Original Study

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Combined Angiogenesis and Proliferation Markers' Expressions as Long-Term Prognostic Factors in Renal Cell Cancer

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Abstract

The prognostic role of MIB-1, BCL-2, VEGFR3, and CD31 expression was retrospectively evaluated in 224 renal cell cancer (RCC) patients. The combination of high MIB-1/low BCL-2 was with poor survival compared with low MIB-1/high BCL-2, and the combination of low VEGFR3/low CD31 was also associated with poor survival compared with high VEGFR3/high CD31. These molecular expressions might be valuable in planning the follow-up for RCC patients.

Objectives: The aim of this study was to evaluate the expression of MIB-1, BCL-2, VEGFR3, and CD31 and their associations with long-term survival in patients with renal cell cancer (RCC). **Patients and methods:** This study consisted of 224 RCC patients who underwent radical nephrectomy from 1985 to 1995. Follow-up continued for up to over 20 years. MIB-1 and BCL-2 expression were analyzed alone, and additionally, the expression of MIB-1, BCL-2, VEGFR3, and CD31 were combined in pairs using the following groups: low/low, low/high, high/low, and high/high. **Results:** Low BCL-2 expression (hazard ratio [HR], 2.16; 95% confidence interval [CI], 1.42-3.31; $P < .001$ compared with high BCL-2 in univariate analysis) and high MIB-1 expression (HR, 2.05; 95% CI, 1.32-3.19; $P = .001$ in multivariate analysis) were found to associate for poorer survival in RCC. In multivariate analysis, the combination of high MIB-1/low BCL-2 was associated with poor survival compared with low MIB-1/high BCL-2 (HR, 3.20; 95% CI, 1.66-6.17; $P = .001$), and the combination of low VEGFR3/high CD31 was associated with poor survival (HR, 2.48; 95% CI, 1.29-4.78; $P = .007$) compared with high VEGFR3/high CD31. **Conclusions:** Compared with high BCL-2 expression in combination with low or high MIB-1, VEGFR3, or CD31 expression, low BCL-2 expression in combination with low or high MIB-1, VEGFR3, or CD31 expression has poorer survival in the long-term follow-up of patients with RCC. Analysis of MIB-1, BCL-2, VEGFR3, and CD31 expression might be a useful additional marker to tailor the follow-up of RCC patients.

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Introduction

The most powerful prognostic factors for patients with localized renal cell cancer (RCC) remain tumor stage, tumor grade, and clinical variables such as performance status and presence or absence of symptoms, and serum markers are used to determine the prognosis of patients with metastatic RCC (mRCC).[1-3] Previously, several possible immunochemical markers have been studied to predict the survival of patients with RCC, but none has attained status as an independent prognostic marker.[4-10]

Vascular endothelial growth factor receptor 3 (VEGFR3) is important for lymphangiogenesis in normal situations, and it is also

activated in cancer and inflammation.[11] It maintains both angiogenesis and the lymphatic system, and therefore, it is considered an interesting therapeutic target.[12] Marked decreased VEGFR3 plasma levels have been shown in patients with RCC after treatment with multi-targeted tyrosine kinase inhibitor compared with stable or progressive mRCC.[13] Studies of VEGFR3 expression and its association with tumor stage, grade, and survival in patients with RCC have shown conflicting results.[7,9,14,15]

CD31 is a member of an immunoglobulin superfamily of cell adhesion molecules that are expressed on the surfaces of several blood and endothelial cells.[16,17] Higher expression of CD31 has been associated with a lower tumor grade and better survival in patients with RCC,[5] and our previous study also showed an association between CD31 expression and survival but not tumor grade or stage.[9] Microvessel density has shown to correlate negatively to prognosis in at least RCC studies.[18-20] In addition, CD31 expression is found to associate with undifferentiated microvessels.[21]

MIB-1 is a well-known cell proliferation marker, while BCL-2 is a marker of cell death.[22,23] The expression of these markers has been studied in patients with RCC. Higher MIB-1 expression was found to be independently associated with poorer survival and with a recurrence in these patients.[4,24] Low BCL-2 expression was associated with a higher tumor stage and a poorer prognosis in patients with RCC.[6] On the contrary, 2 studies did not find association with BCL-2 expression and a prognosis in RCC.[8,25]

Although the all markers mentioned above have been studied, none has been identified as an independent prognostic factor. Therefore, the aim of this study was to explore VEGFR3, CD31, MIB-1, and BCL-2 expression separately and in combination and to determine their associations with long-term survival in patients with RCC.

