

ORIGINAL PAPER

CLINICAL UTILITY OF MCM2 AND CD44 EXPRESSION IN CLEAR CELL RENAL CELL CARCINOMA

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Clear cell renal cell carcinoma (ccRCC) has an unpredictable clinical consequence even with the use of usual prognostic factors.

To determine whether utilization of cell proliferation and cell adhesion by using MCM2 and CD44 immuno-expression could predict the biological performance of ccRCC.

MCM2 and CD44 protein expression levels in tumor tissues from 120 ccRCC patients were evaluated by immunohistochemistry. The relationships between MCM2 and CD44 expression and clinicopathological parameters were evaluated. The overall survival (OS) was computed by the Kaplan-Meier method. The role of MCM2 and CD44 in the prognosis was estimated by univariate and multivariate Cox regressions. The results showed high MCM2 and CD44 protein expression levels in 63.3% and 55% of ccRCC cases, respectively. MCM2 and CD44 over-expression was significantly related to tumor grade ($p = 0.001$ and $p = 0.003$, respectively), T stage ($p = 0.005$ and $p = 0.008$, respectively), lymph node status ($p = 0.015$ and $p = 0.040$, respectively), AJCC stage ($p = 0.000$ and $p = 0.002$, respectively) and OS ($p = 0.019$ and $p = 0.001$, respectively). Multivariate Cox regression showed that high MCM2 and CD44 expression levels were independently associated with prognosis of ccRCC cases (HR = 2.687, 2.810, 95% CI: 1.217-5.920, 1.267-6.233, respectively).

MCM2 and CD44 are considered as independent risk and poor prognostic factors for the prognosis of ccRCC patients.

Key words: ccRCC, MCM2, CD44, immunohistochemistry, prognosis.

Introduction

Renal cancer accounts for about 3% of all adult malignant tumors. It is the 12th most common malignancy worldwide [1]. Renal cell carcinoma (RCC) represents about 85% of all renal cancers. The majority of RCC are clear cell renal cell carcinoma (ccRCC) [2].

Although most of the patients with RCC are diagnosed with an early stage tumor, still about 25%

of cases present with a locally advanced and/or metastatic disease. A proportion of patients who underwent nephrectomy developed either recurrence or metastasis [3]. The clinical outcomes of RCC differ widely, indicating the need for appropriate and accurate prognostic parameters. To date, the best prognostic system for overall survival (OS) is the TNM staging system; however, it isn't enough to significantly enhance the management of patients. There-

fore, detection of novel reliable prognostic factors is vital for improving therapeutic strategies in order to prolong the survival of RCC patients [4, 5].

Minichromosome maintenance (MCM) protein 2 is one of 6 proteins that form the MCM complex. It has a vital role in DNA replication. MCM2 and other MCM proteins are targets of ATR (ataxia telangiectasia and Rad3 related) and ATM (ataxia telangiectasia mutated) genes that inhibit DNA replication and initiate repair [6].

MCM proteins stay stable throughout the cell cycle but their levels decrease during cellular differentiation. This could be attributed to the presence of the pre-replication complex throughout the cell cycle. This makes these proteins suitable candidates as indicators of proliferation [7].

Previous studies had observed that MCM2 serves as a proliferation marker of malignant cells. High expression levels of it in malignant tumors were associated with several clinico-pathological characteristics such as advanced stage, high grade and poor prognosis [8, 9, 10].

CD44 is a transmembrane glycoprotein with multiple isoforms, among them CD44 standard (CD44s). The extracellular domain of CD44 is the principal receptor for hyaluronic acid. In tumors, CD44s bound with hyaluronic acid targets numerous processes such as progression, proliferation, invasion and metastasis [11, 12].

CD44 is an important cancer stem cell marker and a poor prognostic marker in various malignancies. It helps in various steps which are fundamental in extravasation and migration of neoplastic cells. The interactions between CD44 cytoplasmic tail and actin cytoskeleton could be stimulated via CD44-HA binding; so, migration of tumor cells is initiated [13, 14].

