytic index was calculated as the number of phagocytosed CFSE + cells per 100 macrophages.

Transfection

IOMM-Lee cells were digested to form a single-cell suspension and were divided into three groups including short interfering (si)-CD47, scrambled si-RNA (si-NC) and control group. Then cells were plated in 6-well plates until the cells entered the logarithmic growth phase. When the cell density reached 70 ~ 80%, 20 nM si-CD47 or a scrambled si-RNA (si-NC) was transfected into the cells using Lipofectamine

2000 (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. The sequences of siRNAs are: si-CD47, 5'-AGAUUUGACUUUACUAAGCAG-3' and si-NC, 5'-UUCUCCGAACGUGUCACGUTT-3'. After 24 h of transfection, the mRNA expression of CD47 was determined by reverse Q-PCR.

Statistical Analysis

Statistical Analysis was performed using GraphPad Prism 5.0 (GraphPad software). All the data were analyzed by SPSS 19.0 software. The quantitative data were represented as mean ± SEM. The χ^2 test was used to analyze the difference of immune checkpoint and infiltrated immune cells expression rate in different grades of meningiomas. The one-way ANOVA test was used to analyze the difference of immune checkpoint expression intensity in different grades of meningiomas and the differences between anti-CD47 group, NC group and IgG group. Overall survival analysis was performed using Kaplan-Meier analysis. All experiments were repeated three times.

Results

The malignant grade of meningioma is closely related to the expression of CD47 in tumor cells

The cohort included 190 meningioma patients including WHO grade , and (Table 1). IHC analysis of CD47 and PD-L1 in the tumor tissues showed that CD47 mainly expressed in a cytoplasmic and membranous, while PD-L1 in membranous. The expression of CD47 and KI-67was significantly higher in WHO grade III ($p < 0.001$) compared with PD-L1 (Fig. 1D-I). The expression intensity of CD47 in WHO grade meningioma were generally stronger than in grade and compared with PD-L1 (Fig. S1). It also showed the expression intensity of CD47 in malignant meningioma was similar to that in breast cancer and lung cancer (Fig. S2). Meanwhile, Double-label CD47 and EMA in malignant meningioma showed CD47 was mainly expressed on the membrane in tumor cells (Fig. S3). The differences had statistically significance (Table 2). Q-PCR and Western blot (WB) showed that the transcriptional and expression level of CD47 in WHO grade was higher than in grade and (Fig. 1J-K). Kaplan-Meier analysis also showed a significant difference in overall survival between high-CD47 group and low-CD47 group in malignant meningioma (Fig. 1L, $p = 0.035$).

Table 2

Expression of CD47 and PD-L1 at immune checkpoints in 190 cases of meningioma scored by Image J (separated into low and high expressors-relative to the median score for each marker for the entire cohort; specimens were classified according to pathological types)

Pathological		CD47				PD-L1				Ki-67			
types	**Cases**	**Low**	**High**	**Percent**	P-value	**Low**	**High**	**Percent**	**P-value**	**Low**	**High**	**Percent**	**P-value**
WHO grade I	128	81	47	36.72%	0.001	94	34	26.56%	0.004	105	23	17.97%	*< 0.001*
Meningothelial	25	16	9			14	11			21	4		
Fibrous	32	20	12			23	9			26	6		
Transitional	61	37	24			51	10			50	11		
Angiomatous	6	5	1			4	2			5	1		
Psammomatous	3	2	1			2	1			2	1		
Metaplastic	1	1	0			0	1			1	0		
WHO grade II	37	22	15	40.54%	0.015	26	11	29.73%	0.039	18	19	51.35%	*0.003*
Atypical	29	17	12			22	7			16	13		
Chordoid	8	5	3			4	4			2	6		
WHO grade III	25	6	19	76.00%		11	14	56.00%		3	22	88.00%	
Anaplastic	16	1	15			5	11			1	15		
Rhabdoid	5	2	3			4	1			2	3		
papillary	4	3	1			2	2			0	4		

The numbers of CD47, PD-L1 and Ki-67 positive cells were counted using Image J software. The percentage of cases with low or high infiltration are presented for each marker (< or > the median score across all of the samples in the cohort for each marker, respectively).

Characterization of the immune infiltrate of meningioma in tissue specimens

IHC were performed to detect the infiltration of TAMs and lymphocytes in three grade of meningioma specimens (Table 3). We found that the immune cells infiltrate in WHO grade meningioma was mainly composed of macrophages (CD68) ($p = 0.006$ comparing grade I with grade) (Fig. 2A-R) with a significant decrease of CD3 + cells ($p = 0.008$ comparing grade I with grade) and CD20 + cells. IF co-staining showed the expression of CD68 was much higher than that of CD3, CD4 and FoxP3 lymphocytes (Fig. S4). Also, we verified the expression of CD68, CD47 and PD-L1 in tumor tissue sections of mice (Fig. S5). It indicated the limitations on immunotherapy associated with PD-L1 which worked with the involvement of T lymphocytes.