Patients and Methods

Patient Population

This study included patients with RCC who underwent nephrectomy from 1985 to 1995. The surgeries were performed at Tampere University Hospital or at Tampere Hospital, Tampere, Finland. All of the tumor samples were reclassified and re-evaluated using the Heidelberg classification and Fuhrman grading system[26,27] by an experienced uropathologist (P.K.). A total of 202 (90.2%) clear cell renal cell carcinoma (ccRCC), 12 (5.4%) papillary RCC (pRCC), 5 (2.2%) chromophobe RCC, 2 sarcomatoid RCC (0.9%), and 1 (0.4%) unclassified RCC were included in this study. The tumor samples were classified as Fuhrman grade 1 to 2 (22; 9.8%), grade 3 (114; 50.9%), and grade 4 (88; 39.3%). Clinical stage was assessed according to the TNM 2002 Classification of Malignant Tumors,[28] and 79 (35.3%), 43 (19.2%), 61 (27.2%), and 39 (17.4%) of patients with RCC were TNM stages 1, 2, 3, and 4, respectively. The patients' basic characteristics are described in a table in our previous study.[9]

Due to poor immunostaining, 13 (5.8%) patients in the MIB-1 group, 3 (1.3%) in the BCL-2 group, 15 (6.7%) in the MIB-1/BCL-2 group, 21 (9.4%) in the MIB-1/VEGFR3 group, 21 (9.4%) in the MIB-1/CD31 group, 15 (6.7%) in the BCL-2/CD31 group, and 14 (6.3%) in the VEGFR3/CD31 group were excluded from the analyses.

After surgery, the patients' follow-up and treatment were performed according to standard clinical practice at that time. The median follow-up time was 5.4 years, with a range of 0 to 21.7 years. Tampere University Hospital and the National Board of Medicolegal Affairs approved the research protocol and use of the tumor samples.

Immunostainings

Sections were deparaffinized, and antigen retrieval was achieved by heating the sections in a microwave oven for 2×7 minutes in 10 mM tris/10 mM ethylenediaminetetraacetic acid (pH 9.0). For acid Ki-67 antigen immunostaining, the monoclonal antibody MIB-1 (IgG1, Immuno-tech S. A., Mareille, France) was used at 1:110 dilution. Counterstaining was accomplished using 0.4% ethyl green in acetate buffer. The staining of MIB-1 was evaluated by visual estimation and by using a computer-assisted image analysis system (CAS-200 Software; Becton Dickinson, Parsippany, NJ, USA). The MIB-1 index was defined as the percent of cells with immunopositivity in the nuclei. We first evaluated patients between 1990 and 1995 by visual estimation; only definitely brown nuclei were recorded as positive, and the same samples were evaluated using a CAS-200. Spearman's correlation between the visual estimation and CAS-200 software was excellent (0.826; $P < .001$). Samples from patients seen between 1985 and 1990 were analyzed by CAS-200, and the results of computer-assisted image analysis were used for statistical analysis.

Monoclonal mouse antihuman BCL-2 oncoprotein clone 124 (Dako, Glostrup, Denmark) was used at 1:60 dilution. Sections were slightly counterstained by hematoxylin, and staining for BCL-2 was analyzed semiquantitatively. Stainings were quantitated by intensity (0-3) and the percent area of expression (0%-100%) and by multiplying these figures to obtain staining scores (0-300).

Immunohistochemistry for CD31 (1:200, Novocastra Laboratories Ltd, Newcastle upon Tyne, UK) was performed on formalin-fixed, paraffin-embedded tissue sections as a part of a tissue microarray. Briefly, the sections were deparaffinized with xylen and rehydrated in graded alcohol, treated in an autoclave in 10 mmol/l sodium citrate (pH 5.0) for 2 minutes, and washed with phosphate buffered saline. Primary antibody was incubated at 4°C overnight, and antibody binding was detected by Vectastain ABC kit reagents (Vector Laboratories, Burlingame, CA, USA). Diaminobenzidine was used as the chromogen. The slides were counterstained with hematoxylin and eosin, and mounted.

