

The predictive value of semaphorins 3 expression in biopsies for biochemical recurrence of patients with low- and intermediate-risk prostate cancer

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The class-3 semaphorins (Sema3A-F, Sema3s) are initially identified to play an important role in axonal guidance and cell migration. Our previous studies showed that Sema3s are also involved in the lymph node metastasis of prostate cancer, and are likely to modulate the behavior of prostate cancer with a pro-tumoral or an anti-tumoral effect, depending on their subtypes. However, no study has critically investigated the value of Sema3s expression in preoperative biopsy samples for the prediction of biochemical recurrence (BCR) after radical prostatectomy. In this study, we evaluated Sema3s expression by immunohistochemistry on 198 prostate biopsies with low- and intermediate-risk localized prostate cancer. The median follow-up was 42 months (range, 6-60) for all patients. Our results showed that Sema3A (OR: 0.19, $P<0.001$), Sema3B (OR: 0.38, $P=0.003$), Sema3E (OR: 0.39, $P=0.007$), and Sema3C (OR: 2.31, $P=0.014$) staining were independent predictors of BCR on multivariable analysis. Sema3A, 3B, 3C and 3E expression demonstrated potential values in predicting BCR upon survival analysis ($P=0.001$, $P=0.003$, $P=0.029$, $P=0.037$, respectively, Log-rank test). Our findings suggested that Sema3A, 3B, 3C, and 3E immunostaining in prostate biopsies, as supplements to clinicopathological parameters, could be used for predicting BCR in low- and intermediate-risk prostate cancer patients after radical prostatectomy. Specially, concurrent Sema3C-positive and Sema3A-negative, 3B-negative, 3E-negative staining is associated with an adverse prognosis. Further prospective studies in larger patient populations are needed to validate the current observations.

Key words: biochemical recurrence, biopsy, class-3 semaphorins, immunohistochemistry, prostate cancer, radical prostatectomy

Prostate cancer is the most commonly diagnosed solid organ malignancy among males and is the second leading cause of cancer-related deaths, accounting for approximately 30,000 deaths per year in the United States [1]. Preoperative risk stratification is crucial to guide the therapeutic decision-making process in prostate cancer. Clinical and pathological characteristics such as prostate specific antigen (PSA) level, clinical tumor stage, and Gleason score are currently used for prognosis and risk assessment. However, prostate cancer is a heterogeneous disease and the biological behavior can be evaluated only to a limited extent using these parameters. Some prostate cancer cases classified as low- and intermediate-risk

diseases show more aggressive behavior and more chance of recurrence after radical treatment [2, 3]. Thus, identification of reliable biomarkers in prostate biopsy samples in parallel with other existing parameters for prediction of postoperative recurrence, especially for patients with low- or intermediate-risk disease, is urgently needed.

The class-3 semaphorin family members (Sema3A-F, Sema3s) is the only protein family comprised of secreted vertebrate semaphorins, which were initially identified to play an important role in axonal guidance and cell migration [4]. Interestingly, our previous studies show that Sema3s are also involved in the lymph node metastasis of prostate cancer, and are likely to modulate the behavior of cancers with a pro-tumoral or an anti-tumoral effect, depending on their subtypes [5-8]. Sema3A, 3B, 3D, and 3F act as anti-tumorigenic agents, since they increase apoptosis and inhibit cell migration

Abbreviations: BCR, biochemical recurrence; CI, confidence interval; IHC, immunohistochemistry; OR, odds ratio; PSA, prostate-specific antigen; Sema3s, class-3 semaphorins.

by activating their specific receptors in multiple tumor cells, including prostate cancer [9-11], whereas Sema3C promotes angiogenesis and enhances invasive and metastatic characteristics [12]. In addition, Sema3E was shown to act both as a pro-angiogenic and an anti-angiogenic factor *in vitro*, either attracting or repelling endothelial cells expressing its receptor, PlexinD1 [5]. However, to our knowledge, no prior study has critically investigated the value of Sema3s expression in preoperative biopsy samples for the prediction of biochemical recurrence (BCR) after radical prostatectomy. In this study, we evaluated Sema3s expression by immunohistochemistry (IHC) on 198 prostate biopsies with low- and intermediate-risk localized prostate cancer to specifically address two questions: (1) the frequency of positive Sema3s staining in prostate biopsies; (2) whether positive or negative Sema3s IHC is associated with the BCR rate after radical prostatectomy.