Table 3

Immune cells infiltrate in 190 cases of meningioma scored by Image J (separated into low and high expressors - relative to the median score for each marker for the entire cohort; specimens were classified according to pathological types)

Pathological		**CD3**				**CD4**				***CD8***			
types	**Cases**	**Low**	**High**	**Percent**	P-value	**Low**	**High**	**Percent**	**P-value**	Low	**High**	**Percent**	**P-value**
WHO grade I	128	50	78	60.93%	*0.008*	55	73	57.03%	*0.003*	53	75	58.59%	*0.038*
Meningothelial	25	10	15			8	17			11	14		
Fibrous	32	8	24			10	22			13	19		
Transitional	61	27	34			33	28			26	35		
Angiomatous	6	2	4			1	5			2	4		
Psammomatous	3	2	1			3	0			1	2		
Metaplastic	1	1	0			0	1			0	1		
WHO grade II	37	13	24	64.86%	*0.025*	18	19	51.35%	*0.031*	17	20	54.05%	*0.07*
Atypical	29	11	18			12	17			14	15		
Chordoid	8	2	6			6	2			3	5		
WHO grade III	25	16	9	36.00%		19	6	24.00%		17	8	32.00%	
Anaplastic	16	10	6			*12*	4			11	5		
Rhabdoid	5	2	3			*4*	1			3	2		
papillary	4	4	0			*3*	1			3	1		

Pathological		CD68				CD20				FoxP3			
types	Cases	Low	High	Percent	P-value	Low	High	Percent	P-value	Low	High	Percent	P-value
WHO grade I	128	52	76	59.38%	*0.006*	90	38	29.69%	*0.866*	88	40	31.25%	*< 0.001*
Meningothelial	25	16	9			11	14			18	7		
Fibrous	32	22	10			26	6			16	16		
Transitional	61	11	50			47	14			46	15		
Angiomatous	6	2	4			3	3			5	1		
Psammomatous	3	1	2			2	1			2	1		
Metaplastic	1	0	1			1	0			1	0		
WHO grade II	37	14	23	62.16%	*0.025*	25	12	32.43%	*0.710*	18	19	51.35%	*0.008*
Atypical	29	11	18			18	11			16	13		
Chordoid	8	3	5			7	1			2	6		
WHO grade III	25	3	22	88.00%		18	7	28.00%		4	21	84.00%	
Anaplastic	16	1	15			13	3			1	15		
Rhabdoid	5	1	4			3	2			2	3		
papillary	4	1	3			2	2			1	3		

The numbers of CD3, CD4, CD8, CD68, CD20 and FoxP3 positive cells were counted using Image J software. The percentage of cases with low or high infiltration are presented for each marker (< or > the median score across all of the samples in the cohort for each marker, respectively).

Anti-CD47 inhibits the proliferation, migration and invasion of meningioma in vivo and vitro

IOMM-Lee cells were divided into three groups. The experimental group was treated with anti-CD47 mAbs while the control group treated with IgG and blank control group without any treatment.

The cell scratch assay showed the growth rate of tumor cells in anti-CD47 group was significantly lower than control groups (Fig. 3A, F). Invasion assays showed that anti-CD47 suppressed the migration and invasion ability of malignant meningioma cells (Fig. 3B-E). In addition, CCK-8 assays showed that the absorbance of cells treated with anti-CD47 was significantly lower than control groups (Fig. 3G). All the above experiments were repeated three times. The differences were statistically significant ($P < 0.05$).

In vivo, we observed the subcutaneous malignant meningioma formation in nude mice. The mice with established tumors were randomized into three groups including group with injection of anti-CD47, control group with IgG and blank group with PBS (Fig. 4A). The changes of tumor tissues sizes in three groups showed that tumors in anti-CD47 group was significantly smaller than in other two groups ($p < 0.05$) (Fig. 4B). The above results confirmed that blocking CD47 can effectively inhibit the proliferation of meningioma cells and suppressed the migration and invasion of tumor cells (Fig. 6).

Anti-CD47 induces macrophage-mediated phagocytosis in malignant meningioma

According to previous studies, co-culture of tumor cells with macrophages under the intervention of anti-CD47 could promote tumor recognition and phagocytosis of macrophages [23]. Thus, malignant meningioma cells and macrophages were co-cultured. Then, we used IF to detect the difference of ability of phagocytosis between the control groups and the experimental group treated with anti-CD47 which showed that the phagocytosis rate of macrophages increased in anti-CD47 group (Fig. 4C, D). The difference was statistically significant.

CD47 participates in multiple signaling pathways to affect meningioma progression

Various signaling pathways associated with CD47 have been extensively studied [1]. However, signaling pathways in malignant meningioma are still limited.

Therefore, we examined the expression of EGFR, ERK and β-catenin in meningioma from WHO grade - by IHC. The results showed ERK, EGFR and β-catenin were expressed in all grades of meningiomas with the highest expression in WHO grade meningioma (Fig. 5A-F). Then we used si-RNA targeted to downregulate CD47 expression. The results showed a significantly decrease in EGFR expression (Fig. 5G, H). Meanwhile, si-CD47 treatment also showed there was a close relationship between the expression of CD47 and EGFR.

Discussion

At present, many studies have reported the progress of tumor immunotherapy [24]. Classical immune checkpoint such as PD-L1 has been reported in meningioma [15]. It was found that expression of PD-L1 was associated with increased tumor aggressiveness [25]. Many PD-L1 inhibitors are showing good results in other tumors [26]. However, we performed IHC and found that compared with PD-L1, the expression of CD47 was higher in malignant meningioma which showed PD-L1 checkpoint blockade not to be effective on malignant meningioma.

