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Overexpression of Arginase-1 is an indicator of poor prognosis in patients with colorectal cancer

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ABSTRACT

Aim: Arginase-1 (Arg-1) metabolizes L-arginine to L-ornithine and urea. It has been documented to have a role in various malignancies. However, the relationship between Arg-1 expression and clinicopathological characteristics of colorectal cancer (CRC) patients remains to be elucidated. The present study aimed to analyze the expression and prognostic value of Arg-1 in patients with CRC.

Material and methods: The mRNA and protein expressions of Arg-1 in fresh colorectal cancer tissue specimens and the corresponding noncancerous tissue specimens were examined by RT-qPCR (n = 24) and western blot analysis (n = 17). Arg-1 expression levels were determined in paraffin-embedded CRC tissue specimens (n = 236) by immunohistochemistry. The associations of Arg-1 expression and clinicopathological features and clinical prognosis in 236 CRC patients were analyzed.

Results: The expression levels of Arg-1 were significantly higher in the CRC tissues compared with the matched noncancerous tissues, and elevated Arg-1 expression was remarkably associated with stage III-IV tumors ($P = 0.007$), lymph node metastasis ($P = 0.019$) and a plasma albumin concentration < 35 g/l ($P = 0.022$). Kaplan-Meier analysis indicated that Arg-1 overexpression was associated with adverse prognoses for overall survival (OS) ($P < 0.001$) and disease-free survival (DFS) ($P < 0.001$) in all cases. Further analysis revealed that the patients with high Arg-1 expression had significantly shorter OS and DFS at the advanced stages (III + IV) ($P = 0.032$ for OS, and $P = 0.012$ for DFS) but not at the early stages (I + II) ($P = 0.194$ for OS, and $P = 0.065$ for DFS). Multivariate analysis revealed that Arg-1 overexpression was an independent prognostic factor for OS ($P = 0.002$) and DFS ($P < 0.001$) in patients with CRC.

Conclusion: The data indicated that Arg-1 overexpression in CRC may be a marker that can discriminate subgroups of patients with a poor prognosis.

1. Introduction

Colorectal cancer (CRC) is the third most common cancer and the second leading cause of cancer-associated death [1–3]. Despite the improvement in diagnosis and treatment, the prognosis for patients with CRC remains far from satisfactory. Postoperative recurrence and metastasis remain the two most challenging obstacles in cancer treatment. Given this information, discovering novel biomarkers that can predict the prognosis of CRC may aid in developing therapeutic strategies and improving patients' survival.

L-Arginine, belonging to semiessential amino acid with important functions in different biological and metabolic systems, hydrolyzes L-arginine into the products L-ornithine and urea. It has been reported to play a key part in urea cycle [4] and associate with several disorders, including asthma and cancer [5–8]. Two arginase isoenzymes, namely, Arginase-1 (Arg-1) and Arginase-2, have been identified in mammals.

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These enzymes catalyze the same biochemical reaction but are encoded by different genes and differ in their cellular and subcellular distributions [6,9]. Arginase is involved in several essential metabolic pathways, L-ornithine can be further metabolized into polyamines that are necessary for DNA integrity. Hence, the enzyme participates in a variety of fundamental cellular functions, including cell proliferation and differentiation. Previous research has identified elevated arginase activity in certain patients with cancer, and this increase in enzymatic activity is considered to be responsible for producing the necessary polyamines required to maintain tumor growth [10–12]. High levels of arginase activity have been detected in the serum, tumor tissue and myeloid-derived suppressor cells (MDSCs) of patients with cancer [13,14].

Certain researchers consider Arg-1 to be a useful immunohistochemical marker of hepatocytes, and this enzyme may be used to distinguish hepatocellular carcinoma cells from metastatic tumor cells in the liver because the expression of Arg-1 is highly specific to the human liver [15,16]. Thus far, the role of Arg-1 in patients with CRC remains unclear, and conflicting opinions have been published. Several studies described the arginase activity in the serum and cancer tissues of patients with CRC to be significantly higher than that in the serum and normal tissues of healthy subjects [17–19]. Therefore, the present study aimed to explore the expression of Arg-1 in patients with CRC and the associations between Arg-1 expression and clinicopathological characteristics.