Patients and methods

Patients. This study was approved by the Institutional Review Board of the Third Affiliated Hospital of Sun Yat-sen University. Written informed consent was obtained from all patients. Between January 2007 and December 2010, a total of 235 cases were defined as low- and intermediate-risk prostate cancer according to the D'Amico risk group, i.e. clinical stage T1c or T2a or T2b, PSA \leq 20 ng/mL, Gleason score on biopsy \leq 7. [13]. All these 235 patients underwent laparoscopic radical prostatectomy as previously reported by one single surgeon (X.G.) [14]. Lymphadenectomy was usually performed in patients with intermediate risk. Those with positive surgical margin or positive lymph node were excluded. In total, 198 patients remained for the present analysis. Table 1 summarizes the clinical and pathological characteristics of patients included in this study.

For all patients enrolled, postoperative follow-up was performed quarterly in the first year, semiannually for the second year, and annually thereafter by clinical evaluation, measurement of total serum PSA, and by other measures as indicated. BCR was defined as two consecutive PSA levels of >0.2 ng/ml after surgery [15]. Postoperative endocrine therapy was not administrated until BCR. The median follow-up was 42 months (range, 6-60) for all patients.

Hisopathologic analysis. All patients underwent at least 10-core transrectal ultrasound-guided biopsies, and at least 10 paraffin blocks per patient were prepared. The morphologic diagnosis was confirmed on H&E slides by 2 independent urological pathologists (D.He and C.-K. Shao) who were blinded to the results of Sema3s staining IHC. Gleason score and morphologic feature of each case was assessed independent of Sema3s staining evaluation. If necessary, immunostaining for P504S, 34 β E12 and p63, was carried out using an avidin-biotin complex staining procedure as previously reported [16].

Evaluation of Sema3s protein expression via IHC. The biopsy paraffin blocks were available for analysis and all corresponding H&E-stained and immunostaining slides

were reviewed. A representative slide from each patient with carcinoma was selected for evaluation of Sema3s protein expression status by IHC. The selection of the core for IHC analysis was made by the pathologist conducting the diagnosis and the core with the highest proportion of cancer cells was elected. In brief, following deparaffinization, 5- μ m sections were dehydrated and blocked in 1% hydrogen peroxide in methanol for 20 minutes. Sections were pretreated using the microwave antigen retrieval procedure in 10 mmol/L of boiling EDTA (pH 8.0). The tissue sections were then incubated with primary antibodies against Sema3A (sc-28867, Santa Cruz Biotechnology Inc., CA, USA), Sema3B (sc-10720, Santa Cruz), Sema3C (sc-74555, Santa Cruz), Sema3D (sc-68043, Santa Cruz), Sema3E (sc-49733, Santa Cruz) and Sema3F (sc-68796, Santa Cruz) diluted to 1:100 in phosphate-buffered saline. The biotin-streptavidin detection system (Abcam Inc., MA, USA) was used for detecting the signals from the stained tissue sections. Samples were developed with aliquid diaminobenzidine and substrate-chromogen system (Abcam Inc.) and counterstained with hematoxylin to visualize the staining of the Sema3s protein. Following a hematoxylin counterstaining, positive immunostaining was scored by two independent experienced observers (L.-Y. Li and J. Pang) in a blinded fashion. Tumor specimens were scored in a semiquantitative manner with the following scale: (0) less than 10% positive staining cells; (1+) 10-25%; (2+) 25-50%; (3+) more than 50%. Cases with scores of 2+ or 3+ were designated as "positive," whereas cases with scores of 0 or 1+ were designated as "negative" [17, 18]. In case of any discrepant scores, the samples were further reviewed by another urological pathologist (Z.-L. Su), who acted as an arbiter. All 3 reviewers were unaware of patients' clinicopathological parameters.