In the current study we aimed to evaluate the cross-talk between these two markers in ccRCC and to correlate MCM2 and CD44 expression with clinico-pathological parameters and survival of the patients in a trial to explore their significance as suggested indicators for tumor progression, recurrence and patients' prognosis.

Material and methods

Case selection and tissue sample preparation

In this retrospective study, one hundred twenty cases of ccRCC were randomly selected from the archive of the pathology lab of Minia University Hospital and Minia Oncology Center during the period of January 2010 to August 2014. Paraffin blocks with clinicopathological data of the patients were collected including: patient's age, gender, tumor size, AJCC clinical stage, N stage and TNM stage. HE slides were prepared to detect Fuhrman nuclear grade.

Immunohistochemistry

Sections were cut 4 μ m thick on positively charged slides, de-paraffinized with xylene and rehydrated through graded ethanol. Slides were immersed in 3% hydrogen peroxide for 30 min to quench endogenous peroxidase then rinsed in PBS solution. Treatment in citrate buffer (pH 6.0) was done for antigen retrieval using the microwave. Afterwards, slides were left to cool at room temperature and washed in PBS solution. Mouse monoclonal anti-MCM2 (BioSpring) and mouse monoclonal anti-CD44 (Thermo-Fisher) antibodies were added. Sections incubated overnight at 4°C in a humidity chamber and then rinsed with PBS before treatment with secondary antibody for 30 min. After a wash in PBS, the streptavidin-biotin complex reagent was added for 30 min. Brownish color was developed by using 3,3-diaminobenzidine tetra hydrochloride (DAB), then slides were washed in distilled water, stained with hematoxylin, dehydrated, cleared with xylene, and coverslipped.

Evaluation of immunostaining

Slides were examined by two pathologists (M. Gayyed and M. El-Husseiny), independently who were blinded to clinicopathological data of the cases.

Concerning MCM2, nuclear staining was considered positive. For quantitative analysis hot spots were detected by low power. Then stained cells were counted in 10 high-power fields chosen randomly. The LI was expressed as the percentage of positively stained cells based on a count of at least 1,000 cancer cells. A labeling index more than 20% was used as a cutoff point [7, 15].

The CD44 was stated as percentages of the CD44-positive cells by counting at least 1000 tumor cells at $\times 400$. CD44 was considered positive when membranous/cytoplasmic expression was detected in > 5% of the stained cells [16]. Sections were scored for the CD44 staining patterns as follows: the staining extent was scored as 0 (0-5% staining of tumor cells), 1+ (> 5% - < 25% staining of tumor cells), 2+ (25-50% staining of tumor cells), 3+ (50-75% staining of tumor cells) or 4+ (> 75% staining of tumor cells). Scores of 0 and 1+ were regarded as exhibiting low expression and scores of 2+, 3+ and 4+ were judged as exhibiting high expression [11].

Statistical analysis

Data were analyzed using SPSS version 20. Chi-square and Fisher's exact tests were used to compare categorical variables. Correlation between MCM2 and CD44 was evaluated using Spearman's correlation coefficient. Analysis of OS was examined by the Kaplan-Meier method. The effect of MCM2 and CD44 on the prognosis of ccRCC patients was assessed via univariate and multivariate Cox regression.

Hazard risk (HR) and relative 95% confidence interval (CI) were analyzed. Results were considered statistically significant when p-value ≤ 0.05 .

Results

This study was performed on 120 patients with ccRCC. The age of patients ranged from 29 years to 79 years with a mean (\pm standard deviation: SD) of 52.72 ± 1.28 years and a median of 54 years. The tumor size ranged from 3 cm to 16 cm with a mean (\pm standard deviation: SD) of $7.016 \text{ cm} \pm 2.6$ and a median of 7 cm. Other patients' characteristics are shown in Table I.