2. Materials and methods

2.1. Patients and tissue samples

A total of 236 formalin-fixed paraffin-embedded specimens were collected from patients with CRC. All patients underwent curative surgery between January 2005 and December 2012. For western blot analysis, fresh CRC specimens and their corresponding normal adjacent tissue were obtained by surgery from 17 patients between January 2014 and April 2014, and for RT-qPCR analysis, samples were similarly obtained from 24 patients in January 2019. The normal adjacent tissue samples were taken at a distance > 6 cm from the tumor border. All fresh specimens were confirmed by pathological examination and snap-frozen in liquid nitrogen until utilization.

None of the patients received preoperative treatment (chemotherapy and/or radiation therapy). Postoperative adjuvant therapy was performed according to the National Comprehensive Cancer Network (NCCN) guidelines [20,21]. All enrolled patients received surgery at The Affiliated Tumor Hospital of Harbin Medical University (Harbin, China), and all specimens were confirmed via pathological examination. The present study was approved by the Harbin Medical University Ethics Committee, and all patients provided written informed consent. The tumor-node-metastasis (TNM) staging of the CRC samples was performed according to the seventh edition of the American Joint Committee on Cancer criteria [22]. The patients were evaluated every 3–6 months for the first 2 years, then every 6 months up to 5 years after surgery, and annually thereafter, according to the NCCN criteria. Overall survival (OS) was calculated as the period between the date of surgery and date of mortality or the most recent date of follow-up. Disease-free survival (DFS) was defined as the interval between the date of surgery and clinically proven recurrence/metastasis/the most recent date of follow-up.

2.2. Western blot analysis

Total protein was extracted from fresh tumor and corresponding healthy tissue samples using a lysis buffer (cat. no. P0013; Beyotime Institute of Biotechnology, Shanghai, China). Protein concentrations were determined using a colorimetric bicinchoninic acid assay reagent. Equal amounts (30 μg) of protein were electrophoresed in 10% Tris-glycine gels and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% milk powder in TBS containing 0.1% Tween-20 for 1 h at 25 °C and incubated with an anti-human Arg1 antibody (1:500; cat. no. ab96183; Abcam, Cambridge, MA, USA) overnight at 4 °C. Subsequently, the membranes were washed with Tris-buffered saline containing Tween-20 (TBST) and incubated with a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (cat. no. A0208; Beyotime Institute of Biotechnology) at a dilution of 1:2000 for 1 h at 25 °C. Protein bands were detected using a FluorChem HD2 Western Blotting Detection system (ProteinSimple, San Jose, CA, USA). Equal-protein sample loading was monitored using an anti-GAPDH antibody (dilution, 1:1000; cat. no. AF-0006; Beyotime Institute of Biotechnology).

2.3. Quantitative real-time PCR (qPCR) assay

Total RNA was extracted from fresh frozen tissue by using Trizol reagent (Thermo Fisher Scientific, Waltham, MA, USA), and the reverse-transcription reactions were performed using an M-MLV Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA). Real-time PCR was performed using a standard SYBR Green PCR kit (Toyobo Life Science, Shanghai, China). qPCR was performed to detect *ARG1* and *ACTB* (β-actin). *ACTB* expression was used as a reference to determine the fold changes in the expression of the target genes using the comparative Ct method. Primer sequences were as follows (52 to 32): *ARG1*-F: TGGA CAGACTAGGAATTGGCA, *ARG1*-R: CCAGTCCGTCAACATCAAAACT; and *ACTB*-F: CATGTACGTTGCTATCCAGGC, *ACTB*-R: CTCCTTAATGT CACGCACGAT.