The expression of CD47 has been reported in many tumors such as breast cancer, thyroid cancer, etc [8–10]. CD47 mainly binds the ligands SIRPα [7]. Blocking CD47 with antibody turns off "don't eat me" signal and favors phagocytosis [12, 27].

And the experiment results showed that the expression of CD47 was higher in WHO grade meningioma than in grade and . The detection of immune cells infiltrated in tumor tissue showed that there were decreased expression of CD3+, CD4 + and CD8 + cells in malignant meningioma which implied that blocking PD-L1 may not have obvious effect for malignant meningioma. Previous studies demonstrated the therapeutic effect of CD47 blockade requires functional DCs [28]. Meanwhile, we found that macrophages (CD68) had an increased expression in malignant meningioma, which indicated blocking CD47 may be a potential therapy target.

Previous studies have shown that anti-CD47 can inhibit the proliferation, invasion and migration of tumor cells [9, 11, 14]. However, no one has reported the effect on malignant meningioma. So we conducted experiments above and found that anti-CD47 could inhibit the proliferation, migration and invasion ability of malignant meningioma. Furthermore, we observed the ability of anti-CD47 in vivo. The results showed that anti-CD47 could inhibit the progression of meningioma.

CD47 on the tumor surface produces corresponding signals by binding to SIRPα on macrophages, which makes macrophages unable to recognize and phagocytize tumor cells [29]. Tumor cells and macrophages were co-cultured, and treated with anti-CD47 or IgG. IF and FACS results showed that the activation and phagocytosis rate of macrophages in anti-CD47 group were significantly higher than in control group, which confirmed the suggestion above.

According to previous studies, there were various signaling pathways related to CD47 [30, 31]. The most closely related to CD47 were EGFR, ERK and Wnt signaling pathways [32–34]. Here, we observed these pathways in malignant meningioma, which showed that ERK, EGFR was highly expressed in malignant meningioma compared to β-catenin. And the expression of EGFR was higher in high expression of CD47 group, which suggesting CD47 may affect the progression of meningioma by participating in EGFR pathway. Moreover, meningioma cells are capable of autocrine expression of the ligands of EGFR, such as TGF-α and EGF [1]. Previous studies showed that B6H12 down regulated EGFR expression at the mRNA and protein levels [32]. These suggest that therapeutic CD47 antibodies may be effective against tumors with high EGFR expression when used alone or in combination with EGFR inhibitors., which may be a novel treatment direction for malignant meningioma.

However, in clinical trials, anemia and thrombocytopenia have become the dose-limiting toxicity of CD47 because it is also involved in the clearance of red blood cells in the human body. More importantly, the large number of red blood cells in the body will be the best cover for tumor cells, and CD47 drugs will be depleted by red blood cells before they reach the tumor cells.

Conclusion

To sum up, we should realize that anti-CD47 plays a practical and effective role in eliminating tumor immune escape and inhibiting the proliferation and migration of malignant meningioma. At the same time, we should also consider that the core of CD47 drug development is how to kill tumor cells to the maximum extent while protecting red blood cells from accidentally injure [35]. It is believed that with the development of targeting technology and drug targeting carriers, anti-CD47 drugs will be targeted and quantitatively acted on local tumor tissues, especially tumors such as malignant meningioma, so as to achieve the effective cure of tumors.

Declarations

ACKNOWLEDGEMENTS

We thank staff and patients who participated in the clinical study. We thank other members at the Nanfang Hospital for participating in useful discussions.

AUTHOR CONTRIBUTIONS

Conception and design: Xiaotong Liu, Huarong Zhang; Development of methodology: Xiaotong Liu, Huarong Zhang, Chaohu Wang; Acquisition of data: Xiaotong Liu, Huarong Zhang, Chaohu Wang, Zhiyong Li, Qianchao Zhu, Yiwen Feng, Jun Fan; Analysis and interpretation of data: Xiaotong Liu, Huarong Zhang, Chaohu Wang; Writing, review and/or revision of the manuscript: Xiaotong Liu; Administrative, technical or material support: Songtao Qi, Yi Liu, Zhiyong Wu; Study supervision: Songtao Qi, Yi Liu, Zhiyong Wu. The work reported in the paper has been performed by the authors, unless clearly specified in the text.

COMPETING INTERESTS

The authors declare no competing interests.

AVAILABILITY OF SUPPORTING DATA

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

ETHICS DECLARATIONS

This study was carried out in accordance with the recommendations of the Ethics Committee of Nanfang Hospital with written informed consent from all subjects. Written informed consent or consultee approval to enroll was secured for all study participants. All data were anonymized. Animal studies were carried out under protocols approved by the Southern Medical University Institutional Animal Care and Use Committee.

