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Cytological-Pathologic Correlation

Cytoplasmic expression of B7-H3 and membranous EpCAM expression are associated with higher grade and survival outcomes in patients with clear cell renal cell carcinoma

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ABSTRACT

B7-H3 and EpCAM are overexpressed in cancer and play a role in tumorigenesis and metastasis. In this study, the membranous, cytoplasmic and nuclear expression levels of B7-H3 and EpCAM biomarkers were mapped in three major subtypes of renal cell carcinoma (RCC). Expression of B7-H3 and EpCAM were evaluated using immunohistochemistry in RCC samples on tissue microarrays (TMAs), including clear cell RCCs (ccRCCs), type I and II papillary RCCs (pRCCs), and chromophobe RCCs (chRCCs). The association between B7-H3 and EpCAM expression and clinicopathological features as well as survival outcomes was determined. There was a statistically significant difference between B7-H3 and EpCAM expression among the different RCC subtypes. In ccRCC, higher cytoplasmic expression of B7-H3 was significantly associated with increase in nucleolar grade, lymph node invasion (LNI), invasion of the Gerota's fascia, and tumor necrosis, while no association was found with the membranous and nuclear expression. Moreover tumors with cytoplasmic expression of B7-H3 tended to have a worse prognosis for disease-specific survival (DSS) than those with membranous expression. In case of EpCAM, increased membranous expression of EpCAM was associated with nucleolar grade and tumor necrosis in ccRCC. Additionally, membranous EpCAM expression added prognostic value in patients with ccRCC who had high nucleolar grade versus low nucleolar grade. Moreover, membranous EpCAM expression was found to be an independent favorable prognostic marker for progression-free survival (PFS) in ccRCC. Our results demonstrated that higher cytoplasmic B7-H3 and membranous EpCAM expression are clinically significant in ccRCC and are associated with more aggressiveness tumor behavior.

1. Introduction

Renal cell carcinoma (RCC) is the most common neoplasm of the adult kidney, responsible for approximately 90% of all cases [4]. Initial treatment is most commonly either partial or complete removal of the affected kidney(s) and remains the mainstay of curative treatment [13]. Where the cancer has not metastasized or grown deeper into the tissues of the kidney the 5-year survival rate is 65–90%, a number that is lowered considerably when the cancer has spread [25]. Unfortunately, a considerable number of RCC patients have advanced disease by the time of diagnosis, or in the time after primary surgery [18]. Better prognostic and predictive biomarkers are sorely needed.

RCC is divided into several histological subtypes including clear cell RCC (ccRCC) which is the predominant subtype with a prevalence of 70% of all RCCs, papillary RCC (pRCC; 10–15%) is the most frequent compared to the more rare types of, chromophobe RCC (chRCC; 5%) and collecting duct carcinoma, and other unclassified [24]. Consequently, we focused on the three dominating histological subtypes:

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ccRCC, pRCC, and chRCC and two putative interesting biomarkers in RCC; B7 homolog 3 (B7-H3) (CD276) [19,33] and Epithelial cell adhesion/activating molecule (EpCAM) (CD326) [12,41].

Recently, the immunosuppressive factors belonging to the B7 family of proteins or their corresponding ligands have been rewarded a lot of interest. Several inhibitors, mainly antibodies, counteracting the function of these proteins have been approved for cancer treatment or are in clinical trials [22]. These include targeting of CTLA-4, PD-L1 (B7-H1), PD-1, and B7-H3 in patients with metastatic disease. As in many tumor types, a correlation between expression levels of B7-H3 and advanced disease has been reported in ccRCC, but the effects have been ascribed to its immunological functions [19]. Importantly, however, the current knowledge is not useful in guiding the therapy of individual patients, and the mechanism underlying the effects of B7-H3 is not known.

The EpCAM antigen is an interesting marker as it is one of the most frequently and most highly expressed antigens on epithelial carcinomas, including RCC, and the expression increases as the cancer progress from lower to higher grades. We have recently completed a phase-I clinical study with our anti-EpCAM immunotoxin MOC31PE with some promising anti-tumor effects and without serious adverse events [1]. The clinical study with MOC31PE suggests that it may also be effective in other tumor types, like urothelial cancer.

In this study, for the first time we have mapped the membranous, cytoplasmic and nuclear expression levels of these biomarkers in subtypes of RCC, related to disease stage, aggressiveness and patient survival outcomes. The results provide a basis for future diagnosis, prognosis and clinical choice of targeted therapy in RCC and may help to define subgroups of patients requiring different follow-up strategy.

2. Material & methods

2.1. Patient characteristics and tumor samples

A total of 265 paraffin-embedded tissues from RCC samples, including ccRCC, pRCC (type I and II), and chRCC were examined in this study. These specimens were received from the Hasheminejad hospital, Tehran, Iran, from 2008 to 2015. All samples were collected from patients who had undergone radical nephrectomy and had no history of radiation therapy. The hematoxylin and eosin (H & E) stained slides and medical archival records were retrieved to obtain clinicopathological parameters. Information about patients' outcomes including the time between radical nephrectomy and cancer-related death or last follow-up (if death did not occur) and primary surgery and last follow-up if the patient showed no evidence of disease, recurrence, or metastasis of RCC or cancer-related death was also recorded. In addition, tumor stage and nucleolar grade were defined according to the pTNM classification for RCC [11].

2.2. TMA construction

The renal tissue microarrays (TMAs) were prepared as described previously [38-40]. In brief, hematoxylin and eosin slides were examined and the most representative areas in different parts of the tumor were marked by two experienced pathologists (M.A. and M.A.). Microarray samples of 0.6 mm diameter were punched out from the selected regions of each donor block and precisely transferred into a new recipient paraffin block using tissue arrayer MiniCore (ALPHELYS, Plaisir, France). In the present study, three cores were punched and evaluated from each tumor and scored individually. Then, four micrometer sections were cut from the completed array blocks and transferred to adhesive slides. Next, TMA blocks were constructed in three copies for each specimen. The mean overall histochemical score (H-score) value of three cores was calculated as final scores.

2.3. Immunohistochemistry staining

The expression levels of B7-H3 and EpCAM were determined using TMA-based immunohistochemical (IHC) analysis applying the standard method. Briefly, all TMA sections were deparaffinized. Then unmasking of epitopes were carried out in PT-Link (Dako, Glostrup, Denmark) using target retrieval solution at low pH (citrate, pH 6) for anti-B7-H3 and high pH (Tris/EDTA, pH 9) for anti-EpCAM and rinsed in Dako wash buffer according to the manufacturer's instructions. Sections were incubated with 0.3% hydrogen peroxide (H_2O_2) solution for 5 min to block endogenous peroxidase activity. Sections were then incubated with mouse monoclonal anti-B7-H3 antibody (LifeSpan BioSciences, Seattle, WA), and mouse monoclonal anti-EpCAM (Abcam, Cambridge, UK) antibodies for 1 h at 1:100 dilutions. Sections were then treated as described [2]. Positive controls consisting of colorectal carcinoma were satisfactory in each experiment.

2.4. Evaluation of immunostaining

The stained TMAs slides were scored by the study pathologist (U.A), without previous knowledge of the clinicopathological parameters. The intensity of staining was scored by applying a semi-quantitative system ranging from negative to strong as follows: 0 = negative, 1 = weak, 2 = moderate, and 3 = strong. The percentage of positive cells was categorized according to the positive tumor cells as follows: Group 1: < 25% positive cells, Group 2: 25% to 50% positive cells, Group 3: 51% to 75% positive cells, and Group 4: > 75% positive cells. For comparing all the available data, an overall histochemical score (H-score) was assigned to each case by multiplying the intensity score by the percentage of positive cells, which yielded a range from 0 to 300. In this study, the H-scores were classified into three groups: 0–100 as group 1(low expression), 101–200 as group 2 (moderate expression), and 201–300 as group 3 (high expression).