MCM2 immunoreactivity

MCM2 expression was detected in the nucleus as shown in (Fig. 1). MCM2 overexpression was low in 44 cases (36.7%) and high in 76 cases (63.3%). MCM2 high expression was significantly associated with high tumor grade ($p = 0.001$), tumor stage ($p = 0.005$), nodal status ($p = 0.015$), AJCC stage ($p = 0.000$) and overall survival ($p = 0.019$). No association was found between MCM2 immunoreactivity and other clinicopathological characteristics, as shown in Table II.

CD44 immunoreactivity

The CD44 protein was distributed in the membrane \pm cytoplasm of the tumor cells, as shown in Fig. 1. CD44 expression was low in 54 (45%) cases and high in 66 cases (55%). The CD44 immunostaining was significantly associated with high tumor grade ($p = 0.003$), tumor stage ($p = 0.008$), lymph node status ($p = 0.040$), high tumor AJCC stage ($p = 0.002$) and overall survival ($p = 0.001$). No association was found between CD44 expression and other clinicopathological characteristics (Table II).

MCM2 and CD44

No statistically significant association was found between MCM2 and CD44 ($p = 0.940$ and $r = 0.007$).

Survival analysis

The follow-up ranged from 12 months to 59 months with a mean (\pm standard deviation: SD) of 42.9 ± 1.06 months and a median of 46 months. Overall survival was not significantly associated with any prognostic clinicopathological factors. Regarding marker expression and OS, increased expression levels of MCM2 and CD44 were associated with worse OS ($p = 0.019$ and 0.001 respectively; Fig. 2).

The relationship between the prognosis and the expression of MCM2 and CD44 in ccRCC cases was evaluated via univariate and multivariate Cox

Table I. Clinicopathological characteristics of ccRCC cases (n = 120)

CLINICOPATHOLOGICAL CHARACTERISTICS	No (%)
Age	
≤ 54	53 (44.2)
> 54	67 (55.8)
Gender	
Male	77 (64.2%)
Female	43 (38.5%)
Tumor size (cm)	
$< 7 \text{ cm}$	74 (61.7%)
$\geq 7 \text{ cm}$	46 (38.3%)
Tumor grade	
GI	18 (15%)
GII	41 (34%)
GIII	44 (36%)
GVI	17 (14%)
T Stage	
T1	23 (19.2%)
T2	50 (41.7%)
T3	44 (36.7%)
T4	3 (2.5%)
N Stage	
N0	111 (92.5%)
N1, N2	9 (7.5%)
AJCC Clinical stage	
I	16 (13.3%)
II	46 (38.4%)
III	36 (30%)
IV	22 (18.3%)
Survival	
Censored	66 (55%)
Event	54 (45%)

regression. The univariate regression (Table III) indicated that tumor size (HR = 2.383, 95% CI: 1.189-4.774, $p = 0.014$), grade (grade III: HR = 2.876, 95% CI: 1.107-7.472, $p = 0.030$), AJCC stage (stage III: HR = 2.252, 95% CI: 1.042-4.870, $p = 0.039$), high MCM2 expression (HR = 3.313, 95% CI: 1.019-10.771, $p = 0.046$) and high CD44 expression (HR = 3.456, 95% CI: 1.000-11.947, $p = 0.05$) were all associated with survival status of ccRCC patients. The multivariate regression (Table IV) showed that positive MCM2 and CD44 expression significantly increased the risk of adverse consequences (HR = 2.687, 95% CI: 1.217-5.930,

