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Vascular adhesion protein 1 (VAP-1) as a regulator mediates tumor immune escape in human astrocytomas

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Abstract

Vascular adhesion protein-1 (VAP-1) is the endothelial adhesion molecules which were considered to be an essential component in tumor progression and metastasis, supporting cancer cell extravasation. Rare studies have been performed on analyzing the contribution of VAP-1 in astrocyomas. In previous results, we found VAP-1 expressed different pattern in different grades of astrocytoma that can distinguish the tumor malignancy. It could be a promising prognostic biomarker in astrocytoma patients, but the mechanism in regulating tumor progress is still unclear. This study aimed to achieve the support for our hypothesis that VAP-1 might contribute to astrocytoma progression via mediating tumor immune responses. We investigated the tumor cells and the tumor-associated macrophage (TAM) exhibited the VAP-1 levels that presented the clinical significance and its prognostic value. The expressions of VAP-1 were detected by immunohistochemistry in astrocytoma tissues. The prognostic role of VAP-1was evaluated using univariate and multivariate analysis in 112 astocytoma patients. IHC results revealed significantly higher expressions of VAP-1 and TAM-related proteins (CD68, iNOS, CD163) in patients with malignant astrocytomas. High values for VAP-1 significantly correlated with grade, patient survival, and IDH1 mutant ($p=<0.0001$, <0.0001 and 0.0105, respectively). Overall survival rate was significantly associated with over-expressions of VAP-1 ($p<0.0001$) as well as co-expression of VAP-1and TAM-related proteins ($VAP\text{-}1^{High}/CD68^{High}$, $p=0.0166$; $VAP\text{-}1^{High}/iNOS^{High}$, $p=0.0021$; $VAP\text{-}1^{High}/CD163^{High}$, $p=0.0513$). These results indicate that VAP-1 may have an important role in the regulation of tumor immune via changing the tumor-associated macrophages.

Key words: VAP-1, astrocytoma, malignancy, prognosis

Introduction

Astrocytomas are the most common type of brain tumors, which are classified by World Health Organization (WHO) into four grades according to the degree of malignancy (1). Potential contributors to the pathogenesis of astrocytoma include germline mutations, somatic mutation, variation in copy number of genes, and variation in loci of genes. Ionizing radiation exposure is another well-known risk factor for astrocytoma development (2). An estimated 60 to 70% of malignant astrocytomas are GB (2). The median survival time for newly diagnosed GB patients is only approximately 1 year, mainly because patients respond poorly to current therapeutic modalities. Although significant improvement has been achieved in surgical techniques and adjuvant treatment, the prognosis of patients with astrocytoma remains poor, with a 5-year survival rate of less than 23% (3).

Vascular adhesion protein-1 (VAP-1; AOC3) is one of the endothelial adhesion molecules that are believed to play a role in tumor progression and metastasis, supporting cancer cell extravasation (4). Very few studies have been performed on analyzing the contribution of VAP-1 in brain tumor. Our previous results showed the levels of VAP-1 were significantly higher in malignant astrocytomas. VAP-1 expression was significantly correlated with the overall survival, which as an independent predictive marker for poorer prognosis in astrocytomas (5).

Some studies indicated VAP-1 induces recruitment of tumor associated macrophages (TAM) then promotes cancer cell extravasation (6). In tumor immune, M2-polarized macrophages enhances tumor immune escape through secreting immunosuppressive mediators and shutdown of T cell–mediated immunity by blocking the ability to present antigen to T cells (7). VAP-1 is expressed in blood vessels and contributes to M2 Macrophage infiltration underlies IL-1β–induced lymph- and angiogenesis in cancers (8).

In previous study, we found VAP-1 can be a predict marker in human astrocytoma, but the mechanism regulating tumor progress is still unclear. This present study aimed to achieve the support for our hypothesis that VAP-1 might have contribution to brain tumor progression, by investigating the expression of VAP-1 on different types of the tumor-associated macrophage and its prognostic significance.