VEGFR-3 was stained with the 9D9 antibody (a mouse mAb against the extracellular domain of human VEGFR-3; a kind gift from Professor Kari Alitalo, Helsinki, Finland) at the concentration of 10 μ/L as described in detail previously.[29]

Microvessel density was quantified as the number of CD31-positive or VEGFR-3-positive microvessels per high-power field at ×250 (field of view 0.407 mm^2, including the whole tissue microarray core) using a Leitz Laborlux 12 bright-field microscope (Leitz Wetzlar GmbH, Germany). Two fields with the highest density of vessels were counted, and an average of 2 scores was reported. Scoring was performed in a blinded manner. Specimens were analyzed independently by two investigators.

Statistical Methods

Statistical analyses were performed using IBM SPSS Statistic for Windows version 21.0 (IBM Corp, Amonk, NY, USA; released

2010). The differences between categorical variables were tested using the Pearson χ^2 test. The Cox proportional hazards models were used in age- and gender-adjusted univariate and multivariate survival analyses. In the multivariate model, all dependent variables were entered simultaneously into the model. Furthermore, survival was analyzed using the Kaplan-Meier survival estimation method. All *P*-values under 0.05 were considered statistically significant.

Results

Clinical Characteristics

Our cohort included 132 men (58.9%) and 92 woman (41.1%). The patients' median age at the time of nephrectomy was 65 years (interquartile range, 55.9-71.9 years), and the median survival time was 5.6 years (interquartile range, 1.6-11.9 years).

MIB-1 Expression

Only one sample had negative staining; the remaining samples had positive expression of MIB-1. The median MIB-1 expression value was 1.36, and the highest expression value was 40.9. The MIB-1 expression values were divided into low (< 1.36) and high (≥ 1.36), according to the median staining result. Approximately half of the ccRCC cases (96 samples; 50.3%) showed low expression. Three (30%) papillary RCCs (pRCC) had low and 7 (70%) had high expression. Four (80%) chromophobe RCCs had low and 1 (20%) had high expression. The single collecting duct tumor and the single unclassified RCC showed high expression. One sarcomatoid type of RCC showed low expression, and the other exhibited high expression. There was no association between histologic type of RCC and MIB-1 expression (low or high; *P* = .38). Low expression of MIB-1 was observed in 10 (45.5%) grade 1 to 2 tumors, and high expression was seen in 12 (54.5%); 55 (51.4%) and 52 (48.6%) grade 3 tumors and 39 (47.6%) and 43 (52.4%) grade 4 tumors showed low and high expression of MIB-1, respectively. There was no association between low/high MIB-1 expression and tumor grade (*P* = .81). For tumor stages 1, 2, 3, and 4, the distribution of low/high MIB-1 expression was 43 (57.3%)/32 (42.7%), 23 (54.8%)/19 (45.2%), 23 (40.4%)/34 (59.6%), and 15 (41.7%)/21 (58.3%), respectively (*P* = .19).

BCL-2 Expression

Seventy-five (33.9%) tumor samples had negative staining for BCL-2. The median expression of BCL-2 was 30, and the highest value was 300. BCL-2 staining was divided into low (0-30) and high (31-300) expression groups, based on the median value. Immunostaining of BCL-2 in ccRCC showed no differences; 102 (51.3%) samples had low and 97 (48.7%) had high expression. Ten (83.3%) pRCC had high and 2 (16.7%) had low BCL-2 expression. Three (60%) chromophobe RCCs had low and 2 (40%) had high expression. Both sarcomatoid RCCs had low immunostaining, as did the single unclassified RCC, and the single collecting duct RCC had high immunostaining. Fisher exact test showed *P* = .037 on cross-tabulation between low/high BCL-2 expression and different histological types of RCC. For tumor grades 1 to 2, 3, and 4, the BCL-2 expression was low in 6 (28.6%), 49 (43.8%), and 56 (63.6%) cases and high in 15 (71.4%), 63 (56.3%), and 32 (36.4%) cases, respectively (*P* = .002). Low expression was observed in 30 (39.0%), 19 (43.2%), 37 (60.7%), and 25 (65.8%)

tumor samples in tumor stages 1, 2, 3, and 4, respectively, and high expression was observed in 47 (61.0%), 25 (58.8%), 24 (39.3%), and 13 (34.2%) samples, respectively (*P* = .011).