2.5. Statistical analysis

All data were analyzed using the “statistical software SPSS, version 20.0. Armonk, NY: IBM Corp”. We reported the categorical data by N (%), valid percent and quantitative data as follows, mean (SD) and median (Q1, Q3).The comparisons of B7-H3 and EpCAM expression in ccRCC, pRCC (type I and II), and chRCC samples were done using Kruskal-Wallis and Mann-Whitney *U* tests, for pairwise comparison between groups. Moreover, Pearson's $\chi 2$ test was used to analyze the significance of association and correlation between B7-H3 and EpCAM expression and clinicopathological parameters. Disease-specific survival (DSS) was measured from the date of nephrectomy to the date of death caused due to RCC. Progression-free survival (PFS) was defined as the interval between primary surgery and the last follow-up visit without evidence of disease, recurrence or progression. The DSS and PFS were estimated using Kaplan–Meier method with 95% confidence intervals (CI) and compared across the groups using the log-rank test. The Cox proportional hazards regression model was applied to determine which variables influenced the DSS and PFS. Variables that significantly influenced survival in univariate analysis were included in multivariable analysis. A P value of < 0.05 was considered statistically significant.

3. Results

3.1. Patient characteristics

3.1.1. Stained by B7-H3 antibody

Of 265 cases, 225 RCC patients was evaluated which, include 147 (65.3%) ccRCC, pRCC type I 23 (10.24%) and type II 21 (9.36%), and 34 (15.1%) chRCCs. Technical problems led to a loss of some cases; the punches contained no tumor tissue at all. The patients'

Table 1
Patients and tumor pathological characteristic of the study population.

Patients and tumor characteristics	Total samples N(%)	RCC			
		ccRCC N(%)	pRCC N(%)		chRCC N(%)
			Type I	Type II	
Number of tumor samples	225	147(65.3)	23(10.24)	21(9.36)	34(15.1)
Mean age, years (range)	55(25–82)	57(25–82)	56(29–76)	51(25–73)	50(27–76)
≤Mean age	108(48.0)	77(52.4)	13(56.5)	10(47.6)	18(52.9)
> Mean age	117(52.0)	70(47.6)	10(43.5)	11(52.4)	16(47.1)
Gender					
Male	158(70.2)	100(68.0)	21(91.3)	16(76.2)	21(61.8)
Female	67(29.8)	47(32.0)	2(8.7)	5(23.8)	13(38.2)
(Male/female)	2.35	2.12	10.5	3.2	1.61
Tumor size (cm)					
0–4	44(19.6)	31(21.1)	6(26.1)	3(14.3)	4(11.8)
4.1–7	84(37.3)	51(34.7)	10(43.5)	8(38.1)	15(44.1)
7.1–10	46(20.4)	35(23.8)	3(13.0)	3(14.3)	5(14.7)
> 10.1	51(22.7)	30(20.4)	4(17.4)	7(33.3)	10(29.4)
Nucleolar grade					
I	9(4.0)	8(5.4)	1(4.3)	0(0.0)	0(0.0)
II	101(44.9)	83(56.5)	13(56.5)	5(23.8)	0(0.0)
III	70(31.1)	45(30.6)	9(39.1)	16(76.2)	0(0.0)
IV	11(4.9)	11(7.5)	0(0.0)	0(0.0)	0(0.0)
Primary tumor (PT) stage					
pT1	63(28.0)	45(30.6)	12(52.2)	1(4.8)	5(14.7)
pT2	20(8.9)	12(8.2)	4(17.4)	2(9.5)	2(5.9)
pT3	124(55.1)	78(53.1)	7(30.4)	13(61.9)	26(76.5)
pT4	18(8.0)	12(8.2)	0(0.0)	5(23.8)	1(2.9)
Microvascular invasion (MVI)					
Present	48(21.3)	30(20.4)	1(4.3)	7(33.3)	10(29.4)
Absent	176(78.2)	117(79.6)	22(95.7)	14(66.7)	23(67.6)
Not identified	1(0.4)	0(0.0)	0(0.0)	0(0.0)	1(2.9)
Lymph node invasion (LNI)					
Involved	12(5.3)	9(6.1)	18(78.3)	3(14.3)	0(0.0)
None	186(82.7)	118(80.3)	5(21.7)	16(76.2)	34(100.0)
Not identified	27(12.0)	20(13.6)	0(0.0)	2(9.5)	0(0.0)
Renal vein invasion					
Present	21(9.3)	15(10.2)	3(13.0)	3(14.3)	0(0.0)
Absent	204(90.7)	132(89.8)	20(87.0)	18(85.7)	34(100.0)
Histological tumor necrosis					
Present	93(41.3)	54(36.7)	14(60.9)	15(71.4)	10(29.4)
Absent	129(57.3)	92(62.6)	9(39.1)	6(28.6)	22(64.7)
Not identified	3(1.3)	1(0.7)	0(0.0)	0(0.0)	2(5.9)
Renal sinus fat invasion					
Present	130(57.8)	81(55.1)	5(22.7)	18(85.7)	26(76.5)
Absent	95(42.2)	66(44.9)	17(77.3)	3(14.3)	8(23.5)
Renal pelvis invasion					
Present	22(9.8)	12(8.2)	3(13.6)	7(33.3)	0(0.0)
Absent	203(90.2)	135(91.8)	19(86.4)	14(66.7)	34(100.0)
Perirenal fat invasion					
Present	47(20.9)	30(20.4)	5(21.7)	5(23.8)	7(20.6)
Absent	178(79.1)	117(79.6)	18(78.3)	16(76.2)	27(79.4)
Gerota's fascia invasion					
Present	13(5.8)	11(7.5)	2(8.7)	0(0.0)	0(0.0)
Absent	212(94.2)	136(92.5)	21(91.3)	21(100.0)	34(100.0)
Distant metastasis					
Present	48(21.3)	38(25.9)	2(8.7)	4(19.0)	4(11.8)
Absent	177(78.7)	109(74.1)	21(91.3)	17(81.0)	30(88.2)
Tumor recurrence					
Yes	35(15.6)	30(20.4)	1(4.3)	0(0.0)	4(11.8)
No	190(84.4)	117(79.6)	22(95.7)	21(100.0)	30(88.2)

RCC indicates renal cell carcinoma.
ccRCC; clear cell renal cell carcinoma, pRCC; papillary renal cell carcinoma, chRCC; chromophobe renal cell carcinoma.

clinicopathological features for samples from the study population stained with B7-H3 antibody are summarized in Table 1. It was agreed that nucleolar grade for chRCC should not be given [11,23]. In addition, the majority of the cases that were categorized as pT3 in our study had involvement of renal sinus fat or loose connective tissue. This category also included some cases that had renal vein invasion and a few cases with renal sinus vessel invasion without involvement of renal sinus fat or connective tissue.

3.1.2. Stained by EpCAM antibody

For EpCAM expression, 222 cases were evaluated. The patients' clinicopathological features for the study population with samples stained by EpCAM antibody are summarized in Table 2.

3.2. Comparison of B7-H3 and EpCAM expression in RCC subtypes

The expression levels of B7-H3 and EpCAM markers were assessed using IHC on TMA sections by three scoring methods, namely intensity

Table 2
Patients and tumor pathological characteristics of the study population.

