Original Article CD44v6 down-regulation is an independent prognostic factor for poor outcome of colorectal carcinoma

Lili Wang^{1,2}, Qin Liu^{1,2}, Dongliang Lin³, Maode Lai^{1,2}

¹Department of Pathology, School of Medicine, Zhejiang University, Hangzhou 310058, Zhejiang, China; ²Key Laboratory of Disease Proteomics of Zhejiang Province Hangzhou 310058, Zhejiang, China; ³Department of Pathology, The Affiliated Hospital of Qingdao University, Qingdao University, Qingdao, China

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Abstract: We aim to investigate the variation of CD44v6 expression in the normal-adenoma-primary carcinoma-liver metastasis sequence and its prognostic impact on colorectal carcinomas. The difference in CD44v6 expression between the tumor center and invasive front was also assessed. Immunohistochemistry was performed for CD44v6 on two cohorts. The first was tissue microarrays including 402 primary CRCs sampled from the tumor center and the invasive margin. The second was whole-tissue sections, consisting of 217 adenomas, 72 primary carcinomas, and the corresponding metastatic carcinomas. In the first cohort, we found that CD44v6 down-regulation was inclined to lymph node metastasis and perineural invasion, and had an unfavorable prognosis compared with CD44v6 up-regulation. In the second cohort, CD44v6 expression was predominant in adenoma over primary carcinoma and liver metastasis in multiple steps (normal < adenoma > primary carcinoma and liver metastasis). In addition, our analysis showed that CD44v6 expression of CD44v6 in adenoma plays a crucial role in colorectal carcinogenesis, while loss of CD44v6 expression on the cell surface of the tumor edge enhances the progression of metastasis. CD44v6 down-regulation is an independent prognostic factor for strikingly worse disease-specific survival.

Keywords: CD44v6, adenoma, colorectal carcinoma, metastasis, immunohistochemistry

Introduction

CD44v6, an alternative splicing of the CD44 gene, is a member of the CD44 family of glycoproteins [1, 2]. Overexpression of CD44v6 is well-documented in colorectal carcinomas (CRCs) by immunohistochemistry, but its prognostic role in CRC remains controversial. Many investigations proved increased CD44v6 expression was a risk factor on survival time in patients [3-9], while some showed opposite results [10-14]. Moreover, no relationship between the expression and prognosis was also observed in several studies [15-22]. It is accepted that adenoma is an essential part of multiple steps of CRC, and CD44v6 expression in adenoma has been inconsistently reported [15, 23-26]. Hence, the function of CD44v6 expression in CRC needs to be evaluated.

In our study, we evaluated the expression of CD44v6 by immunohistochemistry analysis in order to establish its prognostic effect on 402

primary CRCs, define the expression pattern in the adenoma-primary carcinoma-liver metastasis sequence in 217 adenomas, 72 primary carcinomas, and matched liver metastasis, and investigate the expression difference between the tumor center and invasive front in 72 primary and corresponding metastatic carcinomas.

Materials and methods

Clinical data

Two cohorts of CRC tissues were constructed in this study. The first cohort was tissue microarrays (TMAs) including 402 primary colorectal tumors from the tumor center and the invasive margin. These tissues were from patients with CRC who underwent intentionally curative surgical resection at the People's No. 1 Hospital of Xiaoshan, Hangzhou, between 1991 and 2006. We collected the tissue blocks from the Department of Pathology of that hospital. The

Clinicopathological Feature	n (%)
Age	
< 60	147 (36.6)
≥ 60	255 (63.4)
Sex	
Male	219 (54.5)
Female	183 (45.5)
Location	
Colon	209 (52)
Rectum	193 (48)
Histotype	
Tubular	336 (83.6)
Mucinous, signet ring and undifferentiated	66 (16.4)
pT stage	
pT1-2	80 (19.9)
рТЗ-4	322 (80.1)
pN stage	
pNO	215 (53.5)
pN1-2	187 (46.5)
pM stage	
рМО	364 (90.5)
pM1	38 (9.5)
Vascular invasion	
Negative	344 (85.6)
Positive	58 (14.4)
Perineural invasion	
Negative	267 (66.4)
Positive	135 (33.6)
Tumor budding	
Negative	316 (78.6)
Positive	86 (21.4)

 Table 1. Clinicopathological characteristics of the patients

Table 2. CD44v6 expression during the
colorectal adenoma-carcinoma-metastasis
sequence in the second cohort