Statistical analysis. Continuous parametric variables were reported as the median values and range. Mann-Whitney *U*-test and chi-square tests were used to compare continuous and categorical variables as appropriate. A logistic regression model, including age, PSA, clinical T stage, biopsy Gleason score, and Sema3s immunostaining, was used to perform univariable and multivariable analyses in a stepwise manner. The predictive values of Sema3s expression status were tested using Kaplan-Meier analysis with the Log-rank test for assessing differences. SPSS 16.0 software package (SPSS Inc., Chicago, IL, USA) was used for all the analyses and a two-tailed test with $P < 0.05$ was considered statistically significant.

Results

Sema3s were diffusely expressed on cancer cells with membranous and/or cytoplasmic immunoreactivity in biopsy samples (Fig.1). Among these 198 cases, 34 with BCR were observed during a median follow-up of 42 months (range, 6-60). The overall BCR-free survival rate was 82.8%. The positive percentages of Sema3A-F expression in the BCR group and the BCR-free group were listed in Table 1. Semiquantitative analysis revealed that the BCR-free group had significantly

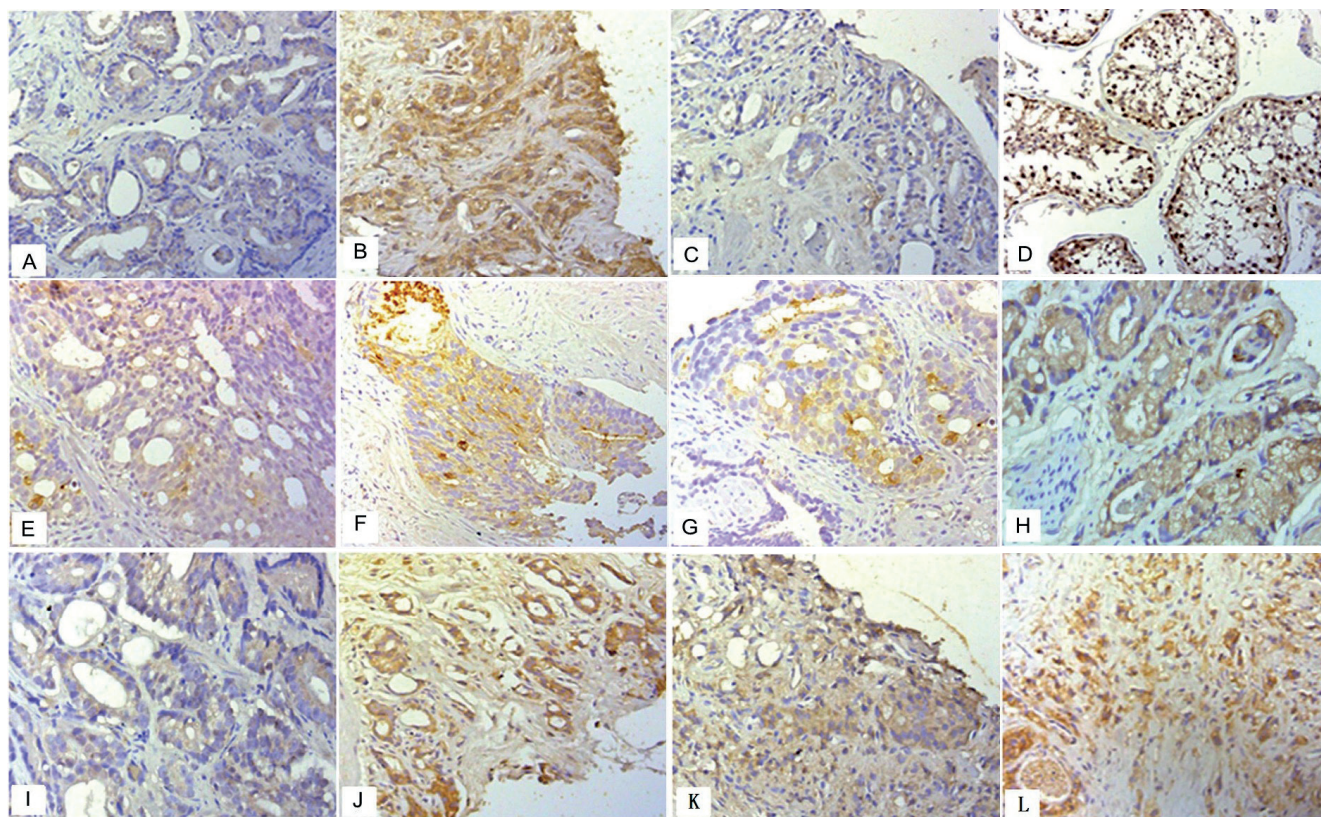


Figure 1. Representative images of the immunohistochemical staining of Semaphorin 3s proteins in biopsy samples: Semaphorin 3s are diffusely expressed on cancer cells with membranous and/or cytoplasmic immunoreactivity. A/B, negative/positive Semaphorin 3A expression; C/D, negative/positive Semaphorin 3B expression; E/F, negative/positive Semaphorin 3C expression; G/H, negative/positive Semaphorin 3D expression; I/J, negative/positive Semaphorin 3E expression; K/L, negative/positive Semaphorin 3F expression. Original magnification of H&E images, $\times 200$.

higher positive percentages of Semaphorin 3A, Semaphorin 3B, and Semaphorin 3E, but had significantly lower expression of Semaphorin 3C, relative to the BCR group (Table 1 & Fig. 1). The median relapse time was 17 months (range, 9-32). 11 cases with BCR occurred in 12 months, 14 cases had the relapse between 12 to 24 months, and 9 patients recurred 24 months later.