Patients and tumor characteristics	Total samples N(%)	RCC			
		ccRCC N(%)	pRCC N(%)		chRCC N(%)
			Type I	Type II	
Number of tumor samples	222	165(74.3)	13(5.88)	10(4.52)	34(15.3)
Mean age years (range)	55(25–82)	57(25–82)	58(29–76)	46(25–69)	50(27–76)
≤Mean age	105(47.3)	85(51.5)	6(46.2)	6(60.0)	18(52.9)
> Mean age	117(52.7)	80(48.5)	7(53.8)	4(40.0)	16(47.1)
Gender					
Male	152(68.5)	112(67.9)	13(100.0)	7(70.0)	20(58.8)
Female	70(31.5)	53(32.1)	0(0.0)	3(30.0)	14(41.2)
(Male/female)	2.17	2.11	13.0	2.33	1.42
Tumor size (cm)					
0–4	42(18.9)	36(21.8)	1(7.7)	2(20.0)	3(8.8)
4.1–7	79(35.6)	53(32.1)	7(53.8)	4(40.0)	15(44.1)
7.1–10	53(23.9)	45(27.3)	1(7.7)	2(20.0)	5(14.7)
> 10.1	48(21.6)	31(18.8)	4(30.8)	2(20.0)	11(32.4)
Nucleolar grade					
I	9(4.1)	9(5.5)	0(0.0)	0(0.0)	0(0.0)
II	107(48.2)	97(58.8)	8(61.5)	2(20.0)	0(0.0)
III	59(26.6)	46(27.9)	5(38.5)	8(80.0)	0(0.0)
IV	13(5.9)	13(7.9)	0(0.0)	0(0.0)	0(0.0)
Primary tumor (PT) stage					
pT1	63(28.4)	52(31.5)	5(38.5)	1(10.0)	5(14.7)
pT2	19(8.6)	15(9.1)	2(15.4)	0(0.0)	2(5.9)
pT3	123(55.4)	85(51.5)	6(46.2)	6(60.0)	26(76.5)
pT4	17(7.7)	13(7.9)	0(0.0)	3(30.0)	1(2.9)
Microvascular invasion (MVI)					
Present	47(21.2)	32(19.4)	1(7.7)	4(40.0)	10(29.4)
Absent	175(78.8)	133(80.6)	12(92.3)	6(60.0)	24(70.6)
Lymph node invasion (LNI)					
Involved	10(4.5)	6(3.6)	1(7.7)	3(30.0)	0(0.0)
None	179(80.6)	130(78.8)	8(61.5)	7(70.0)	34(100.0)
Not identified	33(14.9)	29(17.6)	4(30.8)	0(0.0)	0(0.0)
Renal vein invasion					
Present	22(9.9)	16(9.7)	4(30.8)	2(20.0)	0(0.0)
Absent	200(90.1)	149(90.3)	9(69.2)	8(80.0)	34(100.0)
Histological tumor necrosis					
Present	85(38.3)	60(36.4)	7(53.8)	7(70.0)	11(32.4)
Absent	135(60.8)	104(63.0)	6(46.2)	3(30.0)	22(64.7)
Not identified	2(0.9)	1(0.6)	0(0.0)	0(0.0)	1(2.9)
Renal sinus fat invasion					
Present	48(21.6)	88(53.3)	7(53.8)	9(90.0)	25(73.5)
Absent	174(78.4)	77(46.7)	6(46.2)	1(10.0)	9(26.5)
Renal pelvis invasion					
Present	25(11.3)	16(9.7)	3(23.1)	6(60.0)	0(0.0)
Absent	197(88.7)	149(90.3)	10(76.9)	4(40.0)	34(100.0)
Perirenal fat invasion					
Present	48(21.6)	35(21.2)	4(30.8)	2(20.0)	7(20.6)
Absent	174(78.4)	130(78.8)	9(69.2)	8(80.0)	27(79.4)
Gerota's fascia invasion					
Present	17(7.7)	15(9.1)	2(15.4)	0(0.0)	0(0.0)
Absent	205(92.3)	150(90.9)	11(84.6)	10(100.0)	34(100.0)
Distant metastasis					
Present	51(23.0)	42(25.5)	3(23.1)	2(20.0)	4(11.8)
Absent	171(77.0)	123(74.5)	10(76.9)	8(80.0)	30(88.2)
Tumor recurrence					
Yes	38(17.1)	34(20.6)	0(0.0)	0(0.0)	4(11.8)
No	184(82.9)	131(79.4)	13(100.0)	10(100.0)	30(88.2)

RCC indicates renal cell carcinoma.
ccRCC; clear cell renal cell carcinoma, pRCC; papillary renal cell carcinoma, chRCC; chromophobe renal cell carcinoma.

of staining, percentage of positive tumor cells, and H-score. In the RCC samples these markers were expressed at different intensities in the cell membrane, cytoplasm and nucleus each of which, were analyzed separately (Supplementary Tables 1 and 2) (Fig. 1).

3.2.1. B7-H3

Membranous B7-H3 expression was observed in 218 (96.9%) patients, cytoplasmic expression in 178 (79.1%) cases and nuclear expression in 9 (4.0%).

The nonparametric Kruskal–Wallis and Mann–Whitney *U* tests were used to compare the differences between the median expressions of B7-H3 among the three tumor subtypes. The results of Kruskal-Wallis showed a statistically significant difference between the levels of cytoplasmic B7-H3 expressions in different RCC subtypes ($P < 0.001$). With the Mann–Whitney *U* test, significant differences in the median levels of cytoplasmic B7-H3 expression were observed between the ccRCC and pRCC ($P < 0.001$) and also pRCC and chRCC ($P = 0.001$) (Fig. 2A). In contrast, we did not observe a statistically significant difference in median membranes and nuclear B7-H3 expression between the three subtypes of RCC ($P = 0.654$, $P = 0.274$, respectively).