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Figures

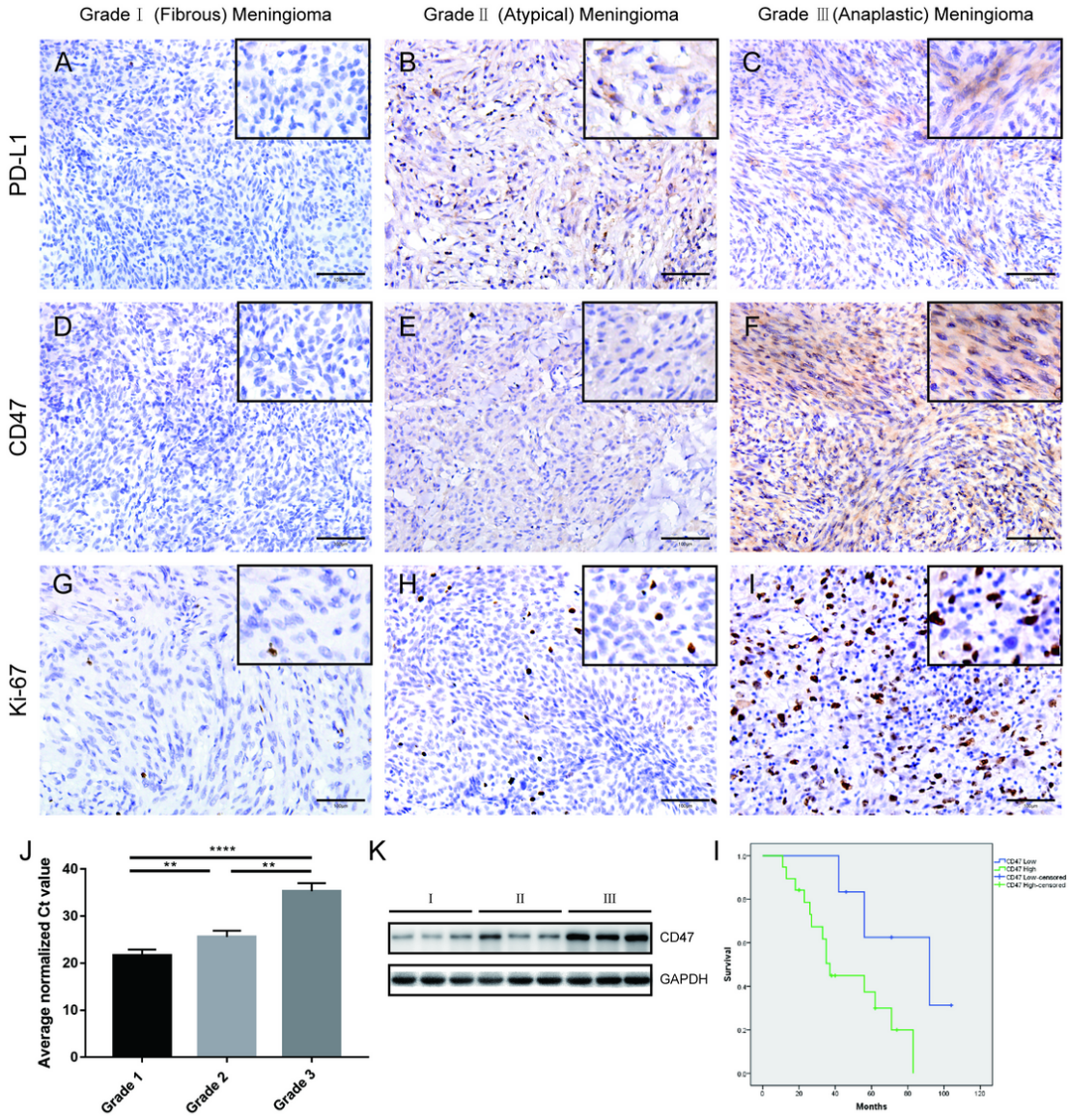

Figure 1

The expression analyses of CD47, PD-L1 and Ki-67 in grade Ⅰ, Ⅱ, Ⅲ meningioma.

A-C IHC showed PD-L1 was expressed higher in grade III. **D-F** CD47 has an increased expression in grade meningioma, and the expression was stronger than PD-L1 in grade meningioma. **G-I** The expression of Ki-67 was increased in grade . **J** CD47 Q-PCR in primary meningioma cells from 9 cases of patients in three grades meningioma. **K** CD47 expression was analyzed by western blot. Results from primary meningioma cells of patients in three grades meningioma. **L** Kaplan–Meier survival curves for malignant meningioma patients comparing those with CD47 expression higher than the mean and those without increased CD47 expression. **J** **p < 0.01, ***p < 0.001 by paired Average normalized Ct value. **A-K** Original magnifications: × 200.