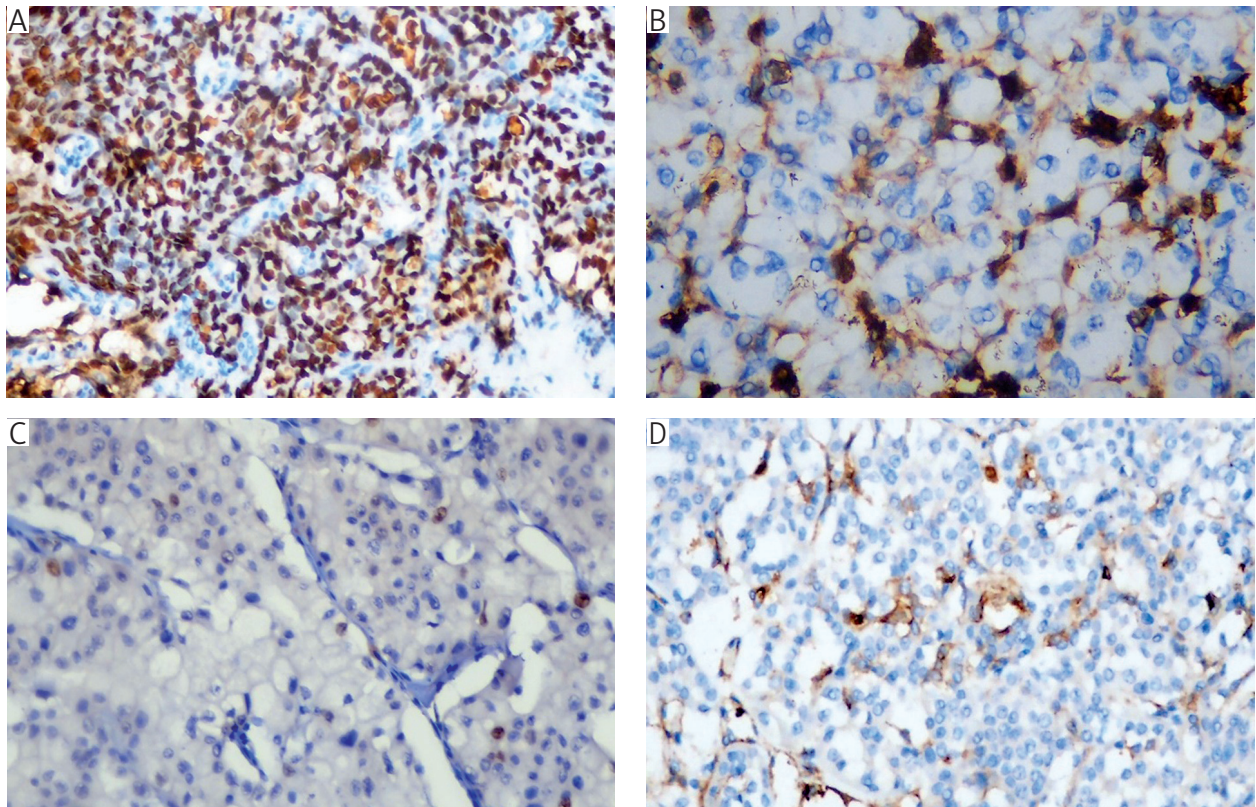


Fig. 1. Immunohistochemical expression of MCM2 and CD44 in ccRCC. Increased nuclear MCM2 expression (A) is associated with increased CD44 expression (B) (the first row). Cases with low MCM2 expression (C) have low CD44 expression (D) (the second row) (magnification 200×)