Study design

Part 1. To Identify the independent predict potential of VAP-1 (AOC3) in human astrocytoma from TCGA database

First, we predicted the potential of VAP-1 expression in astrocytoma form the big database analysis. Relative expression of VAP-1 and Kaplan–Meier (KM) survival were performed with RNA sequencing data of 513 DCGs and 161 GBM samples obtained from The Cancer Genome Atlas (TCGA) website (https://tcga-data.nci.nih.gov). These data were analyzed by UALCAN, an interactive web-portal to perform to in-depth analyses of TCGA data (UALCAN is publicly available at http://ualcan.path.uab.edu). Box-whisker plots showed the levels of VAP-1 in sub groups of LGG and GBM. KM plots depicted association of VAP-1 gene expression with glioma patient survival. A P value less than 0.05 was considered statistically significant.

Part 2. To investigate VAP-1 expression in astrocytoma tissues and the tumor-associated macrophage

First, we selected the specimens from the patients diagnosed with astrocytoma on central pathological review and were analyzed for survival rate. The levels of VAP-1 in the tumor tissue and the tumor-associated macrophage from astrocytoma were detected by immunohistochemistry. We also compare the VAP-1 expression in different types of the tumor-associated macrophages: macrophage (CD68), M1 (iNOS) and M2 (CD163). The correlation between the clinicopathological parameters and the differences of VAP-1 expression were also evaluated using Chi-square test. The survival analysis was performed using Kaplan-Meier method to compare the prognosis of patients in relation to the VAP-1 expression level and the results were then determined by using log-rank test. Cox logistic regression analysis was applied to evaluate the independent influence of various clinicopathological factors on the survival.

Material and methods

UALCAN analysis

Brain lower grade glioma (LGGs) and glioblastoma multiforme (GBM) data sets were obtained from The Cancer Genome Atlas (TCGA) database. Genes that positively correlated with VAP-1 (AOC3) expression in LGG and GBM database were analyzed by UALCAN. UALCAN, an easy to use, interactive web-portal to perform to in-depth analyses of TCGA gene expression data (9), is publicly available at http://ualcan.path.uab.edu. The results were validated using two datasets from 513 LGG cases and 161 GBM cases available from TCGA. The expression of VAP-1 in sub groups of LGG and GBM were performed by box-whisker plots. Kaplan–Meier plots depicted the association of VAP-1 expression levels with patient survival.

Patient selection

The available tumor tissue and clinical follow-up information were included. The clinicopathological informations were obtained from the cancer center and collected from the medical records of patients, including age, gender, WHO grade, the tumor size, and the recurrence status. The overall survival time was calculated from the date of first diagnosis until the last follow-up or until death of the patients, with the maximum follow-up time being 60 months (or 5 years). Patients who had insufficient clinical and/or pathology information were excluded from this study to improve the final result analysis. Approval to link laboratory data to clinical and pathologic data was obtained from the Institutional Review Board (KMUHIRB-E(I)20180113).

Immunohistochemistry (IHC)

3 μm-thick paraffin-embedded sections were de-paraffinized in xylene and dehydrated through graded alcohols. Antigen retrieval was performed in 0.1 M citrate buffer (pH 6.0) at 121˚C for 10 min. The slides were then incubated in 3% hydrogen peroxide at room temperature to quench endogenous peroxidase. The target proteins were detected with anti-human rabbit polyclonal antibodies, including: VAP-1 (1:500; Santa Cruz Biotechnology, Inc., USA), CD68 (1:200; DAKO, Denmark), iNOS (1:500; Sigma, USA) and CD163 (1:200; DAKO, Denmark). Incubation with primary antibodies were performed for overnight at 4℃. The antigen-antibody complexes were visualized using the DAKO REAL Envision Detection System, Peroxidase/DAB, Rabbit/Mouse (DAKO, Denmark), followed by hematoxylin counterstaining and mounting.

Pathologic Evaluation

The immunoreactivity of markers was evaluated on the basis of the proportion score and the intensity score of positively stained tumor cells (21). In scoring the proteins

expression, both the extent and intensity of immunopositivity in the cytoplasm or nucleus were considered. The score for the markers were evaluated by the sum of the percentage of positive cells (0:0%; 1:<10%; 2:10-50%; 3:>50%) and staining intensity graded from 0 to 3 (0, negative; 1, weak; 2, moderate; and 3, strong). Scores for 6 or greater were considered to represent high expression and those of 4 or lower to indicate low expression.