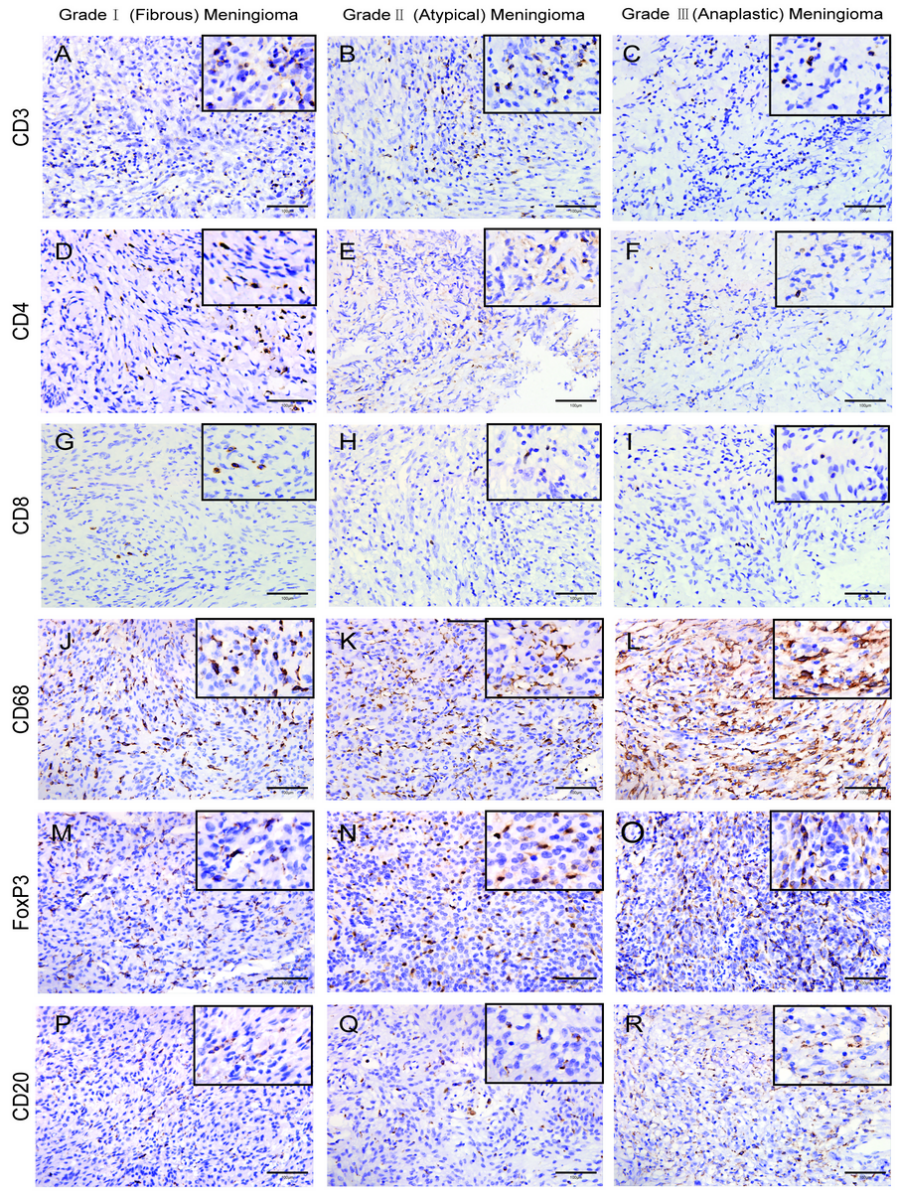

Figure 2

Immune cells infiltrate in FFPE from grade Ⅰ, Ⅱ and Ⅲ meningioma patients.

A-I IHC for CD3, CD4, CD8 (marker of T cells) in grade , and meningioma. **J-R** IHC for CD68(marker of macrophage), CD20(marker of B cells) and FoxP3(marker of Treg cells) in grade , and meningioma. **A-R** Original magnifications: × 200.