2.4. Immunohistochemical analysis

For immunohistochemical analysis, 4-μm-thick sections of tumor specimens were incubated at 60°C for 12h, and the slides were deparaffinized in xylene and rehydrated first in a series of graded (100, 95, 85 and 75%) alcohol solutions and then in ddH_2O. Pressure cooker antigen retrieval was performed in citrate buffer (pH 6.0) for 5 min at 115 °C to expose masked epitopes. The samples were then incubated at 25 °C for 2 h and subsequently washed with PBS (pH 7.4) three times. The activity of endogenous peroxidases was blocked with 3% H_2O_2 at 37 °C for 20 min. Thereafter, the samples were washed three times in PBS and treated with a primary rabbit polyclonal anti-Arg-1 antibody (1:300 in antibody diluent; cat. no. ab96183, Abcam) at 4 °C overnight. Following washing with PBS, the sections were incubated at 37 °C with a secondary rabbit antibody (cat. no. PV-6001; Ori Gene Technologies, Inc., Beijing, China) for 30 min and then washed with PBS once more. Finally, the slides were visualized with 3,3′-diaminobenzidine tetrahydrochloride (Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) for 5 min at 25 °C, observed with a microscope (magnification, 100×) and subsequently washed with H_2O for counterstaining for 3 min with 0.2% Mayer's hematoxylin at 25 °C.

The immunostaining was scored by combining the proportion and intensity of positively stained immunoreactive cells. The staining intensity was classified according to the following criteria: 0, negative; 1, weak; 2, moderate; and 3, strong. The extent of staining was scored as 1, 1–10% of the tumor cells stained; 2, 11–50% of the tumor cells stained; 3, 51–80% of the tumor cells stained; and 4, 81–100% of the tumor cells stained. The two grades were multiplied, and the product of the intensity and extent scores ranged between 0 and 12. For statistical analyses, we defined final staining scores ≤8 as the low expression group and staining scores ≥9 as the high expression group. To avoid artificial effects, cells in areas with necrosis or poor morphology were not counted. The degree of immunostaining was reviewed by two pathologists who were blinded to the clinicopathological data of the patients.

2.5. Statistical analysis

The Wilcoxon signed-rank test was applied to examine the significance of differences in Arg-1 expression between the healthy mucosa and primary tumor. The significance of the associations between clinicopathological variables and Arg-1 expression was determined using the chi-square test. Survival curves were generated according to the Kaplan-Meier estimator method and log-rank test. Single and multiple factor analyses were performed with the Cox proportional hazards regression model. Statistical analyses were performed using SPSS software (version 16.0; SPSS Inc., Chicago, IL, USA), and $P < 0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. Arg-1 is overexpressed in CRC tissue

To evaluate the expression levels of Arg-1 in CRC tissue, western blot, RT-qPCR and immunohistochemical analyses were performed. Arg-1 staining was predominantly observed in the cytoplasm of tumor cells; by contrast, no or weak staining was observed in the peritumoral tissue (Fig. 1). Based on the staining scores, 48.3% (114/236) of all specimens exhibited high Arg-1 expression, whereas 51.7% (122/236) of the specimens exhibited low Arg-1 expression.

Analyses of Arg-1 expression in fresh specimens of tumor tissue and the corresponding normal tissue were performed using samples from 17 patients for western blotting ($P = 0.001$, Fig. 2 A, B) and samples from 24 patients for RT-qPCR ($P < 0.001$, Fig. 2 C), and the analyses revealed higher Arg-1 expression in the tumor tissue compared with the corresponding normal tissue.

3.2. Arg1 expression is remarkably associated with the clinicopathological parameters of CRC

To determine the clinical significance of Arg-1 expression in CRC, the associations between Arg-1 expression and the clinicopathological variables of patients with CRC were analyzed. As demonstrated in Table 1, elevated Arg-1 expression was significantly associated with III + IV stage tumors ($P = 0.007$), a plasma albumin concentration < 35 g/l ($P = 0.022$) and lymphatic metastasis ($P = 0.019$). In contrast, no significant associations between Arg-1 expression and sex, age, tumor differentiation or depth of invasion were observed.