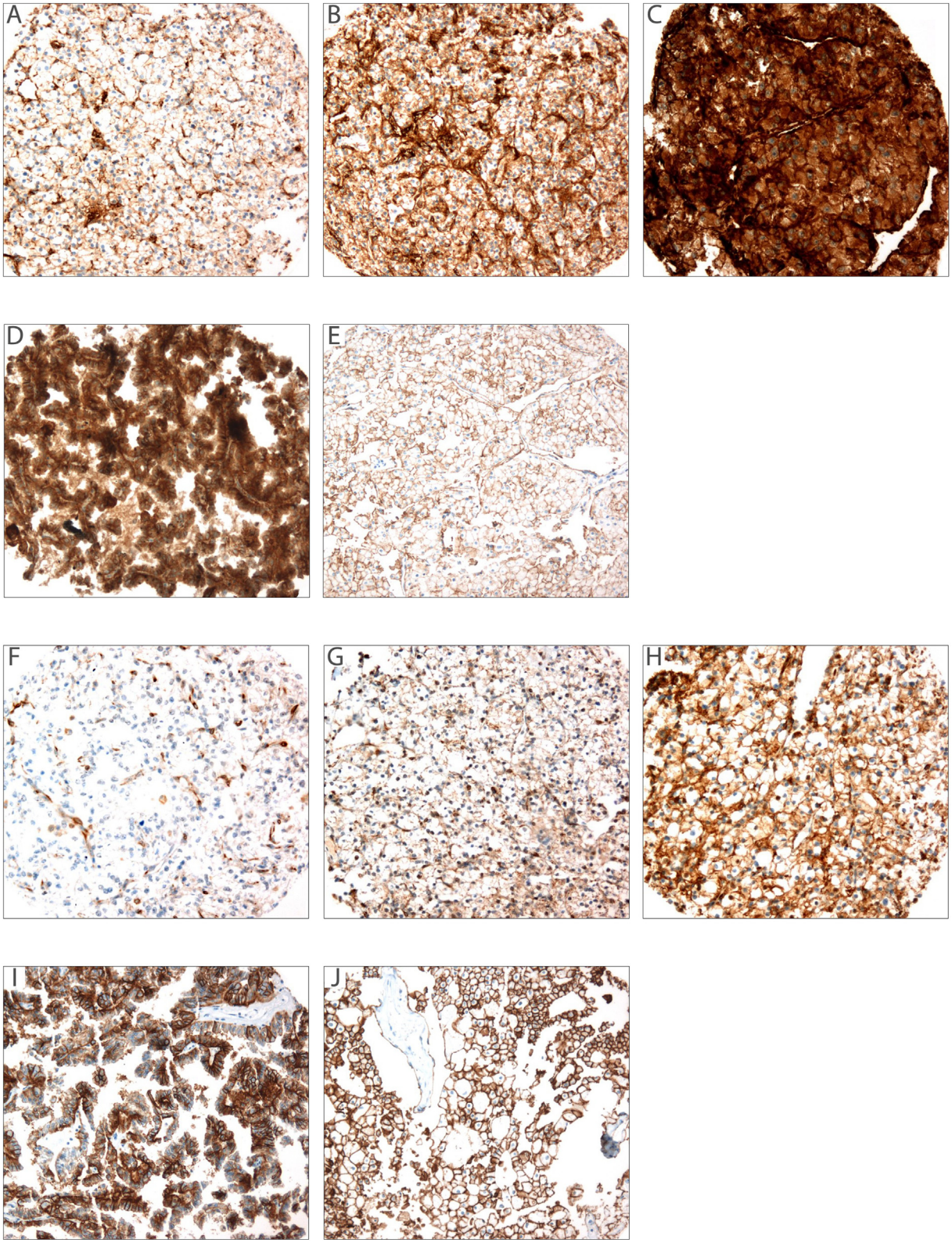

Fig. 1. B7-H3 and EpCAM protein expression in three subtypes of renal cell carcinoma (RCC) samples. B7-H3 expression in clear cell renal cell carcinoma (ccRCC): low expression (A), moderate (B), and strong expression (C). B7-H3 expression in papillary RCC: (D) and in chromophobe RCC (E). EpCAM expression in ccRCC: low expression (F), moderate (G), and strong expression (H). EpCAM expression in papillary RCC: (I) and in chromophobe RCC (J).

3.2.2. EpCAM

Membranous expression of EpCAM was observed in 110 (49.5%) patients, cytoplasmic expression in 28 (12.6%) cases and nuclear expression in 8 (3.6%).

The results of Kruskal-Wallis test indicated a statistically significant difference between the membranous expression of EpCAM in the different RCC subtypes ($P < 0.001$). Thus, with the Mann–Whitney *U* test, a significant difference was observed in membranous EpCAM

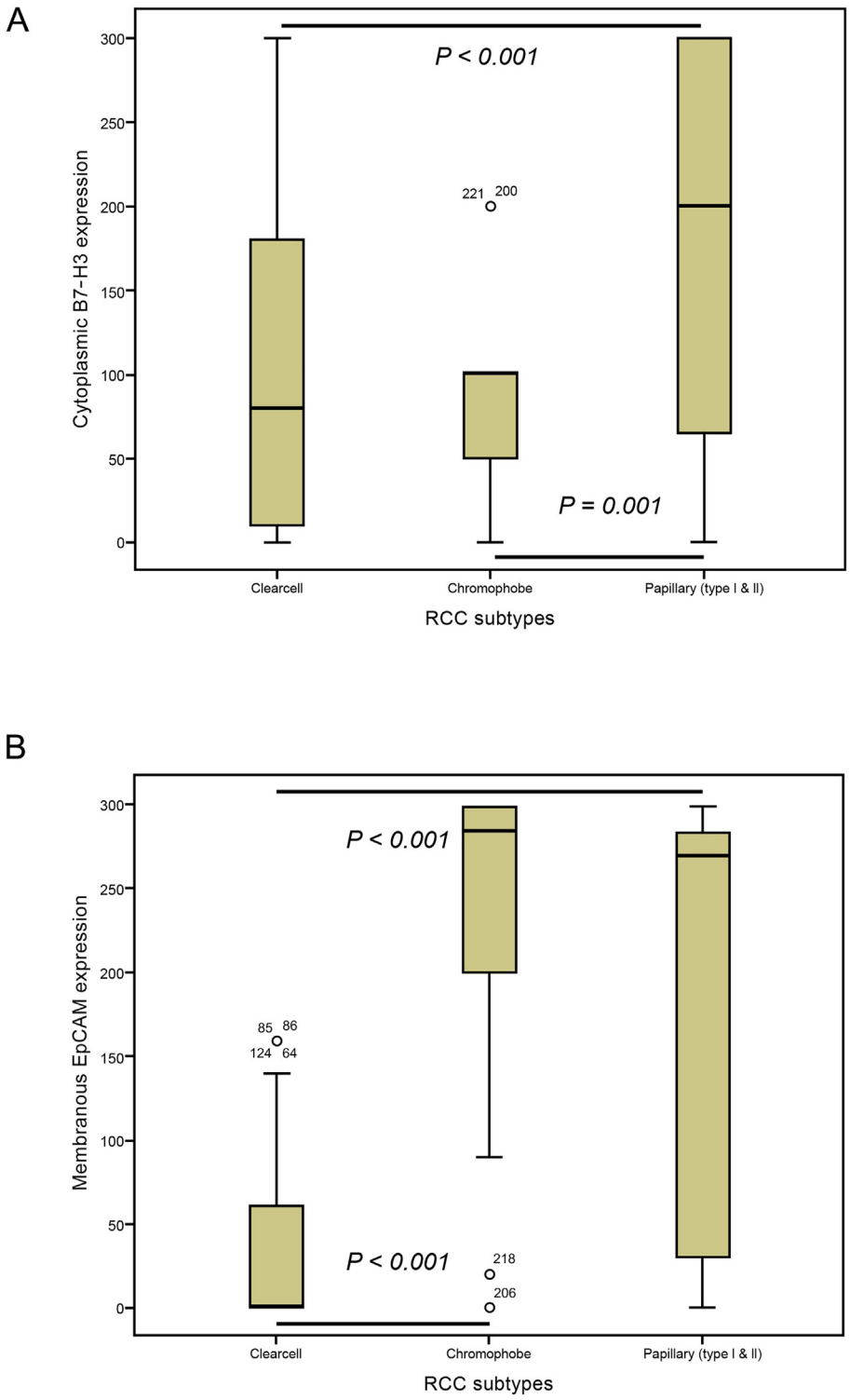

Fig. 2. The median cytoplasmic and membranous expression levels of B7-H3 and EpCAM in different RCC patients using Mann–Whitney *U* test. (A) The results showed there was a statistically significant difference in cytoplasmic B7-H3 expression between ccRCC and pRCC ($P < 0.001$) and between chRCC and pRCC ($P = 0.001$). (B) A significant difference also was seen in membranous EpCAM expression between ccRCC and pRCC cases ($P < 0.001$) and between ccRCC and chRCC ($P < 0.001$) (B). On the basis of the standard definitions, each box-plot shows median (bold line), interquartile line (box), and outlier observation (circle).

expression between ccRCC and pRCC cases ($P < 0.001$) and between ccRCC and chRCC ($P < 0.001$), (Fig. 2B). However, we did not find a statistically significant difference in median cytoplasmic and nuclear EpCAM expression between three subtypes of RCC ($P = 0.254$, $P = 0.240$, respectively).

3.3. Association of B7-H3 expression with clinicopathological parameters

A highly significant association was observed between cytoplasmic B7-H3 expression and nucleolar grades (Pearson's $\chi 2$, $P = 0.045$). Kruskal–Wallis test showed statistically significant differences ($P < 0.001$) in the levels of cytoplasmic B7-H3 expression in various