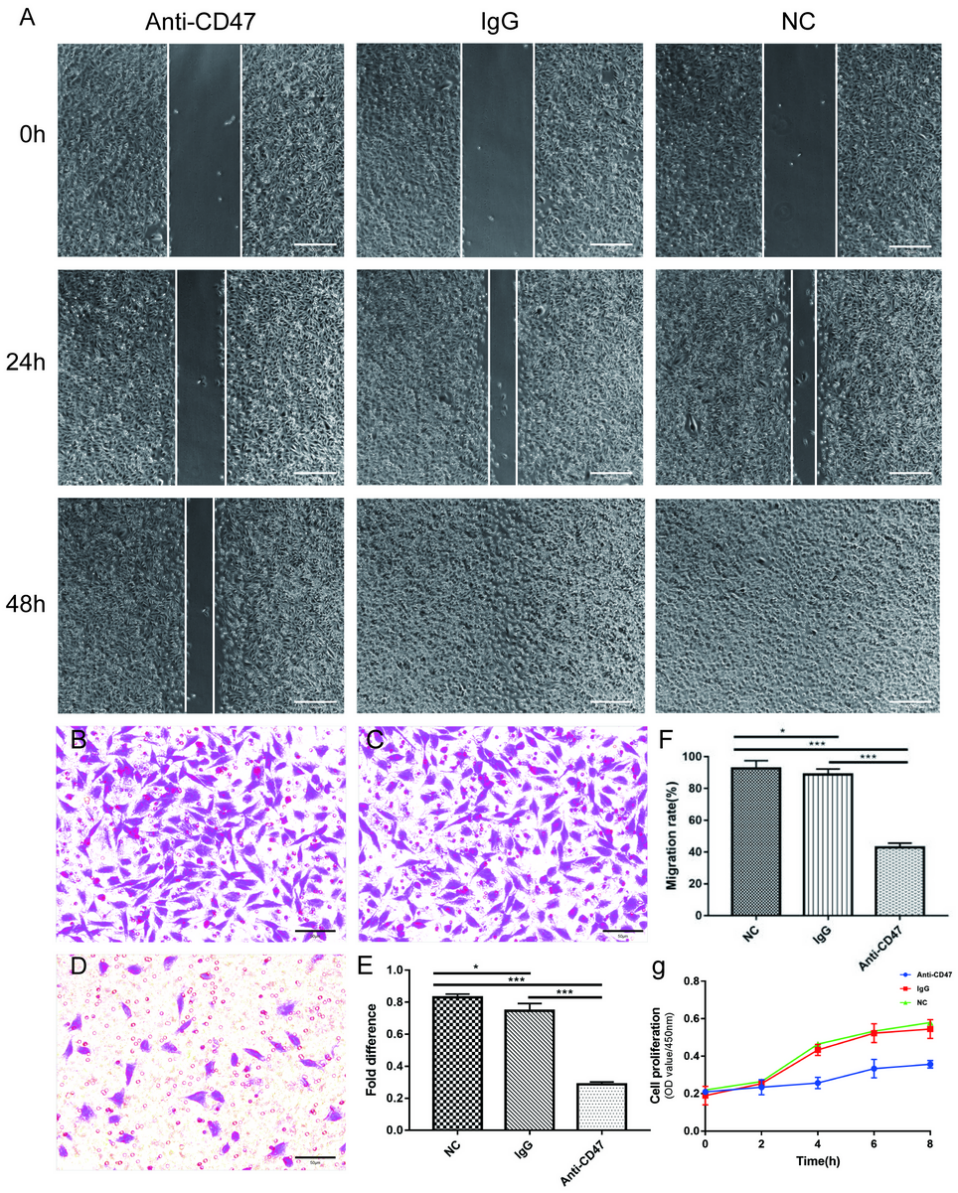

Figure 3

Migration, invasion and proliferation assay of IOMM-Lee cells with anti-CD47, IgG or NC.

A The scratch figure from 0h to 48h taken by microscope every 6h. **B-E** The invasion images of cells the treatment of NC, IgG and anti-CD47. **F**The migration rate of three groups were analysed via SPSS which showed anti-CD47 efficiently inhibited cell proliferation and migration in malignant meningioma. **G** The efficiency of anti-CD47 to the proliferative ability of IOMM-Lee cells. **E** *p < 0.05, ***p < 0.001 by paired Fold difference. **G** The results of cytotoxicity assay were shown by the cell proliferation rate. **A** Original magnifications: × 200, (b-d) × 400.