3.3. Overexpression of Arg-1 may be regarded as an indicator of poor survival

The association between Arg-1 expression and the prognosis of patients with CRC was analyzed by Kaplan-Meier analysis and the log-rank test. The results revealed that the patients with high Arg-1 expression had significantly shorter OS ($P < 0.001$) and DFS ($P < 0.001$) than the patients with low Arg-1 expression (Fig. 3 A, B). The prognostic value of Arg-1 in different subgroups of the patients with CRC stratified according to their clinical staging was further evaluated. It was demonstrated that the patients with high Arg-1 expression had significantly shorter OS and DFS times at the advanced stages (III + IV) ($P = 0.032$ for OS, and $P = 0.012$ for DFS) (Fig. 3 E, F)

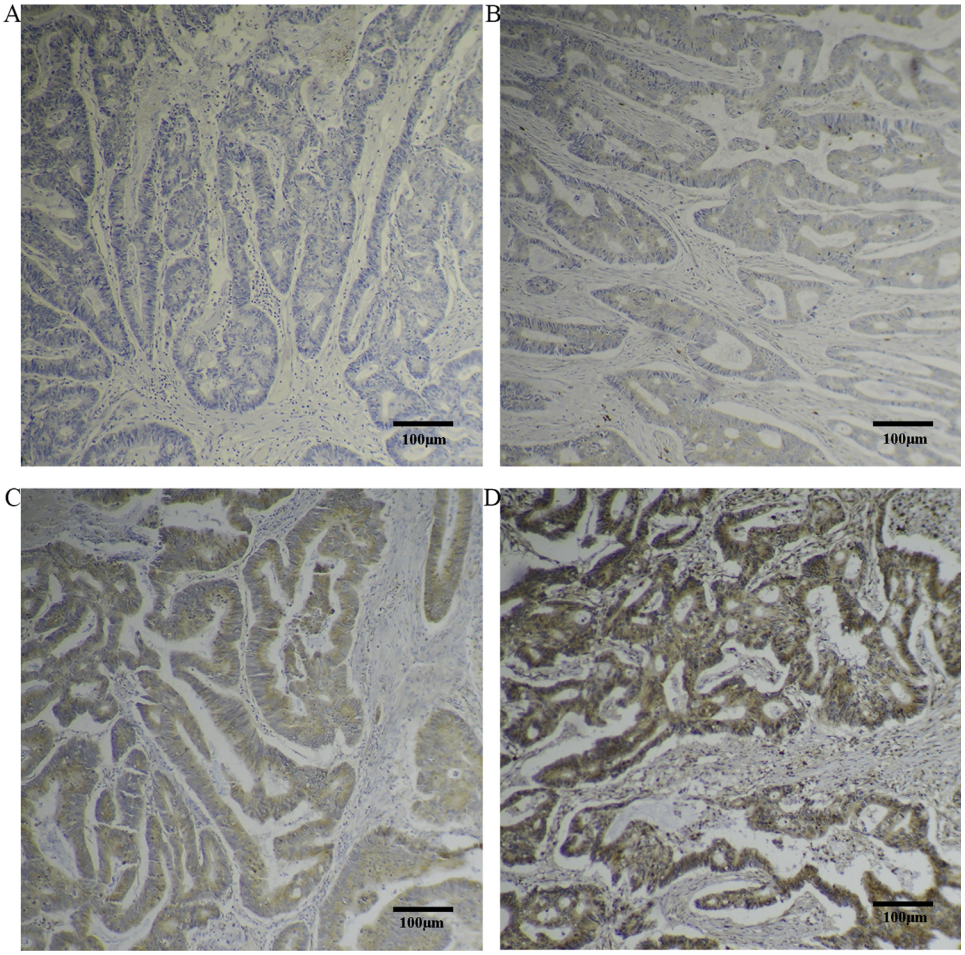

Fig. 1. Arg-1 protein expression in paraffin-embedded CRC tissue samples as determined by immunohistochemistry. CRC tissue samples with low Arg-1 expression (A, B) and high Arg-1 expression (C, D). Original magnification, 100 × . Arg-1, Arginase-1; and CRC, colorectal cancer; the scale bar is 100 μm.