Expressions of VEGFR3 and CD31

The expression of VEGFR3 and CD31 and their distributions with tumor grade and stage were presented in our previous study.[9] Briefly, neither VEGRF3 expressions nor CD31 expressions showed association with tumor stage or grade.

Combinations of MIB-1, BCL-2, VEGFR3, and CD31 Expressions

The expressions of MIB-1, BCL-2, VEGFR3, and CD31 were divided into two groups (low and high). MIB-1, BCL-2, and CD31 were categorized according to their median expression levels of 1.36, 30, and 18, respectively, as follows: MIB-1 low (< 1.35) and high (≥ 1.35); BCL-2 low (0-30) and high (30-300); and CD31 low (≤ 18) and high (> 18). VEGFR3 expression was divided into low (no positive vessels) and high (positive vessels). The cross-tabulation between MIB-1/BCL-2 expression and VEGFR3/CD31 expression is described in Table 1.

Univariate Analysis

Age- and gender-adjusted univariate analysis showed associations between tumor stage, grade, and expressions of MIB-1 and BCL-2 with survival; for stage 4 compared with stage 1 disease: hazard ratio (HR), 13.8; 95% confidence interval (CI), 7.18-26.7; *P* < .001; for stage 3 compared with stage 1 disease: HR, 4.37; 95% CI, 2.29-8.35; *P* < .01; for stage 2 compared with stage 1 disease: HR, 2.62; 95% CI, 1.27-5.41; *P* = .007; for grade 4 compared with grades 1 to 2: HR, 9.31; 95% CI, 2.23-38.8; *P* = .002; for grade 3 compared with grades 1 to 2: HR, 4.91; 95% CI, 1.18-20.4; *P* < .001; for high MIB-1 compared with low MIB-1: HR, 1.76; 95% CI, 1.16-2.68; *P* = .008; for low BCL-2 compared with high BCL-2: HR, 2.16; 95% CI, 1.42-3.31; *P* < .001. Cox regression univariate analysis was also performed for pairs of markers (MIB-1, BCL-2, VEGFR3, and CD31) and all possible variations of their expressions (low/low, low/high, high/low and high/high); the results are summarized in Table 2. Every combination of MIB-1, VEGFR3, or CD31 expression with high BCL-2 expression showed statistically better survival compared with combinations with low BCL-2 expression. All combinations of MIB-1/BCL-2 were significantly associated with survival; for high MIB-1/high

| Table 1 | Cross-Tabulation of MIB-1/BCL-2 Expressions and VEGFR3/CD31 Expressions |

MIB-1/ BCL-2	VEGFR3/CD31				Total
	High/High n (%)	low/low n (%)	High/low n (%)	low/High n (%)	n (%)
Low/high	26 (13)	10 (5)	12 (6)	9 (4.5)	57 (28.5)
High/high	18 (9.0)	6 (3.0)	15 (7.5)	4 (2.0)	43 (21.5)
Low/low	12 (6.0)	13 (6.5)	13 (6.5)	7 (3.5)	45 (22.5)
High/low	10 (5.0)	6 (3.0)	19 (9.5)	20 (10.0)	55 (27.5)
Total	66 (33.0)	35 (17.5)	59 (29.5)	40 (20)	200 (100)

The results are indicated in both numbers and percent of cases, *P* = .002 (Pearson χ^2 test)

Table 2 Age- and Gender-Adjusted Univariate Associations of MIB-1, BCL-2, VEGFR3, and CD31 Expressions