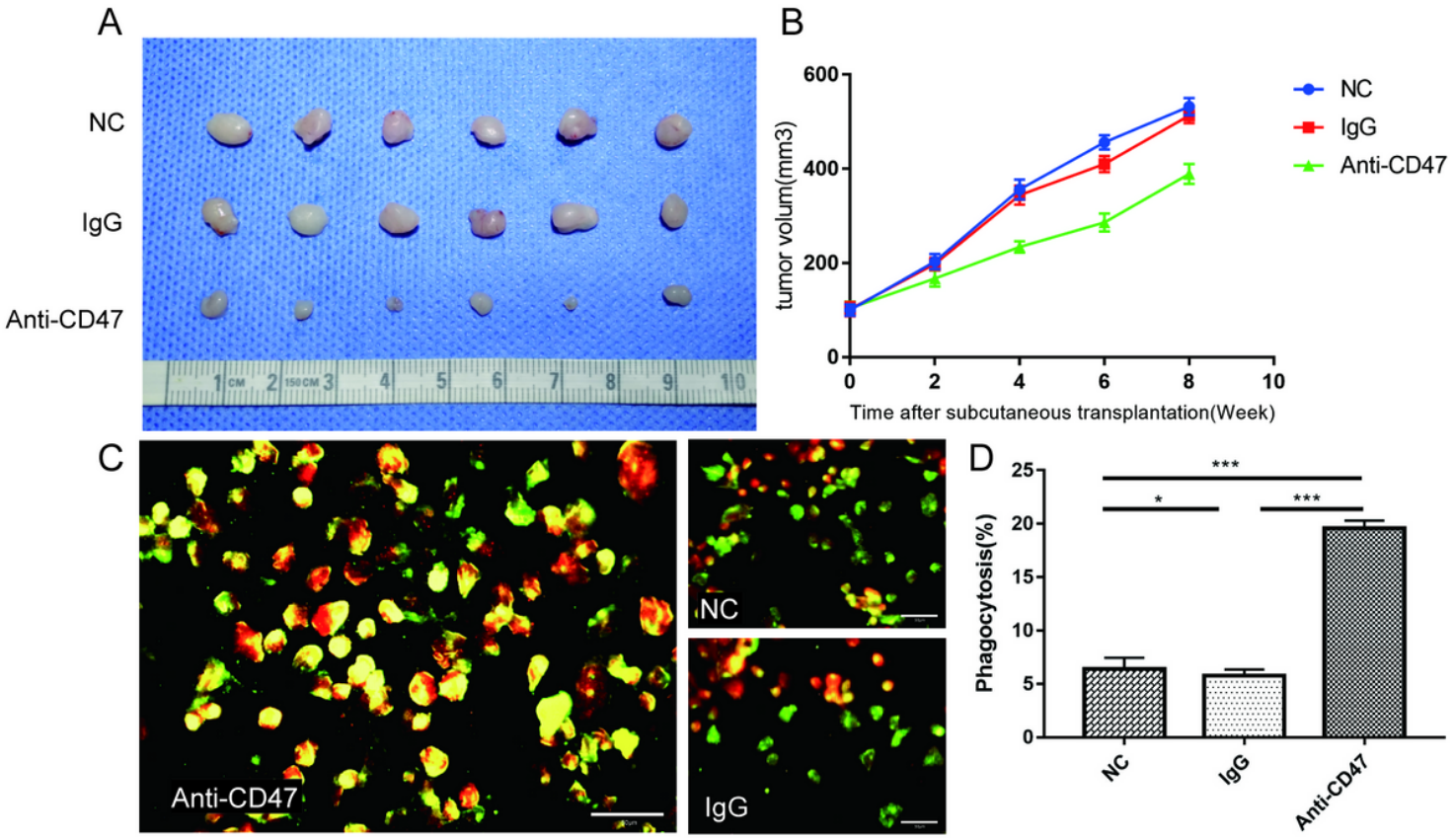

Figure 4

Nude mouse models bearing subcutaneous meningiomas and in vitro phagocytosis assay.

A Volume of subcutaneous tumor in nude mice. Curve of tumor volume changes in nude mice after subcutaneous tumor transplantation. **B** Macrophages were serum-starved for 2 h, followed by co-culture with 1×106 CFSE-labeled effectively phagocytose cancer cells in the presence of IgG, anti-CD47 B6H12 antibody or NC. **C** After 2 h of co-culture, wells were thoroughly washed and multiple fields of view (FOV) were imaged on an inverted fluorescence microscope. Representative overlay images (one section of a FOV) for each condition are shown. RFP+ macrophages were co-cultured with IOMM-Lee cells in the presence of IgG, anti-CD47 B6H12 (blocking) or NC. **D** The percentage of macrophages with ingested CFSE+ tumor cells was known as phagocytosis index. The experiment was performed three times with similar results. *p < 0.05, ***p < 0.001 by paired phagocytosis (%).

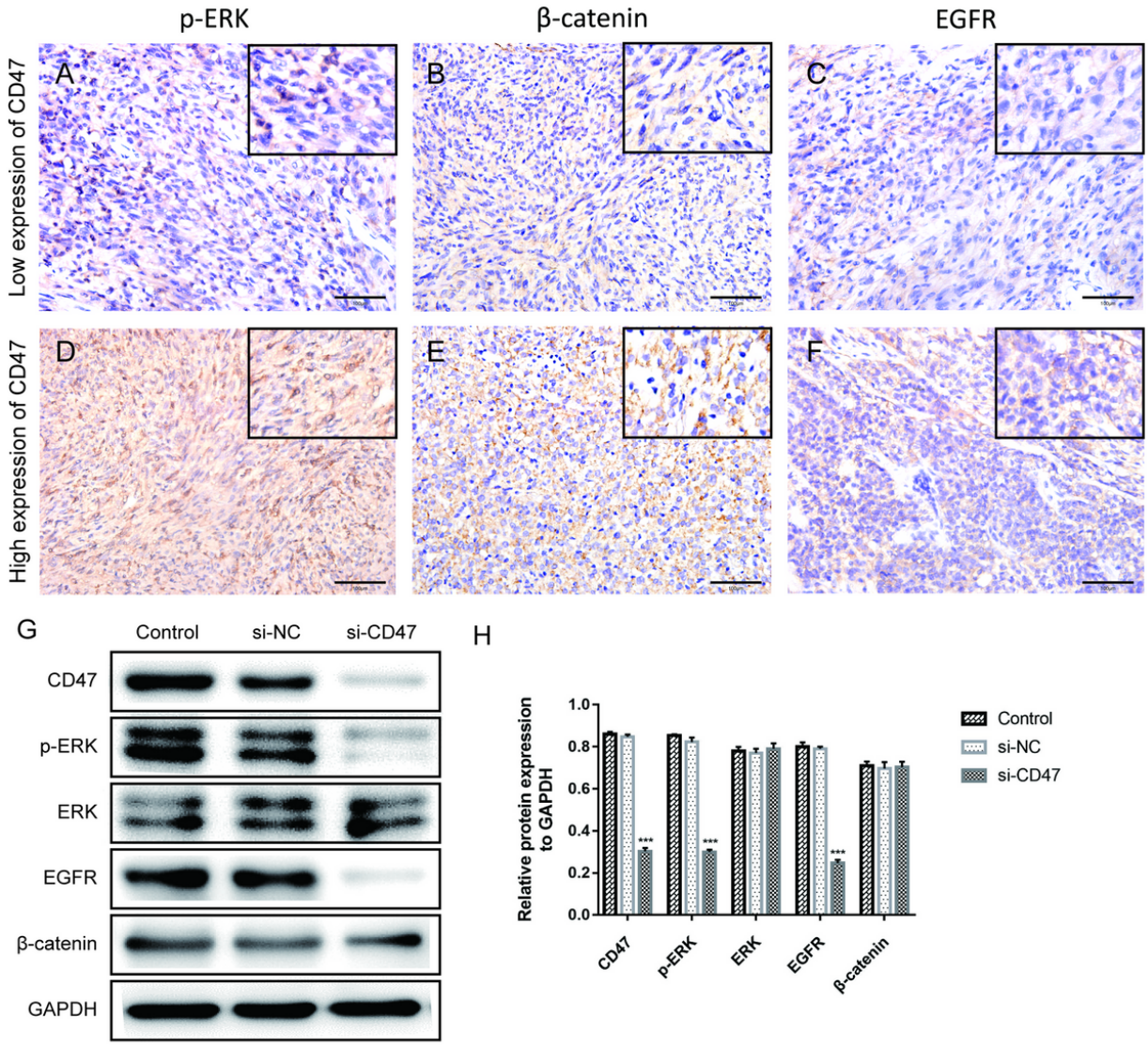

Figure 5

The expression of factors of different pathways in grade Ⅱ meningioma.