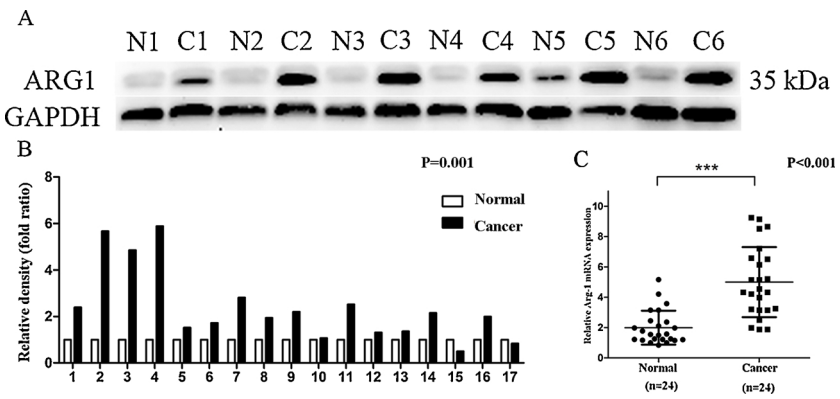

Fig. 2. Arg-1 expression levels in fresh colorectal cancer and adjacent noncancerous tissue samples. (A) Arg-1 protein expression was examined by western blot analysis in fresh colorectal cancer and adjacent noncancerous tissue samples. (B) Analysis on the degree of grayness indicates that Arg-1 expression is significantly higher in the tumor tissue compared with the corresponding normal tissue (P = 0.001). (C) RT-qPCR analysis showed that the mRNA expression of Arg-1 in fresh colorectal cancer tissues was significantly higher than that in adjacent noncancerous tissues (P < 0.001). Arg-1, Arginase-1; N, noncancerous tissue; and C, colorectal cancer tissue.

Table 1
Relationship between clinicopathological variables and Arg-1 expression in patients with colorectal cancer.

Characteristic	Number (n = 236)	Arg-1 expression		P-value[a]
		Low (51.7%) (n = 122)	High (48.3%) (n = 114)	
Age(years)				0.231
≤60	174	94(54.0)	80(46.0)	
> 60	62	28(45.2)	34(54.8)	
Gender				0.216
Male	136	75(55.1)	61(44.9)	
Female	100	47(47.0)	53(52.0)	
TNM stage				0.007
I + II	100	62(62.0)	38(38.0)	
III + IV	136	60(44.1)	76(55.9)	
Depth of invasion				0.185
T1 + T2 + T3	91	52(57.1)	39(42.9)	
T4	145	70(48.3)	75(51.7)	
Lymph node metastasis				0.019
Negative	120	71(59.2)	49(40.8)	
Positive	116	51(43.9)	65(56.1)	
Distant metastasis				0.713
No	165	84(50.9)	81(49.1)	
Yes	71	38 (53.5)	33(46.5)	
Differentiation				0.834
Well and moderately	162	83(51.2)	79(48.8)	
Poorly	74	39(52.7)	35(47.3)	
Histological type				0.161
Adenocarcinoma	189	102(54.0)	87(46.0)	
other[b]	47	20(42.6)	27(57.4)	
Albumin(g/l)				0.022
≥35	194	107 (55.2)	87(44.8)	
< 35	42	15(35.7)	27(64.3)	
CEA(ng/ml)[c]				0.289
≤5	108	51(47.2)	57(52.8)	
> 5	97	53(54.6)	44(45.4)	

[a] $\chi 2$ – test.
[b] Mucinous carcinoma and mixed carcinoma.
[c] Number = 205.

but not at the early stages (I + II) (P = 0.194 for OS, and P = 0.065 for DFS) (Fig. 3 C, D), suggesting that Arg-1 is a valuable prognostic marker for advanced-stage patients.