Statistical Analysis

Descriptive statistics of the study population, including means (with corresponding standard deviations), medians (with corresponding ranges), and proportions, together with 95% CIs were computed. Association between each paired group was tested using chi-squared test. Survival and hazard functions were estimated using the Kaplan-Meier method, and survival between groups was compared using the two sided log-rank test (log-rank test for trend only where ≥ three groups were entered in logical order). The multivariate Cox proportional hazards regression model was used to examine risk factors related to survival after adjusting for other factors. Risk factors included age, tumor size, pathology prognosis, grade, TNM staging and recurrent as well as survival status. Interaction between tumor-associated macropahge marker and VAP-1 related proteins were analyzed. Significance was established at $P \leq 0.05$. Statistical analyses were carried out using SAS 9.3 (SAS Institute, Cary, NC).

Results

The independent predict potential of VAP-1 in human astrocytoma from GEO database

First, we predicted the potential of VAP-1 expression in astrocytoma form the big database analysis. The UALCAN analysis data showed that VAP-1 (AOC3) presented no significant difference among low grade diffuse gliomas (LGGs), normal tissue and glioblasotmas (GBMs). It was also not a significant predictor for glioma patient survival. These results only showed VAP-1 existed in LGG or GBM groups, we can't observe the comparing data involving grade II, III, and IV basing on some limitations from UALCAN analytic platform.

VAP-1 expression correlated with clinicopathological variables in astrocytoma patients

Figure 1 shows representative images of expressions of VAP-1 and TAM-related proteins (CD68, iNOS, and CD163) observed in immunohistochemical analyses of astrocytoma patients. The VAP-1 expression was low in 43(38.39%) patients and high in 69(61.61%) patients. Table 1 shows the results for Chi-square test, which revealed that expression of VAP-1 was significantly associated with tumor grade ($p<0.0001$), survival status ($p<0.0001$), and IDH1 mutant ($p=0.0105$). The relationships between VAP-1 and TAM-related proteins (CD68, iNOS, and CD163) were performed in table2. High VAP-1 expression was significantly correlated with high level of TAM marker, CD68 ($p<0.0001$), M1 macrophage marker, iNOS ($p=0.0050$), and M2 macrophage marker, CD163 ($p<0.0001$). We found high levels of VAP-1 presented in malignant regions and surround blood vessels and exuberant TAM activity also existed in the similar areas.

VAP-1 and TAM-related proteins expression and patient survival

Figure 3A showed overall survival was significantly lower in patients with high VAP-1 expression ($p<0.0001$). The patients coexpressed VAP-1 and TAM-related proteins ($VAP\text{-}1^{High}/CD68^{High}$, $VAP\text{-}1^{High}/iNOS^{High}$, $VAP\text{-}1^{High}/CD163^{High}$) also had poor outcome (Figure 3B-D). According to the univariate analysis for different clinicopathological characteristics, including VAP-1, CD68, CD163, gender, and recurrence status, we found that VAP-1 expression was significantly associated with patient survival ($P = 0.0008$; Table 3). Furthermore, multivariate logistic analysis showed that high VAP-1 expression was a statistically independent predictive factors of poorer prognosis in astrocytoma patients ($VAP\text{-}1^{High}$, HR:1.942, 95% CI: 1.003-3.761; $P = 0.0489$; Table 3).

All these results will be helpful to understand the potential of the VAP-1, a novel

molecular and the associated researches have the novelty and perspicacity in human astrocytoma. We found VAP-1 express in malignant astrocytomas linking TAM activities that may imply VAP-1 regulates tumor immune via changing the tumor-associated macrophages. We will clarify the mechanism under in vitro and in vivo experiments. The design of this project was derived from the original data of PI's laboratory which provided the characteristic of originality and creation as well as the value of clinical application. These results provided an important reference in the future studies and clinical application. All data will be published on the scientific journals and present on the international conferences.