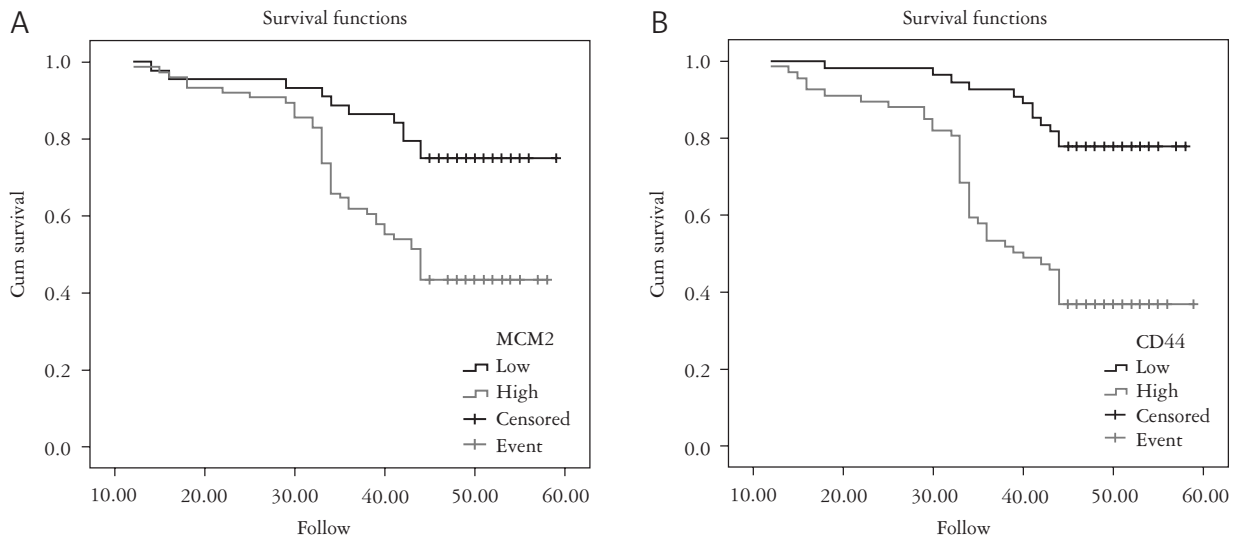


Fig. 2. Kaplan-Meier survival curves for overall survival according to MCM2 and CD44 expression. Shorter OS is associated with high MCM2 and CD44 expression

$p = 0.014$ and $HR = 2.810$, 95% CI: 1.267-6.233, $p = 0.011$; Fig. 3).

Discussion

The clinical outcome of ccRCC is problematic and its prognosis can be diverse even with analogous

pathological descriptions. Therefore, it is important to create a more accurate prognostic model including molecular and genetic biomarkers alongside traditional prognostic markers such as histopathological features and the TNM staging system [17]. Moreover, molecular-based biomarkers not only have benefits in expecting prognosis, but also have promising

Table II. Association between CD44 and MCM2 expression and clinicopathological characteristics of ccRCC cases (n = 120)

CLINICOPATHOLOGICAL CHARACTERISTICS	CD44 EXPRESSION		P-VALUE	MCM2 EXPRESSION		P-VALUE
	Low (%)	High (%)		Low (%)	High (%)	
Age (years)						
≤ 54	21 (39.6%)	32 (60.4%)	0.292	23 (43.4%)	30 (56.6%)	0.174
> 54	33 (49.3%)	34 (50.7%)		21 (31.3%)	46 (68.7%)	
Gender						
Male	36 (46.8%)	41 (53.2%)	0.605	26 (33.8%)	51 (66.2%)	0.378
Female	18 (41.9%)	25 (58.1%)		18 (41.9%)	25 (58.1%)	
Tumor size (cm)						
< 7 cm	27 (36.5%)	47 (63.5%)	0.017*	32 (43.2%)	42 (56.8%)	0.058
≥ 7 cm	27 (58.7%)	19 (41.3%)		12 (26.1%)	34 (73.9%)	
Tumor grade						
GI	18 (100%)	0 (0%)	0.003*	18 (100%)	0 (0%)	0.001*
GII	23 (56.1%)	18 (43.9%)		15 (36.6%)	26 (63.4%)	
GIII	6 (13.6%)	38 (86.4%)		6 (13.6%)	38 (86.4%)	
GIV	7 (41.2%)	10 (58.8%)		5 (29.4%)	12 (70.6%)	
T Stage						
T1	19 (82.6%)	4 (17.4%)	0.008*	19 (82.6%)	4 (17.4%)	0.005*
T2	32 (64%)	18 (36%)		20 (40%)	30 (60%)	
T3	2 (4.5%)	42 (95.5%)		4 (9.1%)	40 (90.0%)	
T4	1 (33.3%)	2 (66.7%)		1 (33.3%)	2 (66.7%)	
N Stage						
N0	53 (47.7%)	58 (52.3%)	0.040*	44 (39.6%)	67 (60.4%)	0.015*
N1, N2	1 (11.1%)	8 (88.9%)		0 (0%)	9 (100%)	
AJCC Clinical stage						
Stage I	14 (87.5%)	2 (12.5%)	0.002*	13 (81.2%)	3 (18.8%)	0.000*
Stage II	31 (67.4%)	15 (32.6%)		20 (43.5%)	26 (56.5%)	
Stage III	7 (19.4%)	29 (80.6%)		6 (16.7%)	30 (83.3%)	
Stage IV	2 (9.1%)	20 (90.9%)		5 (22.7%)	17 (77.3%)	
Overall survival						
Censored	42 (63.6%)	24 (36.4%)	0.000*	33 (50%)	33 (50%)	0.001*
Event	12 (22.2%)	42 (77.8%)		11 (20.4%)	43 (79.6%)	