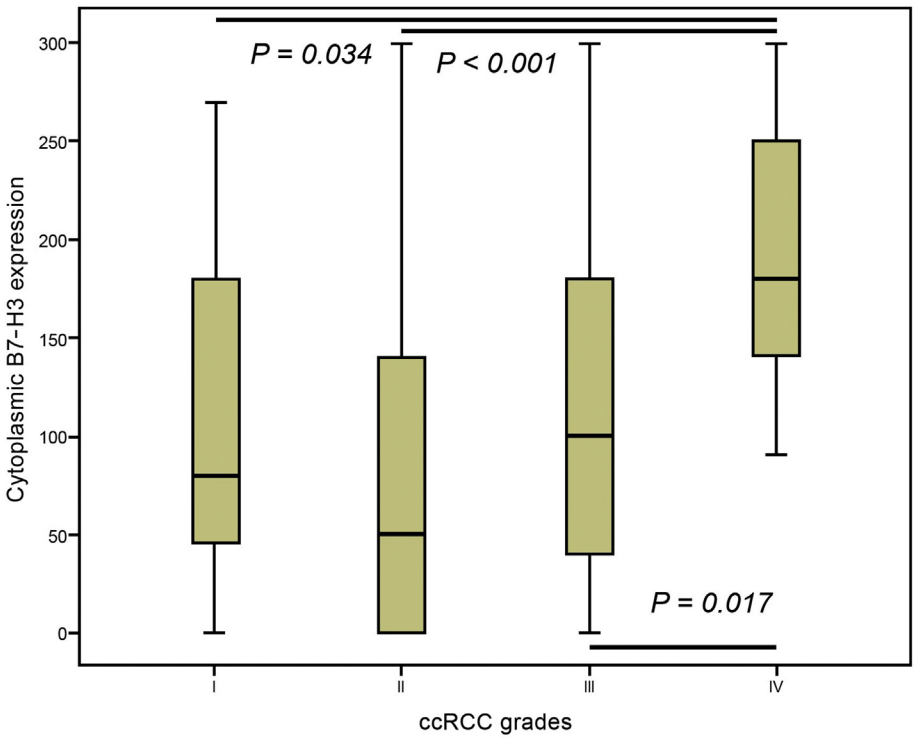

Fig. 3. Box plot analysis of cytoplasmic B7-H3 expression levels in grades in clear cell RCC. The results of Mann–Whitney *U* test showed that there were statistically significant differences in median levels of B7-H3 expression among grade I and IV (P = 0.034), II and IV (P < 0.001), and III and IV (P = 0.017). On the basis of the standard definitions, each box-plot shows the median (bold line) and interquartile lines (box).

nucleolar grades (I–IV). Additionally, highly significant differences were found in the median levels of B7-H3 expression among grade I and IV (P = 0.034), II and IV (P < 0.001), and III and IV (P = 0.017) using Mann–Whitney *U* test (Fig. 3). Moreover, a statistically significant association was observed between the levels of cytoplasmic B7-H3 expression and both lymph node invasion (LNI) (P = 0.037), invasion of the Gerota's fascia (P = 0.048), and histological tumor necrosis (P = 0.014). We also found a significant association among histological tumor necrosis, nucleolar grade (P = 0.002), and tumor size (P < 0.001). We did not find any significant association in membranous and nuclear B7-H3 expression and important clinicopathological features in ccRCC.

In this study, there was no significant association between B7-H3 expression and any clinicopathological parameters in pRCC (type I and II). In chRCC, we found a statistically significant association between cytoplasmic B7-H3 expression and distant metastasis (P = 0.047).

3.4. Association of EpCAM expression with clinicopathological parameters

The highly statistically significant association was observed between membranous EpCAM expression and increased nucleolar grade (P = 0.007) and histological tumor necrosis (P = 0.002). We did not find any statistically significant association between cytoplasmic and nuclear EpCAM expression and any clinicopathological parameters.

In type I and II pRCC, there was no statistically significant association between EpCAM expression and clinicopathological features. In chRCC, a statistical significant association was found only between membranous EpCAM expression and tumor stage (P = 0.037).

3.5. Prognostic value of B7-H3 expression for clinical outcome

Two hundred twenty five RCC samples were included in the present study, of which 171 (76.0%) patients had no history of recurrence, metastasis or cancer-related death, while 54 (24.0%) patients were positive for these parameters. Metastasis and recurrence occurred in 48 (21.3%) and 35 (15.6%) patients, respectively. Cancer-related death was documented in 35 patients (15.6%) during the follow-up period. The mean duration of follow-up time was 49.0 months (SD = 25.6), median was 46.0 months (32, 67); ranging from 1 to 117 months. The main characteristics of patients enrolled for survival analysis according to RCC subtypes is illustrated in Supplementary Table 3.

3.5.1. Clear cell RCC

3.5.1.1. Survival outcomes based on membranous B7-H3 expression. Kaplan-Meier analysis showed that there was no significant differences between DSS or PFS and the patients with high, moderate and low membranous expression of B7-H3 (Log Rank test; P = 0.118 (Fig. 4A), P = 0.281, respectively).

3.5.1.2. Survival outcomes based on cytoplasmic B7-H3 expression. The results of Kaplan-Meier curve demonstrated significant differences between DSS and the patients with high, moderate and low cytoplasmic expression of B7-H3 (P = 0.015) (Fig. 4B). The mean DSS time for patients with high, moderate and, low cytoplasmic expression of B7-H3 was 74 (SD = 11.2), 93 (SD = 7.0), and 98 (SD = 3.5) months, respectively. In addition, the 5-year DSS for patients whose specimens expressed high, moderate, and low cytoplasmic expression of B7-H3 was 59, 81, and 86%, respectively (P = 0.025). Moreover, the results of Kaplan-Meier showed that there was no significant differences between PFS and the patients with high, moderate and low cytoplasmic B7-H3 expression (P = 0.101).

3.5.1.3. Survival outcomes based on nuclear B7-H3 expression. Due to the low number of cases in the group with high nuclear expression of B7-H3 (1case), survival curves and Cox proportional hazards regression analysis were not exploited.

To investigate whether B7-H3 expression was an independent prognostic factor of DSS and to assess the clinical significance of various parameters that might influence survival outcomes in patients with ccRCC, univariate and multivariate analyses were performed. As summarized in Table 3, cytoplasmic B7-H3 expression (P = 0.02), tumor stage (P < 0.001), nucleolar grade (P = 0.003), and tumor size (P = 0.001) were significant risk factors affecting the DSS of patients with ccRCC in univariate analysis. Tumor stage was found as an