Table1. Correlation of VAP-1 and clinicopathological parameters in patients with astrocytoma

Parameters	n	VAP-1		*p* value
		Low(%)	High(%)	
	112	43(38.39)	69(61.61)	
Gender				0.4826
Female	41	14(32.56)	27(39.13)	
Male	71	29(67.44)	42(60.87)	
Age				0.2486
≤45 years	42	19(44.19)	23(33.33)	
>45 years	70	24(55.81)	46(66.67)	
Tumor grade				<0.0001[*2]
II	27	21(48.84)	6(8.70)	
III	36	16(37.21)	20(28.99)	
IV	49	6(13.95)	43(62.32)	
Tumor size				0.1483
< 2 cm	66	29(67.44)	37(53.62)	
≧2 cm	46	14(32.56)	32(46.38)	
Recurrence				0.6809
Absent	65	26(60.47)	39(56.52)	
Present	47	17(39.53)	30(43.48)	
Survival status				<0.0001[*2]
survived	32	22(51.16)	10(14.49)	
died	80	21(48.84)	59(85.51)	
IDH1 mutant				0.0105[*1]
Negative	85	27(62.79)	58(84.06)	
Positive	27	16(37.21)	11(15.94)	

Chi-square test was used for statistical analysis.
[*1]Statistically significant (P < 0.05).
[*2] Statistically significant (P < 0.01).

Table2. Relationships between expressions of VAP-1 and TAM-related markers (CD68, iNOS, and CD163)

Parameters	n	VAP-1		*p* value
		Low(%)	High(%)	
TAM marker				<0.0001[*2]
$CD68^{Low}$	25	20(46.51)	5(7.25)	
$CD68^{High}$	87	23(53.49)	64(92.75)	
M1 marker				0.0050[*2]
$iNOS^{Low}$	37	21(48.84)	16(23.19)	
$iNOS^{High}$	75	22(51.16)	53(76.81)	
M2 marker				<0.0001[*2]
$CD163^{Low}$	31	25(58.14)	6(8.70)	
$CD163^{High}$	81	18(41.86)	63(91.30)	

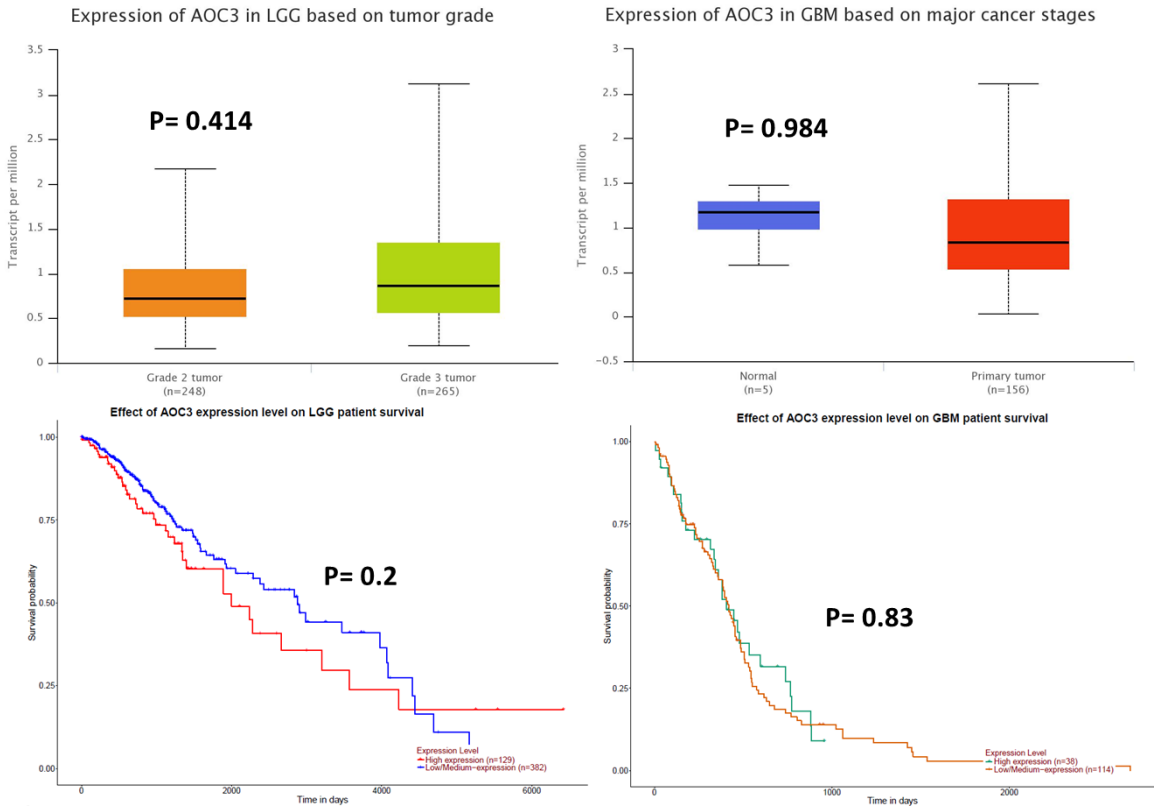
Chi-square test was used for statistical analysis.
[*1]Statistically significant ($P < 0.05$).
[*2] Statistically significant ($P < 0.01$).