Univariate Cox proportional hazard regression analysis demonstrated that Arg-1 expression (P < 0.001 for OS, and P < 0.001 for DFS), TNM stage (P < 0.001 for OS, and P < 0.001 for DFS), depth of invasion (P = 0.039 for OS, and P = 0.033 for DFS), distant metastasis status, (P < 0.001 for OS, and P < 0.001 for DFS) and lymph node metastasis status (P < 0.001 for OS, and P < 0.001 for DFS) were indicators of poor prognosis in patients with CRC. Furthermore, multivariate analysis indicated that Arg-1 expression (P = 0.002 for OS, and P < 0.001 for DFS), TNM stage (P = 0.016 for OS, and P = 0.015 for DFS) and distant metastasis (P < 0.001 for OS, and P < 0.001 for DFS) were independent prognostic factors for patients with CRC (Tables 2 and 3).

4. Discussion

In addition to its role in amino acid metabolism, arginase has also been described to play essential roles in immunobiology and cancer [6–8,23]. We previously reported that Arg-1 gene expression significantly increases in a colitis-associated tumor mouse model [24]. However, the expression pattern of Arg-1 in patients with CRC remains unclear, as two opposite opinions have been published [15,16]. Therefore, the present study aimed to explore the expression levels of Arg-1 in patients with CRC and the associations between Arg-1 expression and clinicopathological characteristics.

In the present study, it was revealed that Arg-1 expression in CRC tissue is significantly higher than that in paracancerous normal tissue. This finding conflicts with previous reports [15,16], and this discrepancy may be due to variations in the race or number of the patients in the populations used. Furthermore, the overexpression of Arg-1 was significantly associated with advanced-stage disease and lymphatic metastasis in the patients with CRC. The possible mechanisms underlying the role of Arg-1 in the development of CRC may include its enzymatic activity, which depletes L-arginine in the tumor microenvironment, severely restraining the responses of T cells during the immunological surveillance of the tumor. It has been reported that L-arginine deficiency leads to the downregulation of the expression of the T cell receptor TCR ζ chain, which is the primary signal-transducing component of the TCR and is required for the assembly and membrane expression of the receptor complex [25,26]. The downregulation of TCR ζ chain expression leads to decreased responses to antigens or mitogens and less robust tumor-specific T cell responses [27,28]. Furthermore, Rodriguez et al. [29] reported that stimulated T cells cultured in the absence of L-arginine are arrested in the G0-G1 phase of the cell cycle. This phenomenon was associated with the inability of T cells to upregulate cyclin D3 and cyclin-dependent kinase 4 expression. Thus, we hypothesized that L-arginine supplementation may increase the immune function of patients with CRC and improve outcomes, but further studies are required in this area.

Thus far, the association between Arg-1 protein expression and the clinical outcomes of patients with CRC has not been reported. The results of the present study revealed that patients with a high Arg-1 level exhibit shorter OS and DFS. Previously, studies reported that L-ornithine, which is generated by Arg-1, is further metabolized into polyamines, which are important for cell differentiation and proliferation, and this increase in enzymatic activity is considered to be responsible for producing the polyamines required to maintain tumor growth [4,30]. Singh et al. [7] demonstrated that breast cancer cell lines with relatively high arginase activity exhibit significantly higher rates of proliferation than cell lines with relatively low arginase activity. The inhibitory effect of an arginase inhibitor on cell proliferation