	N	Age- and Gender-Adjusted Univariate	
		HR [95% CI]	P
MIB-1/BCL-2			
Low/high	58	1.00	
High/high	47	2.13 [1.10-4.12]	.025
Low/low	45	3.02 [1.62-5.62]	.001
High/low	59	3.51 [1.94-6.36]	<.001
MIB-1/VEGFR3			
Low/high	55	1.00	
Low/low	48	1.65 [0.91-3.01]	.10
High/low	46	1.87 [1.02-3.43]	.042
High/high	54	2.36 [1.32-4.22]	.004
MIB-1/CD31			
Low/high	62	1.00	
Low/low	41	1.45 [0.80-2.63]	.22
High/low	59	1.83 [1.07-3.11]	.026
High/high	41	2.14 [1.20-3.82]	.01
BCL-2/VEGFR3			
High/high	60	1.00	
High/low	45	1.10 [0.57-2.13]	.77
Low/low	52	2.37 [1.35-4.15]	.003
Low/high	52	2.71 [1.55-4.76]	<.001
BCL-2/CD31			
High/high	62	1.00	
High/low	44	0.91 [0.46-1.76]	.78
Low/high	41	2.15 [1.21-3.83]	.009
Low/low	62	2.38 [1.41-4.02]	.001
VEGFR3/CD31			
High/high	70	1.00	
High/low	62	1.10 [0.65-1.88]	.75
Low/high	35	2.02 [1.14-3.60]	.017
Low/low	43	2.22 [1.29-3.82]	.004

Cox regression models were used, showing results by hazard ratios (HR) with 95% confidence intervals (CI).

BCL-2: HR, 2.13; 95% CI, 1.10-4.12; P = .025; for low MIB-1/low BCL-2: HR, 3.02; 95% CI, 1.62-5.62; P = .001; for high MIB-1/low BCL-2: HR, 3.51; 95% CI, 1.94-6.36; P < .001, compared with low MIB-1/high BCL-2. The remaining combinations showed no significant association with survival. Univariate analysis of VEGFR3 and CD31 expression with tumor stage and grade showed that low CD31 expression had poorer prognosis in RCC patients but VEGFR3 expression had no association with survival in RCC. The results are described our previous study.[9]

Multivariate Analysis

Multivariate Cox hazard regression analysis was performed including tumor grade, tumor stage, and expressions of MIB-1 and BCL-2 simultaneously into the model with age and gender, described in Table 3. Statistically significant combinations

MIB-1/BCL-2 and VEGFR3/CD31 in age- and gender-adjusted univariate analyses were tested with tumor stage and grade in multivariate analysis. Higher tumor stage showed poorer survival, and the combination of MIB-1/BCL-2 almost reached statistically significant association in survival of all combinations; the results are summarized in Table 4.

Kaplan-Meier Analysis

Kaplan-Meier (KM) survival analysis was used to illustrate MIB-1 and BCL-2 expression and the expression of the combinations described above. Increased BCL-2 expression and low MIB-1 expression showed better survival in KM curves compared with low BCL-2 and high MIB-1 expressions (Figures 1A and 1B). Lower BCL-2 expression in combination with low or high MIB-1, VEGFR3, or CD31 expression showed poorer survival compared with any combinations with high BCL-2 expression. The plateau phase, a time after diagnosis when patients have no RCC-related mortality, was estimated by Kaplan-Meier survival curve analysis and was found to be 1.2 years for low grade (1-2) tumors. For higher tumor grades (3 and 4), the plateau occurred almost 10 years later, at 12.4 and 9.9 years, respectively. For stages 1, 2, 3, and 4, the plateau phases were reached within 4, 8.5, 12.4, and 9.9 years, respectively. No RCC-related deaths were observed after 9.9 and 12.4 years in tumors with low and high MIB-1 expression and after 9.9 and 12.4 years in tumors with high and low BCL-2 expression, respectively. Over half of patients having expression combinations of low MIB-1/high BCL-2, low MIB-1/high VEGFR3, high BCL-2 and low/high VEGFR3, or high BCL-2 and low/high CD31 were alive 10 years after nephrectomy. KM curves of the combinations of MIB-1/BCL-2, VEGFR/CD31, and BCL-2/VEGFR3 expression are shown in Figures 2A-C.

Table 3 Age- and Gender-Adjusted Multivariate Associations of Tumor Grade, Tumor Stage, and Expressions of MIB-1 and BCL-2

	N	Age- and Gender-Adjusted Multivariate	
		HR [95% CI]	P
Grade			
1-2	22	1.00	
3	114	2.51 [0.59-10.69]	.21
4	88	6.45 [1.50-27.8]	.012
Stage			
1	79	1.00	
2	44	2.96 [1.40-6.24]	.004
3	61	3.58 [1.81-7.08]	<.001
4	39	14.51 [7.13-29.55]	<.001
MIB-1			
Low	104	1.00	
High	107	2.05 [1.32-3.19]	.001
BCL-2			
High	110	1.00	
Low	111	1.40 [0.91-2.21]	.12