Table3. Univariate and multivariate logistic analysis of clinicopathological independent prognostic factors for survival of astrocytoma patients

Parameters	Univariate		Multivariate	
	HR (95% CI)	*p* value	HR (95% CI)	*p* value
VAP-1		0.0008[*2]		0.0489[*1]
Low	1		1	
High	2.348(1.424-3.872)		1.942(1.003-3.761)	
CD68		0.0133		0.5314
Low	1		1	
High	2.240(1.183-4.243)		0.752(0.309-1.834)	
CD163		0.0133		0.0062
Low	1		1	
High	2.240(1.183-4.243)		3.204(1.392-7.375)	
Gender		0.8763		0.8011
Female	1		1	
Male	1.037(0.657-1.637)		1.062(0.666-1.693)	
Recurrence		0.6531		0.3632
Absent	1		1	
Present	1.106(0.712-1.719)		0.804(0.502-1.287)	

[*1]Statistically significant (P < 0.05).
[*2] Statistically significant (P < 0.01).

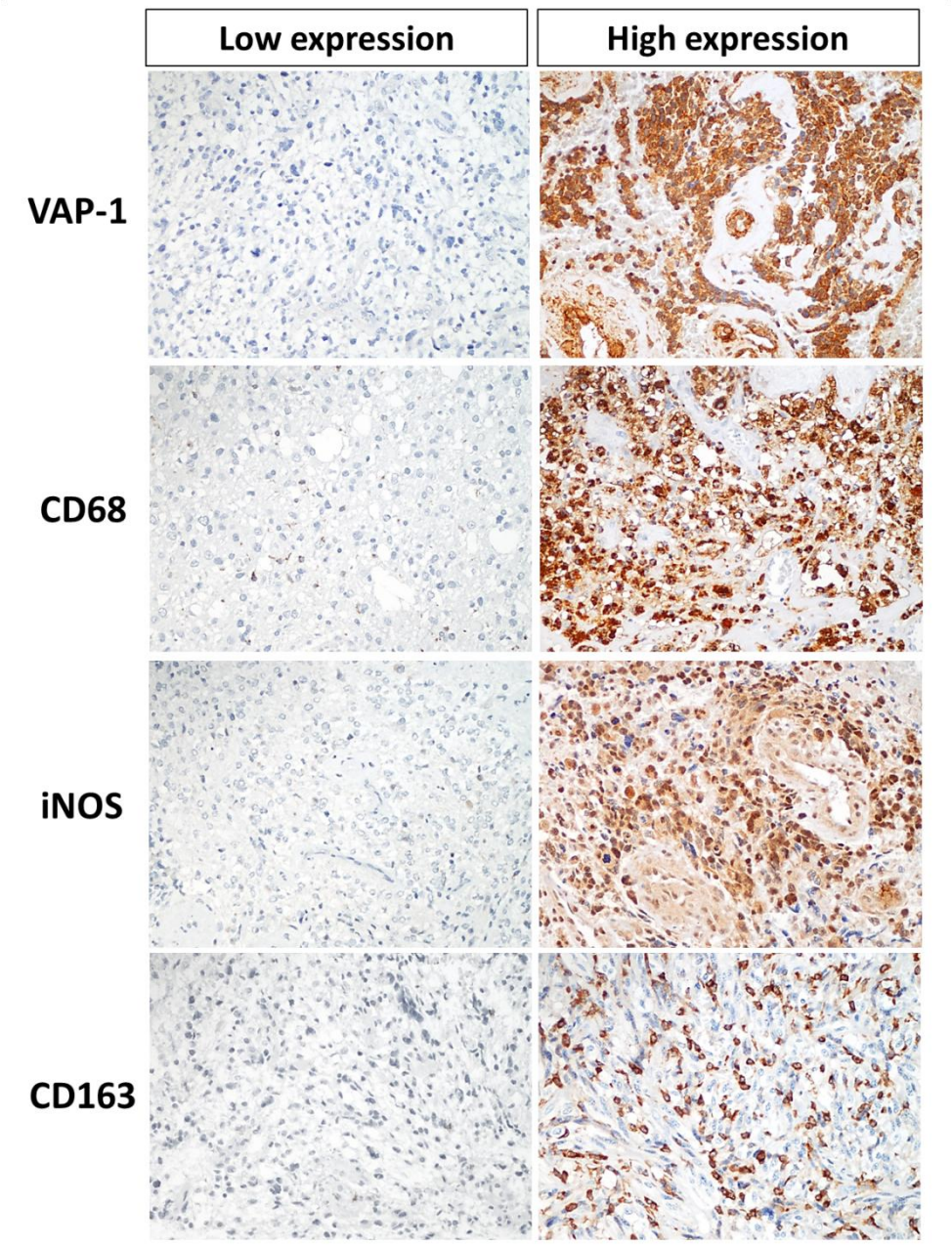
Figure 1.