•		
group 1	group 2	Р
Ν	AD	< 0.001*
AD	CA	0.001*
AD	MT	< 0.001*
Ν	CA	< 0.001§
Ν	MT	< 0.001§
CA	MT	0.372 [§]

N, normal mucosa (n = 72); AD, adenoma (n = 217); CA, primary colorectal adenocarcinoma (n = 72); MT, metastatic colorectal adenocarcinoma (n = 72); *Mann-Whitney non-parametric test for 2-independent samples; [§]Wilcoxon signed ranks test for paired samples.

median age at diagnosis was 65 years (range, 24-91); other clinicopathological features are listed in Table 1. Tumor budding was included in addition to the classical pathological features. This refers to microscopic clusters of undifferentiated cancer cells (\leq 5) ahead of the invasive margin of the cancer. Numbers from 0 to 4 were considered negative; ≥ 5 was considered positive. Tumor budding tends to worsen the prognosis of CRC patients in our previous work [27] and other reports [28]. None of the patients had received chemotherapy or radiotherapy before surgery. The median time of follow-up was 33 months (range 1-186). The second cohort was whole-tissue sections; 217 adenomas, 72 primary colorectal tumors, and matched liver metastases were included. These tissues were collected from the Department of Pathology of Sir Run Run Shaw Hospital, Hangzhou. The 217 adenomas were subdivided into 105 low- and 112 high-grade dysplasias using standard criteria. Seventy-two patients had matched primary and metastatic tumors. This group consisted of 58 synchronous and 14 metachronous metastases. Patients' median age at the time of diagnosis was 58 years (range, 28-78). This study was approved by the Ethics Committee of Biomedicine, Zhejiang University.

Immunohistochemistry

After deparaffinization and rehydration, paraffin-embedded tissue sections (4 μ m thick) were treated with heat-induced antigen retrieval buffer (pH 6.0 citrate buffer) under

high pressure for 2 min. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide in absolute methanol at room temperature for 15 min. Serum blocking was performed using 10% normal goat serum (Liankebio, Hangzhou, Zhejiang, China) for 30 min. Then the tissue sections were incubated with primary antibodies to CD44v6 (clone VFF-18, mouse monoclonal, 1:1000 dilution, Abcam, Cambridge, UK) and E-cadherin (clone NCH-38, mouse monoclonal, 1:100 dilution, Dako Cytomation, Carpinteria, CA, USA) overnight at 4°C. The following day, the slides were washed three times in 0.1 M phosphate-buffered saline (PBS; pH 7.4). Then the signal was amplified using a Polink-2 Plus IHC Detection System using the



Figure 1. Expression of CD44v6 in the normal, adenoma, primary carcinoma, and liver metastasis sequence. CD44v6 immunoreactivity was minimal in normal tissue (A), maximal in adenoma (B), and moderate in primary carcinoma (C) and liver metastasis (D). There was no difference between primary carcinoma and liver metastasis (magnification, 10×).

PV-9001 Reagents kit (GBI, Bothell, WA, USA) two-step method following the manufacturer's instructions. Diaminobenzidine (Zhongshan Goldenbridge Biotechnology, Beijing, China) was used as the chromogenic substrate, and counterstaining was performed with hematoxylin. For negative controls, the primary antibodies were replaced with PBS as a negative. Immunohistochemical scores for each section were scored by two experienced pathologists independently in a double-blinded manner. For CD44v6 and E-cadherin, only membranous staining was evaluated. The staining intensity (SI) was categorized into 4 groups (0-3): none (0), weak (1), moderate (2), and strong staining (3). The proportion of positively-stained cells (PP) was also semi-quantitatively estimated and classified as follows: $0, \leq 5\%$ staining; 1, 6-25%; 2, 26-50%; 3, 51-75%; and $4, \geq 75\%$. SI and PP were multiplied to give an immunohistochemical score (IHS = SI × PP). The expression of CD44v6 and E-cadherin was semi-quantitatively assessed as IHS. Therefore, the combined IHS score ranged from 0 to 12. Based on

	CD44v6 expression (n = 402)		
Eestures	Nega-	Posi-	, Р
	tive n	tive n	Г
Age			
< 60	42	105	0.680
≥ 60	68	187	
Sex			
Male	64	155	0.360
Female	46	137	
Location			
Colon	58	151	0.856
Rectum	52	141	
Histotype			
Tubular	94	242	0.534
Mucinous, signet ring and undifferentiated	16	50	
pT stage			
pT1-2	20	60	0.596
рТЗ-4	90	232	
pN stage			
pNO	52	163	0.125
pN1-2	58	129	
pM stage			
рМО	100	264	0.879
pM1	10	28	
Vascular invasion			
Negative	89	255	0.102
Positive	21	27	
Perineural invasion			
Negative	66	201	0.094
Positive	44	91	
Tumor budding			
Negative	90	226	0.335
Positive	20	66	

 Table 3. Associations between CD44v6 expression and clinicopathological parameters in CRC patients

receiver-operating characteristic curve analysis, cases were categorized into two groups: IHS = 0 (negative) and HIS \geq 1 (positive).