Cox regression models were used, showing results by hazard ratios (HR) with 95% confidence intervals (CI).

| Table 4 | Age- and Gender-Adjusted Multivariate Associations of MIB-1/BCL-2 and VEGFR3/CD31 Expressions With Stage and Grade |

		Age- and Gender-Adjusted Multivariate	
	n	**HR [95% CI]**	**P**
MIB-1/BCL-2			
Low/high	57	1.00	
Low/low	45	1.60 [0.83-3.08]	.16
High/high	42	2.14 [1.07-4.29]	.032
High/low	55	3.20 [1.66-6.17]	.001
VEGFR3/CD31			
High/high	66	1.00	
Low/low	58	0.92 [0.52-1.63]	.79
High/low	40	1.28 [0.70-2.43]	.46
Low/high	35	2.48 [1.29-4.78]	.007
Stage			
1	67	1.00	
2	40	2.60 [1.28-5.25]	.008
3	57	2.58 [1.40-4.73]	.002
4	35	8.85 [4.47-17.5]	<.001
Grade			
1-2	20	1.00	
3	102	1.44 [0.48-4.24]	.51
4	78	3.25 [1.09-9.73]	.035

Cox regression models were used, showing results by hazard ratios (HR) with 95% confidence intervals (CI).
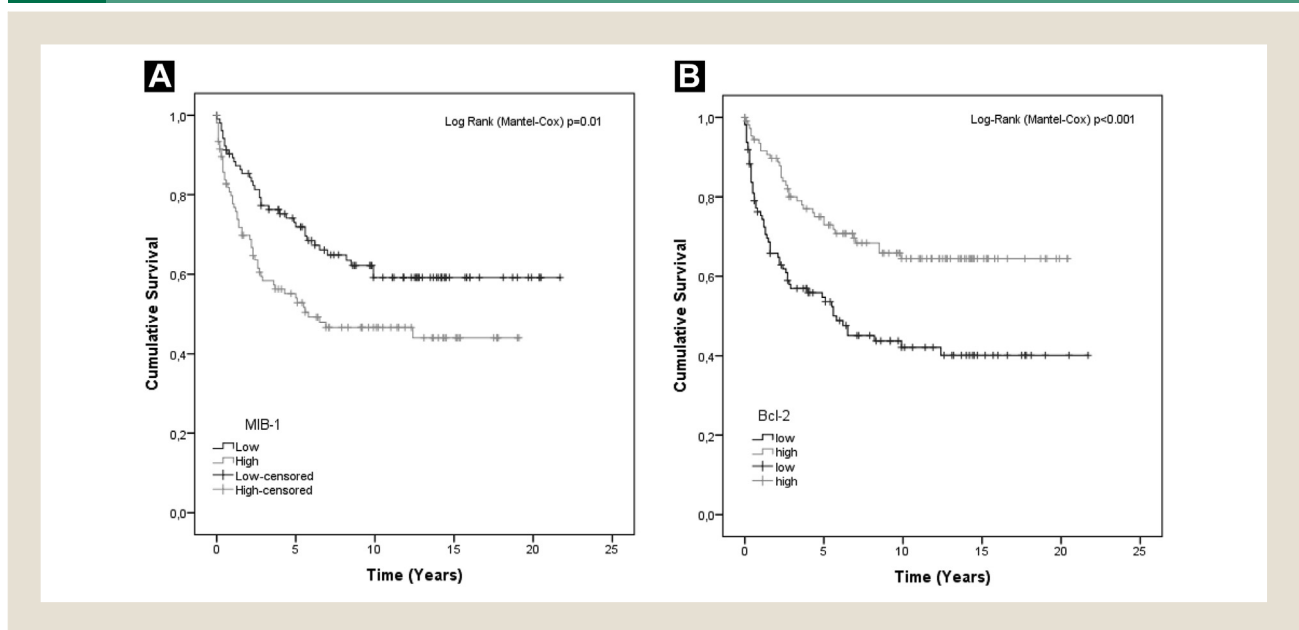
Discussion

In this study, we explored the expression of MIB-1, BCL-2, VEGFR3, and CD31 and their associations with survival in patients with RCC. Our study consisted of 224 patients, for whom all tumor samples were re-evaluated and reclassified by a qualified uropathologist (P.K.). All data were collected directly from the original medical records. The survival data were obtained from the Finnish Cancer Registry; thus were very reliable.