Test of significance: χ^2 and Fisher exact tests p-value < 0.05 is considered significant.

possibility for usage as a postoperative targeted therapy in high-risk patients.

MCM2-7 are the main players in the initiation of DNA replication by formation of what is called a pre-replicative protein complex. They wind down the DNA helix, an essential step for the initiation of DNA manufacture. MCM2 only lets this replication occur when the cell cycle is completed [18]. This gene plays an essential role in the development of many types of cancers and has been linked to enhanced proliferation in cancer [19].

To our knowledge, this is the first study to evaluate the expression of MCM2 in clear cell variant of RCC. In the current study, high MCM2 expression was detected in 63.3% of ccRCC. Also, we found a significant association between MCM2 immuno-expression and the clinical prognostic factors tumor grade, T stage, lymph node status, AJCC stage and OS ($p = 0.001$, $p = 0.005$, $p = 0.015$, 0.000 and 0.019 respectively). There was no statistically significant difference in MCM2 expression in relation to the other clinicopathological features of the cases.

Table III. Univariate Cox regression analysis of relationship between clinicopathological characteristics and prognosis in cases of ccRCC

	B	SE	WALD	P VALUE	EXP(B)	95.0% CI	
						LOWER	UPPER
Age	-0.228	0.303	0.567	0.451	0.796	0.440	1.441
Sex	0.178	0.332	0.288	0.592	1.195	0.624	2.288
Size	0.868	0.355	5.996	0.014	2.383	1.189	4.774
Grade			8.789	0.032			
Grade I	-2.044	1.388	2.167	0.141	0.130	0.009	1.968
Grade II	0.132	0.486	0.074	0.785	1.142	0.440	2.959
Grade III	1.056	0.487	4.704	0.030	2.876	1.107	7.472
T			6.244	0.100			
T1	-0.673	1.042	0.417	0.518	0.510	0.066	3.935
T2	-0.328	0.814	0.163	0.687	0.720	0.146	3.551
T3	0.840	0.795	1.116	0.291	2.317	0.487	11.017
LN	-0.655	0.431	2.305	0.129	0.520	0.223	1.210
AJCC stage			15.611	0.001			
Stage I	-1.371	0.884	2.404	0.121	0.254	0.045	1.436
Stage II	-1.048	0.497	4.443	0.035	0.351	0.132	0.929
Stage III	0.812	0.393	4.261	0.039	2.252	1.042	4.870
MCM2	1.198	0.602	3.966	0.046	3.313	1.019	10.771
CD44	1.240	0.633	3.839	0.050	3.456	1.000	11.947