Identify the independent predict potential of VAP-1 (AOC3) in astrocyomtas from UALCAN analysis.

Relative expression of VAP-1 and Kaplan–Meier (KM) survival were performed with RNA sequencing data of 513 DCGs and 161 GBM samples obtained from TCGA website and data were analyzed by UALCAN perform. Box-whisker plots showed the levels of VAP-1 in sub groups of LGG and GBM. KM plots depicted association of VAP-1 gene expression with glioma patient survival. *Statistically significant (p < 0.05).

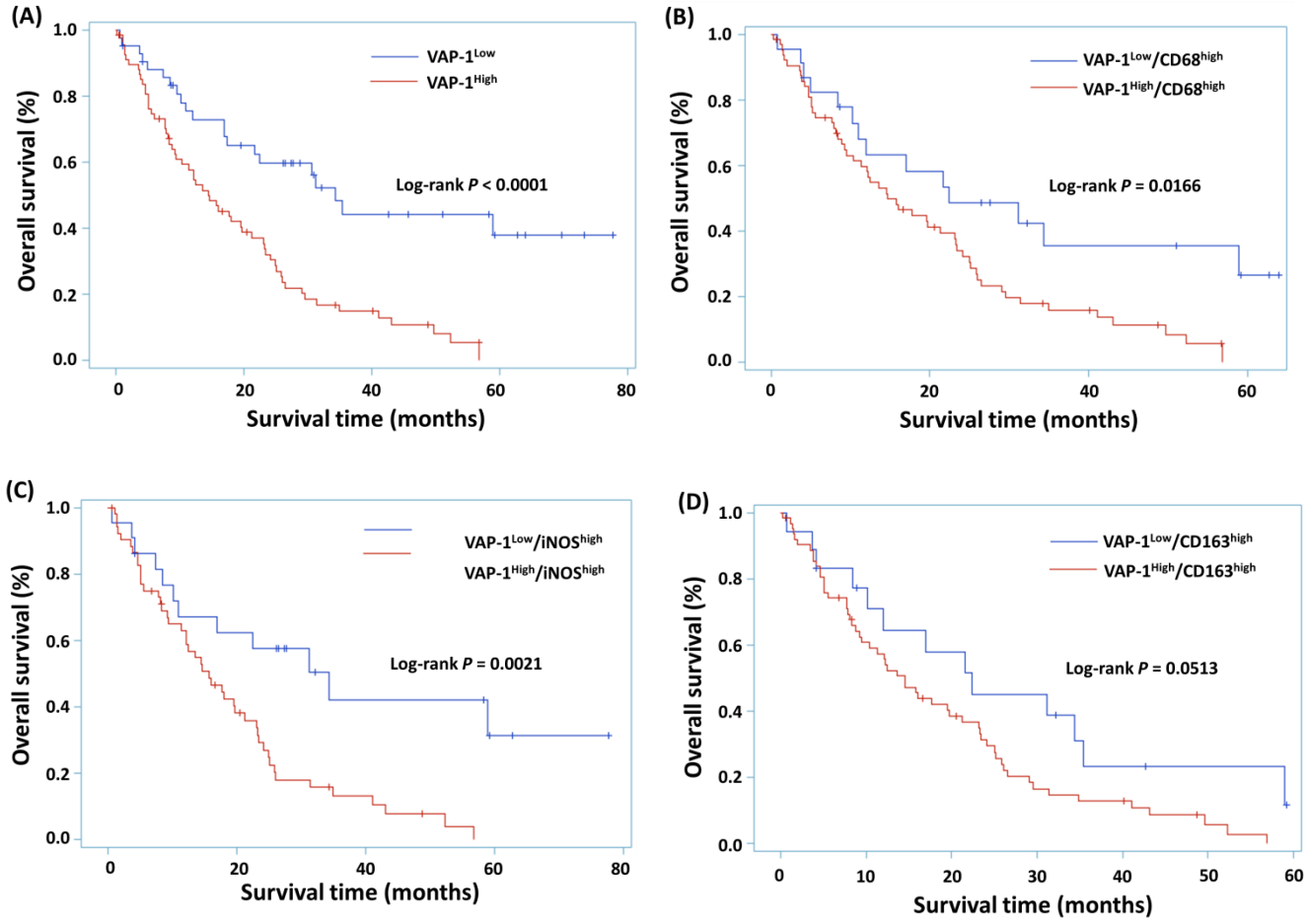
Figure 2.