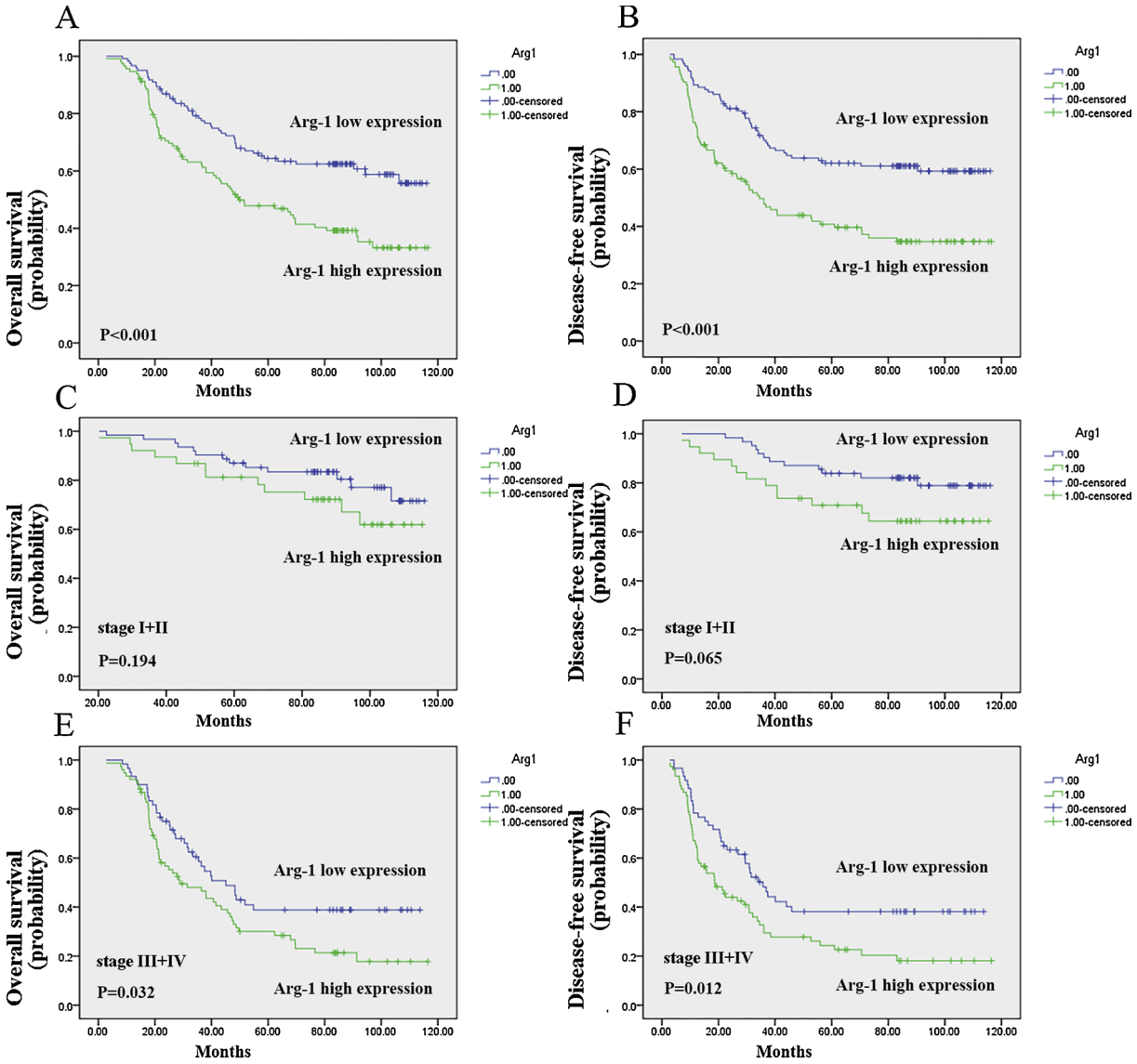

Fig. 3. Association between Arg-1 expression and the prognosis of patients with CRC. Kaplan-Meier curves showed that patients with high Arg-1 expression had significantly shorter OS and DFS than those with low Arg-1 expression in all cases (A, B). Patients with high Arg-1 expression had significantly shorter OS and DFS at the advanced stages (III + IV) (E, F), but not at the early stages (I + II) (C, D). Arg-1, Arginase-1; OS, overall survival; DFS, disease-free survival.

was most prominent in the high-arginase activity cell lines, thereby suggesting that arginase directly promotes cancer cell growth in addition to suppressing the immunological surveillance of the tumor. In line with the results of previous studies [18,19], the present data indicated that elevated Arg-1 expression is associated with a poor prognosis in patients with CRC. Taken together, these findings suggest that the overexpression of Arg-1 may play a significant role in cancer progression.