Table IV. Multivariate Cox regression analysis of relationship between clinicopathological characteristics and prognosis in cases of ccRCC

	B	SE	WALD	P-VALUE	EXP(B)	95.0% CI	
						LOWER	UPPER
Size	-0.546	0.321	2.893	0.089	0.579	0.309	1.087
Grade	0.114	0.207	0.306	0.580	1.121	0.748	1.681
AJCC stage	0.239	0.194	1.528	0.216	1.270	0.869	1.856
MCM2	0.988	0.404	5.986	0.014	2.687	1.217	5.930
CD44	1.033	0.406	6.462	0.011	2.810	1.267	6.233

In line with our findings, Dudderidge *et al.* detected a statistically significant association between MCM2 expression and tumor grade. They also found a strong hint suggesting that increased MCM2 expression was linked to reduced disease-free survival in RCC [20].

Also, Zhong *et al.* (2017) stated that high expression of the MCM2 gene in either primary RCC or its metastasis was significantly associated with a shorter disease-free survival time [21]. Another study done by Giaginis *et al.* (2009) showed that a statistically significant relation was determined between MCM2 expression and tumor grade and stage in colon cancer [22].

Cancer stem cells (CSCs) have been identified in several tumors including hepatic, ovarian, prostatic, bladder, breast and pancreatic cancers [23]. They are characterized by extensive self-renewal ability and pluripotent differentiation ability. There is strong evidence for crosstalk between tumor progression, metastasis and stem cells; however, the importance of stem cell marker overexpression in cancer is still vague and needs further clarification [24]. Cancer stem cells are believed to be resistant to chemotherapy as well as radiotherapy, although the mechanisms are not fully understood [25]. So, they could be the therapeutic target of cancers [26].