VAP-1 and macrophage-related proteins (CD68, iNOS, CD163) were detected in astrocytoma tissues.

Representative immunostaining photomicrographs of high and low proteins expression. IHC staining results for expressions of VAP-1, CD68, iNOS and CD163 in in astrocytoma tumor cells. *Original magnification: 200x.

Figure 3.



Kaplan-Meier overall survival curves for astrocytoma patients with different levels of (A)VAP-1, (B)VAP-1/CD68^{High}, (C)VAP-1/iNOS^{High} and (D)VAP-1/CD163^{High}. *Statistically significant ($p < 0.05$).

References

1. Louis DN, *et al.* (2016) The 2016 World Health Organization Classification of Tumors of the Central Nervous System: a summary. *Acta neuropathologica* 131(6):803-820.
2. Campbell CD & Eichler EE (2013) Properties and rates of germline mutations in humans. *Trends in genetics : TIG* 29(10):575-584.
3. Chaichana KL, *et al.* (2013) Factors associated with survival for patients with glioblastoma with poor pre-operative functional status. *Journal of clinical neuroscience : official journal of the Neurosurgical Society of Australasia* 20(6):818-823.
4. Reymond N, d'Agua BB, & Ridley AJ (2013) Crossing the endothelial barrier during metastasis. *Nature reviews. Cancer* 13(12):858-870.
5. Kostoro J, *et al.* (2016) Overexpression of vascular adhesion protein-1 is associated with poor prognosis of astrocytomas. *APMIS : acta pathologica, microbiologica, et immunologica Scandinavica* 124(6):462-468.
6. Strilic B & Offermanns S (2017) Intravascular Survival and Extravasation of Tumor Cells. *Cancer cell* 32(3):282-293.
7. Kerkar SP & Restifo NP (2012) Cellular constituents of immune escape within the tumor microenvironment. *Cancer research* 72(13):3125-3130.
8. Nakao S, *et al.* (2011) VAP-1-mediated M2 macrophage infiltration underlies IL-1beta- but not VEGF-A-induced lymph- and angiogenesis. *The American journal of pathology* 178(4):1913-1921.
9. Chandrashekar DS, *et al.* (2017) UALCAN: A Portal for Facilitating Tumor Subgroup Gene Expression and Survival Analyses. *Neoplasia* 19(8):649-658.