The traditional TNM staging system that clinicians have relied on to predict patient prognoses for decades is necessary but not sufficient. Identifying novel biomarkers that are able to predict the prognosis of CRC patients may aid in the selection of postoperative therapeutic strategies and improvement of patients' survival. In the current study, survival analysis was performed to identify novel prognostic factors. Kaplan-Meier analysis revealed that a high expression level of Arg-1 was predictive of poor OS and DFS in the advanced-stage subgroup of patients. Multivariate analysis further revealed that the expression of the Arg-1 protein was an independent prognostic factor in patients with CRC. The present study results suggest that the evaluation of Arg-1 expression may serve as an efficient and useful biomarker adjunct to traditional prognostic indicators.

In conclusion, the current study demonstrated that Arg-1 was significantly more highly expressed in cancer tissue compared with paracancerous normal tissue. High Arg-1 expression was significantly associated with advanced CRC and may serve as an unfavorable prognostic biomarker in patients with advanced-stage CRC. In the future, the exact mechanism underlying the contribution of Arg-1 to cancer progression and the therapeutic value of this enzyme in CRC should be explored.

Conflicts of interest

The authors declare that they have no conflicts of interest to disclose.

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Table 2
Univariate and multivariate analysis of prognostic factors in CRC patients for OS.

	Univariate analysis			Multivariate analysis		
	HR	95%CI	P	HR	95%CI	P
Arg-1 (high vs low)	0.510	0.352-0.741	< 0.001	0.541	0.369-0.793	0.002
Gender (male vs female)	0.760	0.522-1.108	0.154			
Age(years) (≤60 vs > 60)	0.823	0.549-1.233	0.345			
Depth of invasion (T1 and T2 and T3 vs T4)	0.652	0.435-0.978	0.039	0.776	0.508-1.186	0.241
Lymph node metastasis (negative vs positive)	0.297	0.201-0.440	< 0.001	0.852	0.452-1.603	0.619
Distant metastasis (Yes vs No)	0.227	0.155-0.333	< 0.001	0.328	0.211-0.509	< 0.001
TNM stage (I + II vs III + IV)	0.197	0.126-0.309	< 0.001	0.385	0.177-0.838	0.016
Histological type (adenocarcinoma vs others[a])	1.217	0.758-1.954	0.416			
Tumor differentiation (well and middle vs poor)	0.764	0.512-1.141	0.188			
plasma albumin < 35 g/l vs ≥35 g/l	1.428	0.922-2.211	0.111			

[a] Mucinous carcinoma and mixed carcinoma.

Table 3
Univariate and multivariate analysis of prognostic factors in CRC patients for DFS.

	Univariate analysis			Multivariate analysis		
	HR	95%CI	P	HR	95%CI	P
Arg-1 (high vs low)	0.470	0.325-0.682	< 0.001	0.483	0.330-0.707	< 0.001
Gender (male vs female)	0.823	0.567-1.195	0.306			
Age(years) (≤60 vs > 60)	0.840	0.563-1.252	0.392			
Depth of invasion (T1 and T2 and T3 vs T4)	0.647	0.434-0.965	0.033	0.701	0.460-1.066	0.097
Lymph node metastasis (negative vs positive)	0.298	0.201-0.440	< 0.001	0.875	0.463-1.655	0.682
Distant metastasis (Yes vs No)	0.251	0.172-0.366	< 0.001	0.335	0.216-0.519	< 0.001
TNM stage (I + II vs III + IV)	0.200	0.128-0.314	< 0.001	0.381	0.175-0.827	0.015
Histological type (adenocarcinoma vs others[a])	1.234	0.769-1.978	0.384			
Tumor differentiation (well and middle vs poor)	0.778	0.522-1.160	0.219			
plasma albumin < 35 g/l vs ≥35 g/l	1.332	0.856-2.073	0.204			

[a] Mucinous carcinoma and mixed carcinoma.

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