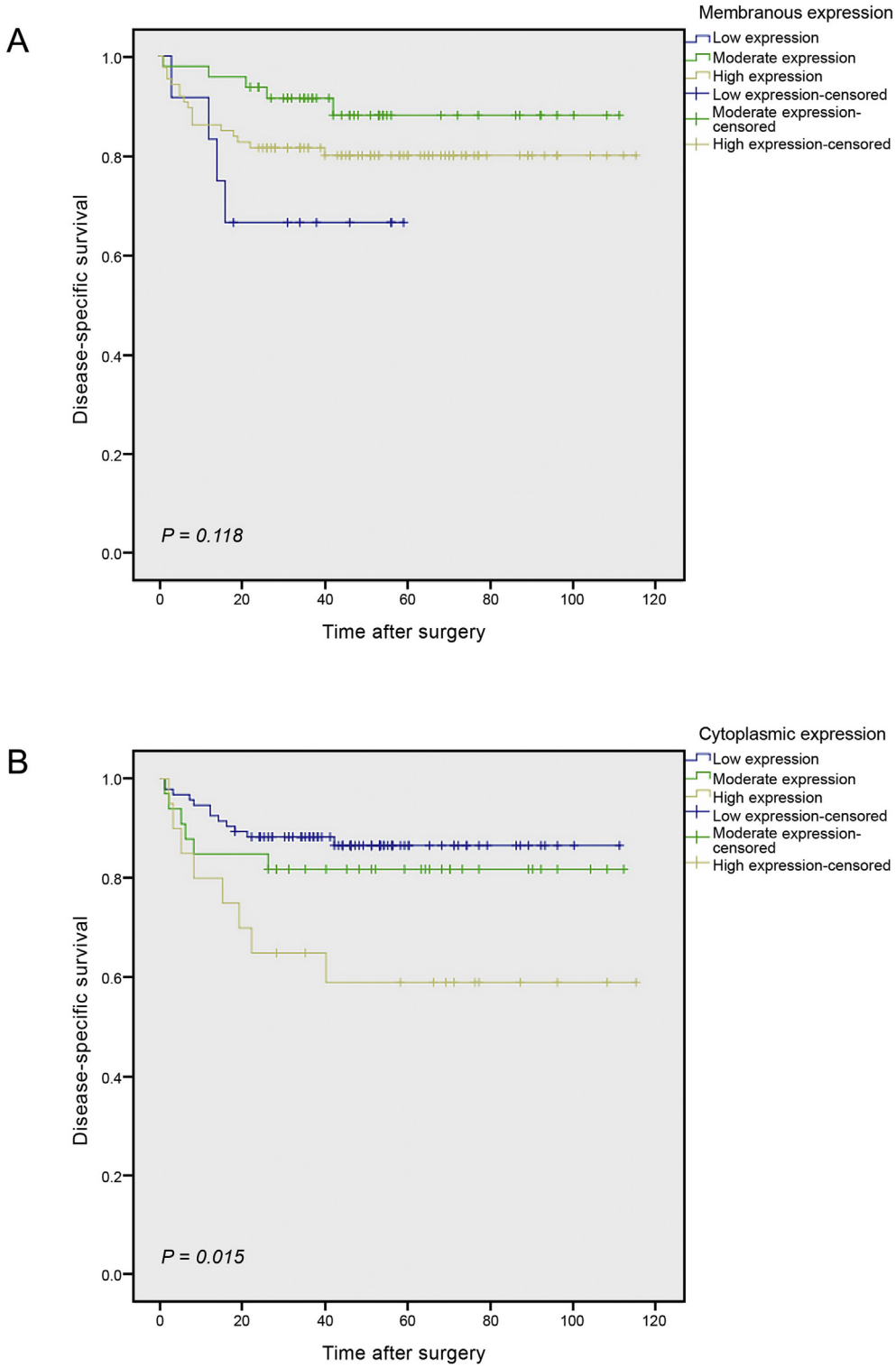

Fig. 4. Kaplan-Meier curves for disease-specific survival (DSS) according to the expression levels of membranous and cytoplasmic B7-H3 in ccRCC. The results showed that patients with high cytoplasmic B7-H3 expression had shorter disease-specific survival (P = 0.015) (B) compared to patients with high membranous B7-H3 expression (P = 0.118) (A). (Membranous and cytoplasmic expressions were grouped into low versus moderate versus high expression levels).

independent prognostic factor in membranous and cytoplasmic B7-H3 expression (P < 0.001, P = 0.002, respectively). In addition, cytoplasmic B7-H3 expression added prognostic value of pT4 stage versus pT1 stage (P = 0.03) in patients with ccRCC. Moreover, nucleolar grade (P = 0.04) and tumor size were independent prognostic factors for DSS in cytoplasmic B7-H3 expression (P = 0.04), but not in membranous expression in multivariate analysis. Other clinicopathologic variables were not significant factors affecting the DSS of patients with ccRCC.

3.6. Prognostic value of EpCAM expression for clinical outcome

Of the 222 RCC samples that were included in this study, 164 (73.9%) patients had no history of recurrence, metastasis, or cancer-related death, while 58 (26.1%) of the patients were positive for these

Table 3
Univariate and multivariate cox regression analyses of potential prognostic factors for disease-specific survival in patients with clear cell RCC.

Disease-specific survival (DSS)						
Covariate	Univariate analysis		Multivariate analysis (Membranous)		Multivariate analysis (Cytoplasmic)	
	HR (95% CI)	P-value	HR (95% CI)	P-value	HR (95% CI)	P-value
Membranous B7-H3 expression[a]		0.13		0.01		
Moderate versus low	0.26 (0.07–0.99)	0.04	0.13 (0.03–0.54)	0.005	–	–
High versus low	0.53 (0.17–1.58)	0.25	0.19(0.05–0.66)	0.009		
Cytoplasmic B7-H3 expression		**0.02**				0.17
Moderate versus low	1.47(0.55–3.93)	0.43	–	–	1.35(0.48–3.81)	0.56
High versus low	3.48(1.42–8.52)	**0.006**			2.46(0.94–6.43)	0.06
Nucleolar grade		**0.003**		0.18		**0.04**
II versus I	0.26 (0.05–1.30)	0.10	0.19(0.03–1.13)	0.06	0.12 (0.01–0.75)	0.02
III versus I	1.18 (0.26–5.27)	0.82	0.47(0.08–2.66)	0.39	0.37 (0.06–2.09)	0.26
IV versus I	2.12 (0.41–10.97)	0.36	0.50(0.07–3.59)	0.49	0.54 (0.07–3.80)	0.54
Primary tumor (PT) stage		< **0.001**		< **0.001**		**0.02**
pT2 versus pT1	1.84 (0.33–10.07)	0.48	0.71 (0.10–4.93)	0.72	0.54(0.07–3.78)	0.53
pT3 versus pT1	1.80 (0.58–5.60)	0.30	1.04(0.24–4.55)	0.95	1.05(0.23–4.68)	0.94
pT4 versus pT1	11.40 (3.40–38.22)	< **0.001**	4.82(0.98–23.67)	0.05	3.83(1.77–18.93)	**0.03**
Tumor size (cm)	2.03(1.35–3.06)	**0.001**	1.65(0.98–2.79)	0.06	1.68(1.00–2.81)	**0.04**

Values in bold are statistically significant.
The variables with P value < 0.05 were included in multivariable analyses.
HR hazard ratio, CI confidence interval.
[a] Low expression level is considered as reference group.

events. Metastasis and recurrence occurred in 51 (23.0%) and 38 (17.1%) patients, respectively. During the follow-up period, cancer-related death was documented in 36 patients (16.2%). The mean and median follow-up duration were 49 (SD = 26.8) and 46 (31, 68) months respectively; ranging from 1 to 118 months. The main characteristics of patients enrolled for survival analysis according to RCC subtypes is illustrated in Supplementary Table 4.

3.6.1. Clear cell RCC

3.6.1.1. Survival outcomes based on membranous EpCAM expression. The Kaplan-Meier curve showed that there was no significant differences between DSS and the patients with high, moderate, and low membranous expression of EpCAM (P = 0.290) (Fig. 5A). In contrast, significant differences was observed between PFS and three groups with membranous expression of EpCAM (P = 0.032) (Fig. 5B). In addition, the mean PFS time for patients with high, moderate, and low membranous expression of EpCAM was 66 (SD = 12.6), 64 (SD = 6.8), and 52 (SD = 2.3) months, respectively.

3.6.1.2. Survival outcomes based on cytoplasmic and nuclear EpCAM expression. Due to the low number of cases in the groups with moderate (1case) and high (1case) in cytoplasmic expression of EpCAM, and low number of cases in nuclear expression (8 cases) and H-score which was < 100 (only one group), survival curves and Cox proportional hazards regression analysis were not exploited.

As shown in Table 4, the membranous EpCAM expression and nucleolar grade were significant risk factors affecting for PFS in univariate and multivariable analyses. In addition, high level membranous EpCAM expression added prognostic value in patients with ccRCC who had nucleolar grades II versus I and III versus I (P = 0.003, P = 0.005, respectively).

3.7. Papillary (type I and II) and chromophobe RCC

In pRCC (type I and II) and chRCC patients, there was no statistical significant association between membranous, cytoplasmic and nuclear B7-H3 and EPCAM expression and patients' survival outcomes.

4. Discussion

RCC has the highest cancer-specific mortality rate among urologic tumors [4]. It is resistant to chemotherapy and radiotherapy, and the efficiency of immunotherapeutic agents, such as interleukin-12 and interferon-α for this type of cancer is also limited [5,8,17]. Therefore, identifying biomolecular markers for targeting of RCC will be highly important in future renal cancer therapy.