Clinicians today have a better understanding of molecular pathway abnormalities, histological subtypes, and new morphological variants of RCC.[30] The improved knowledge might aid in the discovery of new treatments for patients with RCC.[31,32] The standard follow-up practice for patients with RCC is not clear and has been debated in the literature, and the tendency is toward more individual treatment and management of RCC. We urgently need new molecular markers to plan these patients' individual treatments and follow-up.

In the present study, the expression of the proliferation markers MIB-1 and BCL-2 alone and with the angiogenesis markers VEGFR3 and CD31, and their associations with survival, was explored in patients with RCC. We classified the expression of MIB-1, BCL-2, VEGFR3, and CD31 into low and high expression groups according to their immunostaining scores. High MIB-1 expression was associated with poorer disease-free survival and overall survival in patients with RCC in a previous study.[33] Similarly, our study showed that low MIB-1 expression was independently associated with a better prognosis in these patients. Higher BCL-2 expression in combination with any low or high expression of MIB-1, VEGFR3, or CD31 showed better survival compared with combinations with low BCL-2 expression. Previous studies have also found that increased BCL-2 expression was associated with a better prognosis for patients with RCC.[6] Our univariate analysis showed that a combination of the proliferation marker MIB-1 and an anti-apoptosis marker BCL-2 had a statistically significant association with survival in patients with RCC. Anti-apoptotic proteins such as BCL-2 are often overexpressed in cancer, while apoptotic proteins are deregulated.[34] RCC is, in

Figure 1 (A) Kaplan-Meier Curves for MIB-1 Expression (Low/High). (B) Kaplan-Meier Curves for BCL-2 Expression (Low/High)
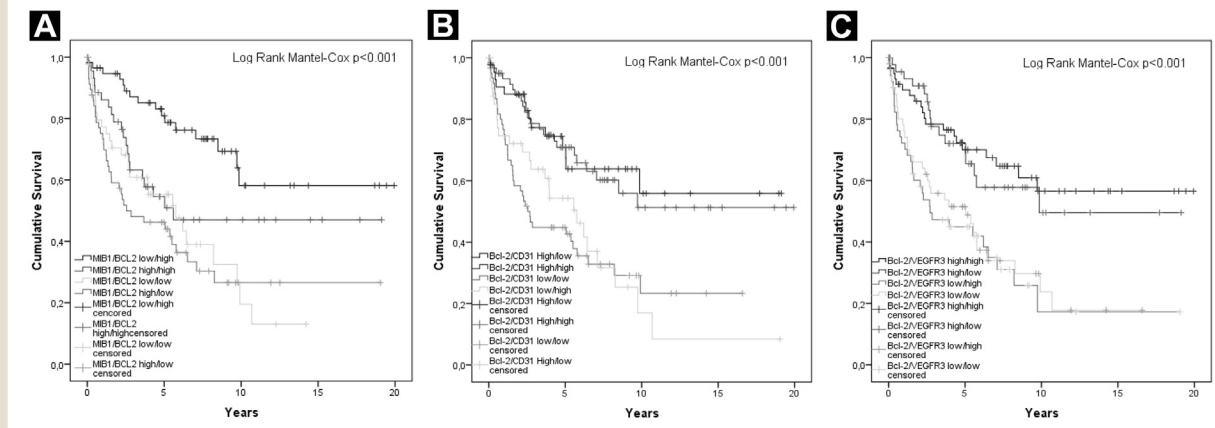
general, a highly vascular tumor, and angiogenesis is very important for tumor growth and spread. Histological tumor necrosis is shown to be associated with poorer survival in RCC.[35] Overexpression of BCL-2 was shown to prevent necrosis,[36] which might be one of the explanatory factors for the improved survival of patients with RCC and higher BCL-2 expression. MIB-1 expression was also significantly associated with survival in multivariate analysis, in which the classical prognostic factors of tumor stage and grade were also included. Our previous study showed that higher CD31 expression was associated with a better prognosis in patients with RCC compared with low CD31 expression.[9] This present study showed that higher CD31 expression had no strong association with survival, whereas high BCL-2 had a strong association with better survival with all combinations of MIB-1, VEGFR3, and CD31 expressions.