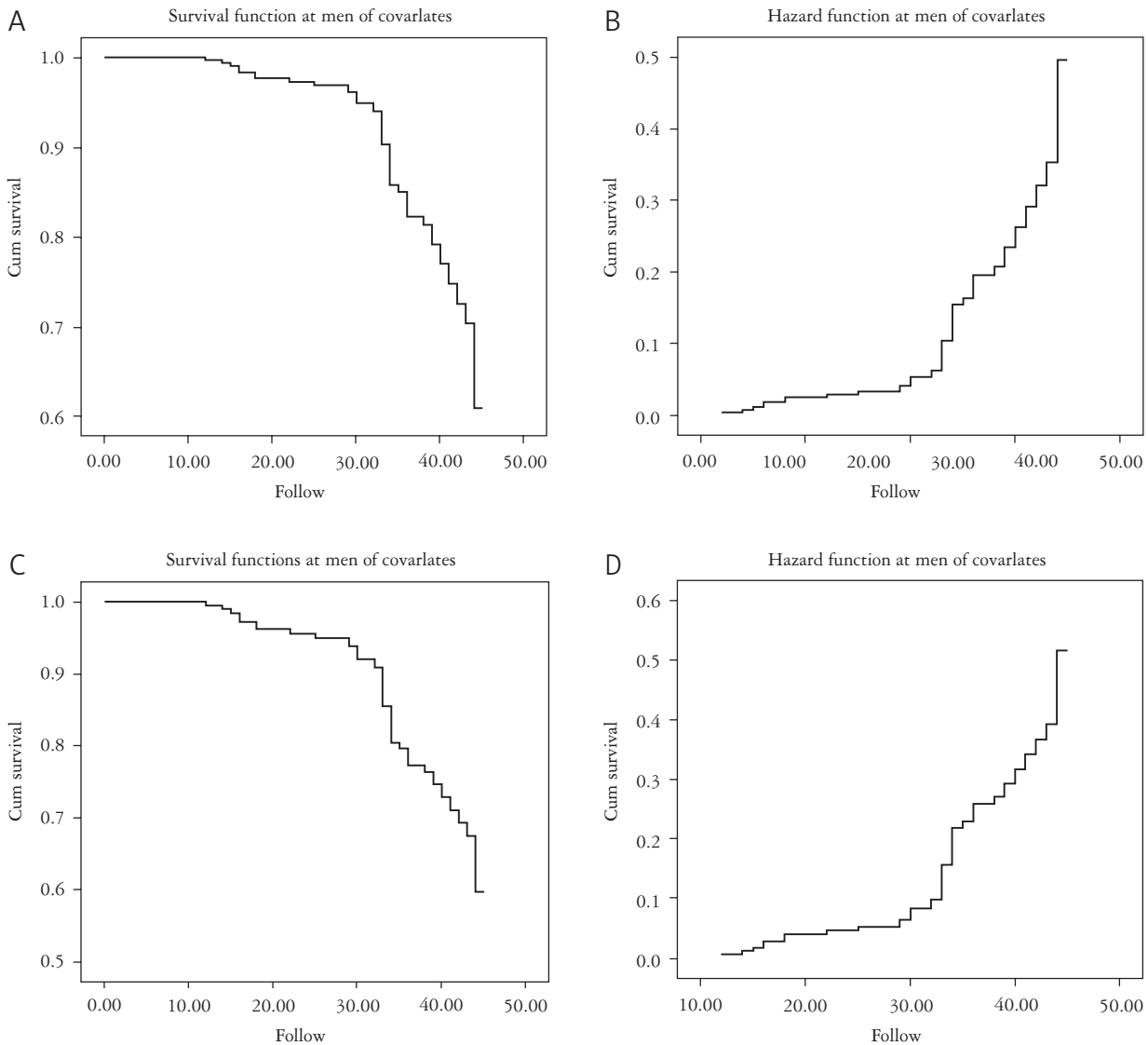


Fig. 3. Univariate (A, B) and multivariate (C, D) Cox regression survival curves according to MCM2 and CD44 expression

CD44 is a cell surface transmembrane glycoprotein that plays a role as a cell adhesion molecule and a receptor for hyaluronic acid. It has a role in tumor cell invasion and metastasis by intermingling with extracellular matrix metalloproteinases. CD44 was used as an indicator of tumor aggressive behavior in malignancy [27]. CD44 was first described as a CSC marker in breast cancer [28]. However, it has limited utility as a CSC marker or prognostic factor in ccRCC.

In this study, we found high CD44 expression in 66 cases (55%), and this was in accordance with what was reported by Qin *et al.*, who found high CD44 expression in 46.67% of cases [29]. In the current work a statistically significant association between CD44 immunostaining and high tumor grade ($p = 0.003$), tumor stage ($p = 0.008$), lymph node status ($p = 0.040$) and high tumor AJCC stage ($p = 0.002$) was detected. These results were in line with Paradis *et al.*, who reported a link between CD44 and grade

and T stage [30]. No statistically significant difference in CD44 expression was found in relation to the other clinicopathological features of the patients.

In the current work a significant association was found between CD44 and OS ($p = 0.001$). This was in line with Qin *et al.*, who reported CD44 as an independent predictor of OS in their research [29], while another study found that it was not an independent predictor of survival but they found a significant association with the poor prognostic factors clinical stage and Fuhrman grade [30].

Multivariate analysis indicated that positive MCM2 and CD44 expressions were independent risk factors of prognosis in ccRCC patients, suggesting that high MCM2 and CD44 expressions are molecular markers of poor prognosis in ccRCC patients.

Our study revealed no significant correlation between MCM2 and CD44. To our knowledge no previous work has evaluated this relation.

In conclusion, this study suggests that increased cell proliferation demonstrated by high immunohistochemical expression of MCM2 and cell adhesion investigated through increased level of CD44 in ccRCC is related to adverse prognosis.

The authors declare no conflict of interest.

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