B7H3 is a member of the B7 family of immune proteins that provide signals for regulating immune responses [6,9,29]. B7-H3 was first identified as a T cell-stimulating protein, but the recent studies describe B7-H3 as a T cell inhibitor that promotes tumor aggressiveness and proliferation, therefore, B7-H3 may be an important immunological target in human malignancies [27]. Previous studies indicate overexpression of B7H3 in various types of cancer, including colorectal [16], melanoma [34], breast [31], prostate [37], leukemia [15], ovarian [36], and pancreatic cancer [7]. Increasing data support an association between increased expression of B7-H3 and worse prognosis and increased potential to metastasize [27]. B7-H3 has also been studied in RCC. Their findings showed that B7-H3 expressed in both tumor mesenchyme and supporting vasculature in ccRCC [10].

EpCAM is a transmembrane glycoprotein with primary cell adhesion characteristics [26,35]. It is demonstrated that EpCAM play a role in tumorigenesis and metastasis of carcinomas, therefore, it can also act as a potential prognostic marker and as a potential target for immunotherapeutic strategies [3]. The association of EpCAM expression with RCC is controversial. Previous studies demonstrated that EpCAM expression is an independent predictor of prolonged DSS in ccRCC and RCC [12,28], while others concluded that overexpression of EpCAM in high grade RCC cases correlated with longer PFS [41]. These lines of evidence show that the information regarding EpCAM is not consistent and therefore further investigations should be performed for clarifying the potential role of EpCAM as a prognostic marker.

In the present study, for the first time, the membranous, cytoplasmic, and nuclear expression levels of B7-H3 and EpCAM were investigated in a well-characterized series of 225 and 222 tissues samples from patients treated with radical nephrectomy in three main subtypes of RCC and the impact of B7-H3 and EpCAM in RCC prognosis was evaluated. Studies on RCC subtypes is important considering the various subtypes of RCC are associated with different biologic behavior

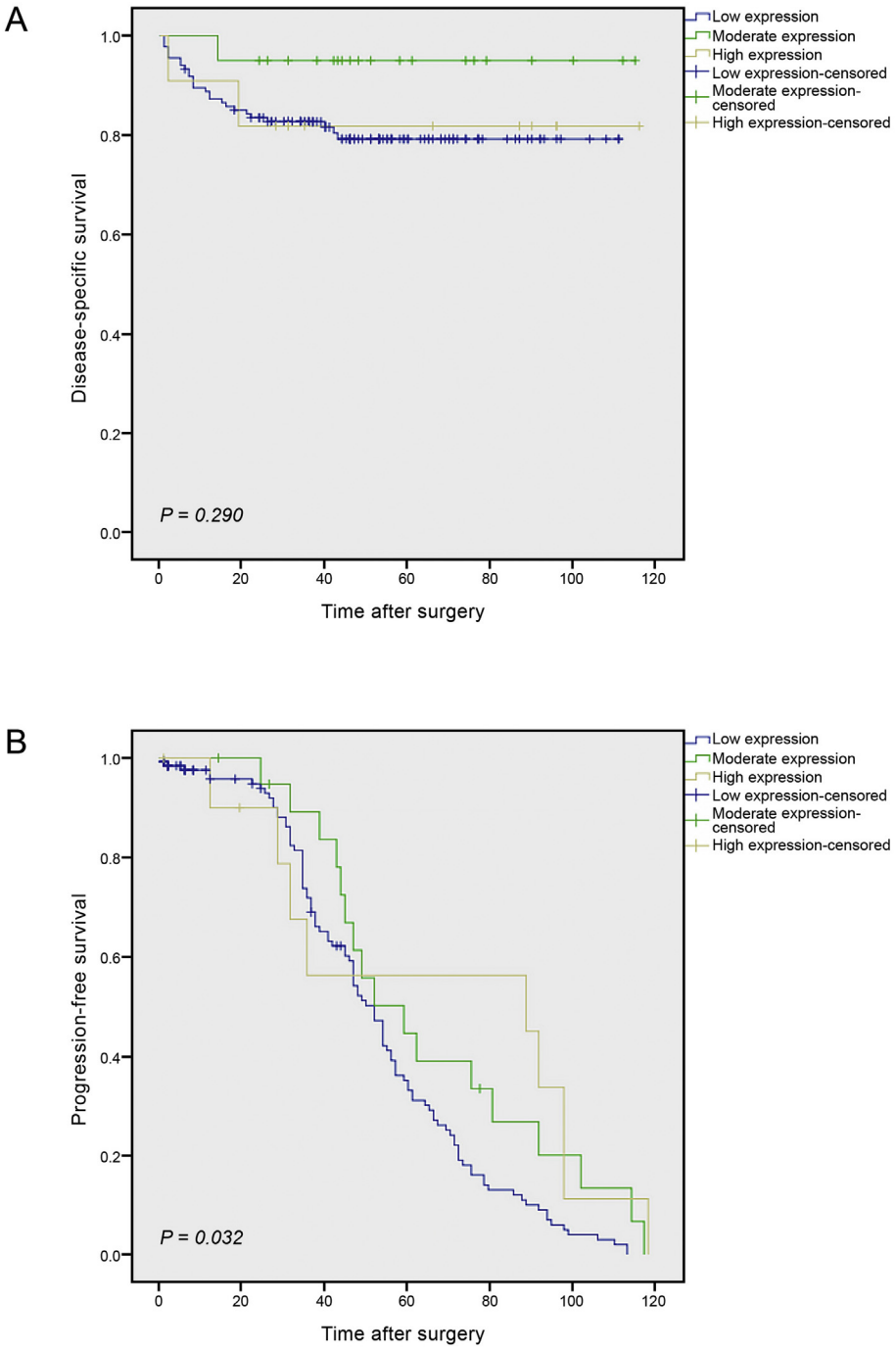

Fig. 5. Kaplan-Meier curves for disease-specific survival (DSS) and progression-free survival (PFS) according to the expression levels of membranous EpCAM in clear cell RCC. The results showed there was no significant differences between DSS and the patients with high, moderate and low membranous expression of EpCAM (P = 0.290) (A) and there was a significant differences between PFS and the patients with high, moderate and low membranous EpCAM expression (P = 0.032) (B). (Membranous expressions were grouped into low versus moderate versus high expression levels).

and prognosis, thus they require different treatment.

The evaluation of staining in each subtype of RCC demonstrated the membranous, cytoplasmic, and nuclear patterns of B7-H3 and EpCAM expression in clear cell, papillary, and chromophobe RCC were heterogeneous, with a range of intensities from weak to strong. Our results indicated a statistical significant association between B7-H3 and EpCAM expression and the RCC subtypes. In addition, a significant difference was found between median levels of cytoplasmic B7-H3 expression between the ccRCC and pRCC (P < 0.001) and also pRCC and chRCC (P = 0.001). Moreover, a statistically significant difference was seen between median levels of membranous EpCAM expression between ccRCC and pRCC cases (P < 0.001) and ccRCC and chRCC (P < 0.001). These findings demonstrate that cytoplasmic expression of B7-H3, and membranous EpCAM expression differs among the subtypes of RCC, and this can affect the prognostic measures and successful treatment of patients.

We found that cytoplasmic B7-H3 expression was positively associated with the advanced nucleolar grade, LNI, histological tumor necrosis, and invasion of the Gerota's fascia in ccRCC which showing that cytoplasmic B7-H3 expression is related to the degree of malignancy and more advanced disease in these cases, while we could not find any association between the membranous and nuclear expression of B7-H3

Table 4
Univariate and multivariate cox regression analyses of potential prognostic factors for progression-free survival in patients with clear cell RCC.