Statistical analysis

Pearson's χ^2 or a 2-independent non-parametric test (Mann-Whitney) was used to evaluate the association between CD44v6 expression and clinicopathological parameters. Diseasespecific survival (DSS) was defined as the time from the date of surgery to the date of death from CRC or last follow-up. Kaplan-Meier method was used to draw the survival curves. The differences between curves were tested by the log-rank test. Multivariate survival analyses were performed using the Cox proportional hazard regression model. The Wilcoxon signed ranks test was used to analyze the paired data among normal mucosa, primary carcinoma, and liver metastasis. The Mann-Whitney non-parametric test was used for 2-independent samples. Significance levels for all tests were set to α = 5% in a two-tailed manner. Statistical procedures were done using IBM SPSS 20.0 statistical software (SPSS Inc. Chicago, IL, USA).

Results

Expression of CD44v6 in adenoma, primary carcinoma, and liver metastasis patients

Immunohistochemical analysis showed that most CD44v6-positive cells were located in the lower half of normal crypts, while the staining of colon carcinomas for CD44v6 was diffuse and strong in TMAs and whole tissues. In the first TMA cohort, the percentage of positive CD44v6 expression was 22.9% in normal tissue and markedly higher in CRCs (72.6%, P < 0.01).

The second cohort was used to identify the variation of CD44v6 expression in the normal-adenoma-primary carcinoma-liver metastasis

sequence. CD44v6 expression in adenoma was maximal, while it was down-regulated in primary carcinoma and liver metastasis. There was no difference between primary carcinoma and liver metastasis. It must be pointed that this reduction was only relative, and the overall CD44v6 expression in primary carcinoma and liver metastasis specimens was still higher compared with normal mucosa (normal < adenoma > primary carcinoma and liver metastasis) (**Table 2**; **Figure 1**). No difference was found between adenomas with low- and high-grade

CD44v6 expression in adenoma and colorectal carcinoma

Variables	Univariate		Multivariate			
	HR	95% CI	Р	HR	95% CI	Р
Age						
< 60	1					
≥ 60	0.093	0.635-1.491	0.901			
Sex						
Male	1					
Female	1.017	0.707-1.620	0.747			
Location						
Colon	1					
Rectum	0.919	0.607-1.391	0.690			
Histotype						
Tubular	1					
Mucinous, signet ring and undifferentiated	0.853	0.622-1.169	0.322			
pT stage						
pT1-2	1					
рТЗ-4	3.448	1.593-7.461	0.002	2.764	1.237-6.177	0.013
pN stage						
pNO	1					
pN1-2	2.192	1.427-3.366	< 0.001	1.129	0.694-1.837	0.625
pM stage						
pMO	1					
pM1	4.154	2.545-6.783	< 0.001	3.864	2.321-6.431	< 0.001
Vascular invasion						
Negative	1					
Positive	1.839	1.119-3.024	0.016	1.154	0.684-1.947	0.592
Perineural invasion						
Negative	1					
Positive	2.517	1.663-3.809	< 0.001	1.584	1.012-2.480	0.044
Tumor budding						
Negative	1					
Positive	3.081	2.019-4.701	< 0.001	2.354	1.486-3.728	< 0.001
CD44v6 expression						
Negative	1					
Positive	0.700	0.455-1.076	0.104*	0.586	0.375-0.916	0.019

 Table 4. Univariate and multivariate analyses of clinicopathological factors in CRC patients with respect to DSS

HR, hazard ratio; CI, confidence interval; P values in bold, statistically significant; *P, borderline significant.

dysplasia (Mann-Whitney non-parametric test, P = 0.936).