A-C IHC for p-ERK, β-catenin, EGFR in grade meningioma with low expression of CD47. **D-F** IHC for p-ERK, β-catenin, EGFR in grade meningioma with high expression of CD47. **A-F** Original magnifications: × 200. **G-H** Western blot analysis showed that CD47 protein level of IOMM-Lee cells transfected with si-CD47 was significantly reducedcompared with that in si-NC and control group. And there were significantly decrease in p-ERK and EGFR, while the expression of β-catenin was not. **p < 0.01 vs the control group.

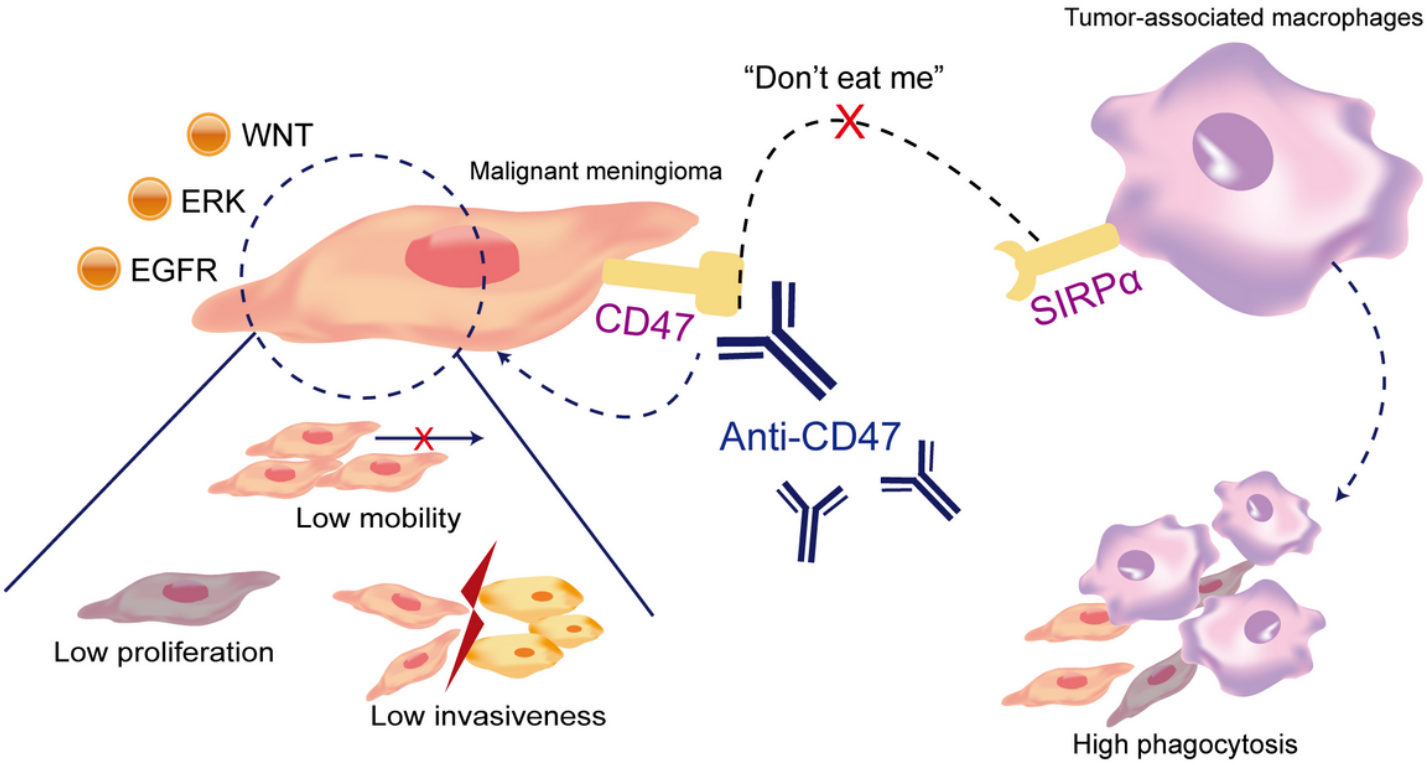

Figure 6

Model diagram of anti-tumor effect of anti-CD47.

Anti-CD47 cuts off the "don't eat me" signal by blocking the binding of the tumor surface protein CD47 to the macrophage surface protein SIRPα. After blocking CD47, the ability of proliferation, migration and invasion of meningioma cells decreased, while the ability of tumor-associated macrophages to phagocytize meningioma cells was enhanced. EGFR, ERK and Wnt pathways may be involved in the above process.

Supplementary Files

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