Progression-free survival (PFS)				
Covariate	Univariate analysis		Multivariate analysis (Membranous)	
	HR (95% CI)	P-value	HR (95% CI)	P-value
Membranous EpCAM expression[a]		**0.04**		**0.04**
Moderate versus low	0.60(0.35–1.02)	0.06	0.50(0.28–0.86)	0.01
High versus low	0.47(0.22–0.99)	**0.04**	0.72(0.33–1.57)	0.41
Nucleolar grade		**0.02**		**0.01**
II versus I	3.22(1.47–7.08)	**0.003**	3.57(1.54–8.22)	**0.003**
III versus I	3.54(1.52–8.24)	**0.003**	3.58(1.46–8.74)	**0.005**
IV versus I	2.34(0.78–7.05)	0.12	2.17(0.69–6.79)	0.18
Primary tumor (PT) stage		0.16		
pT2 versus pT1	0.97(0.51–1.85)	0.94	–	–
pT3 versus pT1	1.51(1.02–2.25)	0.03		
pT4 versus pT1	1.60(0.56–4.52)	0.37		
Tumor size (cm)	1.02(0.86–1.21)	0.78	–	–

Values in bold are statistically significant.
The variables with P value < 0.05 were included in multivariable analyses.
HR hazard ratio, CI confidence interval.
[a] Low expression level is considered as reference group.

with the clinicopathological parameters. Importantly, we found that the median expression of B7-H3 was seen more in the high grade of RCC (nucleolar grade III and IV) compared with the low grade of RCC (nucleolar grade I and II), which depicts the association of cytoplasmic B7-H3 expression with tumor aggressiveness in ccRCC. In EpCAM marker, a significant association was observed between membranous expression of EpCAM and nucleolar grade as well as histological tumor necrosis, while in the case of cytoplasmic and nuclear expression of EpCAM, we did not find any statistically significant association with clinicopathological characteristics in ccRCC samples. Thus, membranous expression of EpCAM showed more aggressive behavior in these cases. Nucleolar grade is one the most powerful prognostic indicators for this malignancy after tumor stage, and tumors with a high nucleolar grade show a more aggressive phenotype and also are associated with local invasion and distant metastasis [20]. A previous study indicated that LNI is the most informative predictor of progression and survival, even in the metastatic setting [21]. Other histological findings, such as tumor necrosis is also helpful for prognostication [20,23]. In this study, we detected an association between histological tumor necrosis and tumor size as well as grade in ccRCC. These evidences demonstrated the prognostic significance of histological tumor necrosis for ccRCC aggressiveness. In addition, we found that, nucleolar grade and tumor size are independent prognostic factors for DSS in cytoplasmic B7-H3 expression but not in membranous expression. Previous studies have shown that tumor size is significantly associated with risk of metastasis in RCC [14,32]. Moreover, cytoplasmic B7-H3 expression added prognostic value in patients with ccRCC who had pT4 stage versus pT1 stage. Therefore, these results exhibited that cytoplasmic B7-H3 expression compared with membranous expression, is related to the degree of malignancy and progression in ccRCC cases.

Our findings showed that tumors with cytoplasmic expression of B7-H3 tended to have a worse prognosis than those with membranous expression. In addition, ccRCC patients, who expressed high level of cytoplasmic B7-H3, had shorter 5-year DSS compared with those with membranous expression. However, the pattern of B7-H3 expression was not a predictor of survival in multivariate analysis. In this study, the number of cancer-related death or event was low, 26 patients (17.7%), hence, the long term follow-up is needed because by extending the follow-up time, the prognostic value of B7-H3 expression may also be increased. In a previous study, expression of B7-H3 was detected in ccRCC tumor cells which was associated with multiple clinicopathological features and survival outcomes as indicated in our own study [10]. However, expression of this marker has not been reported before as membranous, cytoplasmic, and nuclear of the RCC tumor cells separately and this is the first report in this issue. These findings suggest that B7-H3 shift from membranous to cytoplasmic localization is associated with the transition of epithelial cells to a more invasive phenotype in ccRCC. It might be due to changes of biological function of B7-H3 protein based on its site of expression. Further investigations will certainly be required to better understand the precise events that control cytoplasmic B7-H3 expression and its function.

Our results using Kaplan-Meier survival curve showed that higher membranous EpCAM expression was a favorable predictor for PFS in ccRCC. In addition, we found that membranous EpCAM expression increases prognostic value in patients with ccRCC who had nucleolar grades III versus I.

In our study number of events in moderate and high cytoplasmic expression of EpCAM was only one case, therefore, we were not able to draw the Kaplan-Meier survival curve and apply Cox proportional regression analysis. More follow-up time may lead to increase the events and increase the prognosis of cytoplasmic EpCAM expression. This is the first study analyzing the expression and subcellular distribution of EpCAM antigen in RCC tissues, because any protein having different subcellular localization may have a specific function, although the exact biological role of EpCAM is still not clear. In a previous study, Zimpfer et al. [41] found that there is no significant association between overexpression of EpCAM (as membranous and cytoplasmic) and OS in ccRCC, pRCC, and chRCC, which correlate with our findings with DSS. Further, it was significantly correlated with a better PFS in high grade RCC subtypes, while in our study, we found membranous EpCAM expression as an independent prognostic factor for favorable PFS only in ccRCC samples. Our results are in contrast to previous studies which showed that a significantly longer OS and DSS in EpCAM-positive ccRCC [12,28,30]. The discrepancies in the results may be due to the differences in the antibody used, various sensitivity and specificity of the antibodies. The criteria to determine the positive or negative expression of EpCAM varied across the included studies.

In this study, for the first time, we showed membranous, cytoplasmic, and nuclear B7-H3 and EpCAM expression in the other major RCC subtypes. We found no significant association between membranous, cytoplasmic, and nuclear B7-H3 and EpCAM expression and important clinicopathological parameters and patients' outcomes in type I and II pRCC cases. In chRCC, we found a significant association between cytoplasmic B7-H3 expression and distant metastasis and membranous expression of EpCAM and tumor stage. In addition, we did not observe a significant association between B7-H3 and EpCAM expression and survival data in chRCC. Further studies using larger number of cases of each type I and II pRCC subtype and chRCC need to be performed to support these findings.

5. Conclusions

In summary, our results showed that there is a statistically significant difference between cytoplasmic expression of B7-H3 as well as membranous EpCAM expression in various subtypes of RCC. Further, our findings for the first time revealed that increased cytoplasmic expression of B7-H3 rather than its membranous expression is a clinical significance in ccRCC and is associated with increased tumor invasiveness, more advanced disease, and risk of poor prognosis for DSS in univariate analysis. In addition, cytoplasmic B7-H3 expression added prognostic value in ccRCC patients who had pT4 stage versus pT1 stage. Therefore, evaluation of the expression pattern of B7-H3 in the cytoplasm is useful for predicting progression. Our data indicated that membranous expression of EpCAM is associated with more aggressive behavior in ccRCC. Additionally, we observed that membranous EpCAM expression adds prognostic value in patients with ccRCC who

had high nucleolar grade versus low nucleolar grade. Moreover, membranous EpCAM expression was as an independent variable favorably affecting PFS in ccRCC samples.

Abbreviations

RCC	Renal cell carcinoma
ccRCC	clear cell RCC
pRCC	papillary RCC
chRCC	chromophobe RCC
B7-H3	B7 homolog 3
EpCAM	Epithelial cell adhesion/activating molecule
H & E	hematoxylin and eosin
TMA	tissue microarray
IHC	immunohistochemistry
H-score	histochemical score
DSS	Disease-specific survival
PFS	Progression-free survival
CI	confidence intervals
LNI	lymph node invasion
MVI	microvascular invasion

Ethics approval and consent to participate

The study was approved by the Iran University of Medical Sciences Human Research Ethics Committee in Iran (Ref No: IR.IUMS.REC1395.25239). All procedures performed in this study were in accordance with the 1964 Helsinki Declaration and its later amendments. Informed consent was obtained at the time of sample collection in routine consent forms.

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Declaration of competing interest

The authors declare that they have no competing interests.

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Authors' contributions

YA and ØF designed this study; LS, PhD student, gathered the paraffin embedded tissues, collecte the patient data, analyzed them, and wrote the manuscript; ZM, MAa, and MAb examined hematoxylin and eosin slides and marked the most representative areas in different parts of the tumor for preparing the TMAs blocks; UA scored TMAs slides after immunohistochemical staining. AR contributed to gather the paraffin embedded tissues and patients information. All authors read and approved the final manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.anndiagpath.2020.151483.

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