Correlation between CD44v6 expression and clinicopathological characteristics

The correlation between CD44v6 expression and the clinicopathological characteristics is shown in **Table 3**. No statistical associations were found between CD44v6 expression and the different clinicopathological factors analyzed by Pearson's χ^2 test. However, bivariate

independent correlation analysis demonstrated an inverse relationship between CD44v6 expression and lymph node metastasis (P = 0.005), as well as perineural invasion (P = 0.036).

Impact of clinicopathological characteristics and CD44v6 expression on the prognosis of CRC

Univariate survival analyses were used to investigate the prognostic value of clinicopathologi-



Figure 2. Kaplan-Meier analysis for DSS according to CD44v6 expression. Patients with negative CD44v6 expression tended to have a unfavorable prognosis compared with those with positive expression, although the difference was not statistically significant.

cal features and CD44v6 expression on DSS (Table 4). The results showed that depth of infiltration, lymph node metastasis, distant metastasis, perineural invasion, and tumor budding were statistically associated with DSS. Patients with negative CD44v6 expression tended to have an unfavorable prognosis compared with those with positive expression, although the difference was not statistically significant (Figure 2, P = 0.102). All variables that performed prognostic significance in the univariate analysis were entered into the multivariate survival analysis; CD44v6 expression was also included because of the borderline significant *P* value. This study identified CD44v6 down-regulation as an independent prognostic factor for a strikingly worse DSS (P = 0.019), along with depth of infiltration, distant metastasis, perineural invasion, and tumor budding (Table 4).

Correlation between CD44v6 and E-cadherin

Spearman's rank correlation analysis indicated a positive relationship between CD44v6 and E-cadherin (P = 0.034, Rs = 0.011; Figure 3).

CD44v6 expression in the tumor center and invasion front

In the cohort of 402 primary carcinomas, loss of CD44v6 expression was found from the center of the tumor to the invasive front (Z = -3.341, P = 0.001, two-paired sample, Wilcoxon signed

ranks test). In the cohort of 72 matched primary carcinomas/metastases, CD44v6 expression also decreased from the tumor center to the invasive front (Z = -3.566, P < 0.001; **Figure 4**). There was no difference between the center and border in the corresponding liver metastasis (Z = -1.066, P = 0.287).

Discussion

At present, the contribution of CD44v6 to CRC is disputed. Many studies showed that overexpression of CD44v6 indicated a worse clinical outcome, while other studies suggested its down-regulation being a marker for worse prognosis. In order to understand the function of CD44v6 expression in CRC, TMA combined with whole tissues were used in our study, as well as the adenoma-primary carcinoma-liver metastasis assay.

In our study, we assessed the relationship between CD44v6 expression and relevant clinical parameters of CRC, and the value of CD44v6 for the prognosis of CRC. Our data indicated that loss of CD44v6 is associated with a more aggressive tumor phenotype, including worse survival, perineural invasion, and lymph node metastasis. And multivariate analysis suggested that CD44v6 is an independent prognostic indicator. Some studies support our findings; Chen et al. [13] noted that patients with negative CD44v6 expression had a worse clinical outcome than those with positive expression in stage I/II CRC. Although CD44v6 expression was not showed serving as an independent prognosis indicator, the 5-year disease-specific survival rate for patients with negative CD44v6 expression was significantly lower than those with positive expression [14]. Similar results were observed in other types of aggressive carcinoma, including urothelial bladder cancer [29], lung adenocarcinoma [30], and prostate cancer [31]. All together, these studies suggest that decreased CD44v6 expression is linked to a poorer clinical outcome in CRC.

Interestingly, despite the fact that upregulation of CD44v6 expression was associated with a favorable prognosis; the overall expression of CD44v6 in CRC was higher than that in normal mucosa. By studying the serial process of normal-adenoma-primary carcinoma-liver metastasis tumor progression, we assumed that the difference between colorectal carcinogenesis and progression led to this phenomenon.



Figure 3. Immunohistochemical analysis of CD44v6 and E-cadherin (membranous) expression in CRC tissues. Positive and corresponding negative staining for CD44v6 (A and B) and E-cadherin (C and D) (magnification, $10\times$). (A and C) is the same tissue, (B and D) is the same tissue.