VEGFR3 is important for both angiogenesis and lymphatic maintenance,[12] whereas CD31 is involved in tissue regeneration, and its expression has been shown in vascular tumors.[16] This study showed that the combination of high VEGFR3/high CD31 was associated with a better prognosis compared with low VEGFR3/low CD31. This may indicate that patients with better lymphangiogenesis and active tissue regeneration have a better prognosis.

We also examined associations between combinations of MIB-1 and BCL-2 expression, which are related to cell growth and apoptosis, and combinations of VEGFR3 and CD31 expression, which are related to tumor angiogenesis. Cross-tabulation showed a significant association between them, as shown in Table 1. Distributions were in the same line as survival analysis showed; however, 4.5% of cases with low MIB-1/high BCL-2, which was associated with better survival, had low VEGFR3/high CD31, which was associated with poorer survival; in addition, 5% of cases with high MIB-1/low BCL-2, which was associated with a poorer prognosis, had high VEGFR3/high CD31, which was associated with a better prognosis. Based on these results, we cannot clearly indicate clear associations between the expressions of these combinations of

factors; perhaps there are other factors of which we are not yet aware that affect tumor growth, death, and tumor angiogenesis.

This present study included 224 consecutive patients whose RCC was diagnosed from 1985 to 1995. During that time, radiological examinations were performed less frequently than they are currently. Over 50% of RCCs are currently detected incidentally.[30] This might be the reason that our study population consisted of only 22 (9.8%) patients with grade 1 or 2 tumor and 79 (35.3%) patients with stage 1 tumor. Our study showed an association between higher Bcl-2 expression and pRCC, but no association between MIB-1 expression and histological type of RCC. This might be explained by the fact that the majority histological type in our study was ccRCC (90.2%) and this represents a higher proportion than is generally found in RCC.[26,37,38] Due to inadequate immunostaining, we excluded 3 to 21 patients (1.3%-9.4%) from the analyses. The excluded portion was, however, minor and therefore had an insignificant effect on the results.

Our study showed that over 50% of patients with low MIB-1 or high Bcl-2 expression were alive at the end of long-term follow-up. The survival plateau was reached already after 1.2 years in patients with grade 1 to 2 tumors, whereas for other grades and stages, the MIB-1 and Bcl-2 expression groups' plateaus were achieved between 8.5 and 12.4 years. Additionally, 50% of patients with low MIB/ high BCL-2, low MIB-1/high VEGFR3, high BCL-2 and low/high VEGFR3, or high BCL-2 and low/high CD31expression survived 10 years after the diagnosis of RCC. These data showed that all patients with RCC who were alive 10 years after nephrectomy had an excellent prognosis. The follow-up guidelines of RCC have been recently discussed in the literature.[32] Smith et al. showed that nearly one-third of RCC recurrences were missed when patients were followed according to the American Urological Association or National Comprehensive Cancer Network guidelines.[39] New molecular markers or their combinations might be needed to improve the assessment of recurrence risk and to tailor treatment and follow-up for patients with RCC. MIB-1, BCL-2, VEGFR3,

and CD31 might be useful for individual follow-up of patients with RCC and should be tested in prospective trials.

Conclusion

These data showed that low BCL-2 alone or in any combination with low or high MIB-1, VEGFR3, or CD31 expression was associated with poorer survival in patients with RCC. Low MIB-1 expression was an independent predictor of better prognosis in patients with RCC. Low VEGFR3/low CD31 expression was associated with poor survival compared with high VEGFR3/high CD31 expression. These markers might be useful for planning the follow-up of patients with RCC.

Clinical Practice Points

- There are no molecular markers to predict survival in patients with RCC. We evaluated the expressions of MIB-1, BCL-2, VEGFR3, and CD31 and their role in the prognosis of RCC.
- This study showed that low MIB-1 and high BCL-2 expressions were associated with improved survival in RCC.
- High VEGFR3/high CD31 expression showed better survival compared with low VEGFR3/high CD31 expression.
- Molecular markers might be useful for planning patients' follow-up.

Disclosure

The authors have stated that they have no conflicts of interest.

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