In the sequence, CD44v6 immunoreactivity was maximal in adenoma, moderate in primary carcinoma and liver metastasis, and minimal in normal tissue. There was no difference between primary carcinoma and liver metastasis. Similar to our findings, Coppola et al. [15] found that CD44v6 was predominant in adenoma over primary carcinoma and liver metastasis. Contrary to our results, some researchers [23, 25] demonstrated that CD44v6 expression is constant throughout the multiple steps of tumor progression. Both Orzechowski et al. [24] and Weg-Remers et al. [26] considered that CD44v6

expression did not differ between adenoma and primary carcinoma, but was significantly decreased in liver metastasis. In addition, Kim et al. [32] found CD44 expression prior to K-ras mutations in colorectal adenoma. CD44 expression is activated by the oncogene ras in cloned rat embryonic fibroblasts [33] and intestinal epithelial cells [34], indicating that CD44 expression is an early event. Kerr et al. [35] reported that CD44v6 staining is higher in atypical adenomatous hyperplasia (AAH) than that in invasive pulmonary adenocarcinoma. Similar to colorectal adenoma, AAH is regarded as a



Figure 4. Immunohistochemical analysis of CD44v6 expression in the center and the invasive front ofCRC tissues. A. Whole-tissue scan (NanoZoomer Digital Pathology Scan, Hamamatsu, Japan) (magnification, 0.42×). B. Higher CD44v6 expression in the center of the tissue (magnification, 10×). C. Lower CD44v6 expression in the invasion front of the tissue (magnification, 10×).

precursor lesion, which plays an essential role in the progression of pulmonary adenocarcinoma. In any case, significant upregulation of CD44v6 occurs in early adenomas, and may play a crucial role in human colorectal carcinogenesis.

Our analyses showed that CD44v6 expression was lower in the invasion front of the CRC than that in the center, which was in line with many studies [14, 15, 36]. The prognostic effect of CD44v6 downregulation with a poor prognosis in CRC may be due to these results. As a cell adhesion molecule, CD44v6 was significantly associated with E-cadherin expression in our study. And loss of E-cadherin has been described as a predictive factor for an unfavorable prognosis in CRC patients [37]. It is easy for tumor cells to detach from tumors, and gain the ability to invade and metastasize. In our investigation, down-regulation of CD44v6 was associated with lymph node metastasis and perineural invasion. A positive relationship between E-cadherin and CD44v6 has also been reported by Zlobec et al. [14]. In addition, E-cadherin is positively correlated with CD44 in colon cancer [38]. Other than its intercellular adhesion properties, CD44v6 is reported to have a high affinity for hyaluronate [39], which is an important component of the extracellular matrix (ECM). The loss of CD44v6 expression in the front of the tumor leads to defective binding of the tumor cells to the ECM, increasing the metastatic ability [40]. The hypermethylation of the 5' CpG island of the CD44 gene may be another cause of decreased expression of CD44v6 in human CRCs [41].

A number of studies concerning metastasis from CRC have reported that down-regulation of CD44v6 occurs in the metastatic stage [15, 26, 42]. Contrary to these reports, our and other studies [14, 23, 25] found no difference between primary and matched metastatic tumors in CD44v6 expression. Probably, like the case of E-cadherin, re-expression of CD44v6 at the metastatic site may occur during the process of mesenchymal-epithelial transition when metastatic seeding happened [43]. Loss and gain of adhesive functions of the tumor cells may play an important role in the progression of metastatic tumors [44].

The differences in the previous studies are probably due to differences in the primary antibodies used, statistical methods, and study populations. The number of samples is very important, relatively few may lead to a mistaken outcome. The large number of patient specimens in our study could eliminate such potential problems. Combining the TMA with whole tissues strengthens the findings over those obtained by applying these methods alone.

In summary, CD44v6 is upregulated in adenoma, suggesting that the expression of CD44v6 might be an early event in the course of colorectal carcinogenesis which is similar with the genetic mutation of the APC or K-ras genes that have been acknowledged in the colorectal adenoma-carcinoma progress. Once the carcinoma forms in the colorectum, loss of CD44v6 expression at the cell surface of the tumor edge would enhance the progression of metastasis. CD44v6 might play different roles in regulating cell growth and migration. There may be no relationship between metastatic ability and proliferative activity in CRC [45]. So far, no final conclusion has been reached on the role of CD44v6 in CRC. Caution is necessary when considering CD44v6 as a diagnostic and prognostic marker, or as a target for therapeutic strategies in CRC. Further studies with a systematic study including in vivo and in vitro methods are needed to provide definite answers.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Maode Lai, Department of Pathology, School of Medicine, Zhejiang University, 866 Yuhangtang Road, Hangzhou 310-058, Zhejiang, China. Tel: 86-571-8820-8197; Fax: 86-571-8820-8197; E-mail: Imp@zju.edu.cn

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