Table 2 summarizes the univariable and multivariable analyses for BCR. Parameters, including patient age at surgery, preoperative PSA levels, biopsy Gleason score, clinical T stage, and the expression status of Semaphorin 3s were included in these analyses using a logistic regression model. On univariable regression analysis, biopsy Gleason score (odds ratio [OR]: 2.49, $P=0.016$), clinical T stage (OR:2.31, $P=0.007$), Semaphorin 3A (OR:0.20, $P<0.001$), Semaphorin 3B (OR:0.48, $P=0.023$), Semaphorin 3E (OR:0.34, $P=0.02$), and Semaphorin 3C (OR:2.71, $P=0.003$) staining were significantly associated with BCR, while age and preoperative PSA level were not ($P=0.39$, $P=0.21$, respectively). On multivariable analysis, biopsy Gleason score (OR:1.96, $P=0.033$), Semaphorin 3A (OR:0.19, $P<0.001$), Semaphorin 3B (OR:0.38, $P=0.003$), Semaphorin 3E (OR:0.39, $P=0.007$), and Semaphorin 3C (OR:2.31, $P=0.014$) staining were independent predictors of BCR (Table 2).

Moreover, the actuarial probability of remaining free of BCR for this cohort of patients during the follow-up period after surgery was calculated using the Kaplan-Meier method. The BCR rates in Semaphorin 3A-negative, Semaphorin 3B-negative, and Semaphorin 3E-negative patients were significantly higher than those with Semaphorin 3A-positive, Semaphorin 3B-positive, and Semaphorin 3E-positive staining ($P=0.001$, $P=0.003$, $P=0.037$, respectively, Log-rank test) (Fig. 2A, B and E). Patients with Semaphorin 3C-positive tumors were significantly more likely to experience BCR than those with Semaphorin 3C-negative tumors ($P=0.029$, Log-rank test, Fig. 2C). However, Semaphorin 3D or Semaphorin 3F expression alone could not predict BCR with statistical significance ($P=0.71$, $P=0.36$, respectively, Log-rank test, Fig. 2D and F). In addition, we found that patients with concurrent Semaphorin 3C-positive and Semaphorin 3A-negative, Semaphorin 3B-negative, Semaphorin 3E-negative tumors had significantly more chance to experience BCR than those with other patterns of Semaphorin 3s staining ($P<0.001$, Log-rank test, Fig. 2G).

Discussion

BCR is currently one of the major challenges in prostate cancer patients who undergo radical prostatectomy. Although

Table 1. Characteristics of the 198 patients with low- and intermediate risk prostate cancer

Characteristic	BCR	BCR-free	P value
Number of cases	34	164	
Median age (yr, range)	64 (47-73)	63 (50-71)	0.131
Preoperative PSA (ng/ml, range)	9.8 (2.2-16.5)	10.2 (5.2-19.6)	0.215
Clinical T stage, n (%)			0.053
T1c	10(29.4)	67(40.9)	
T2a	7(20.6)	50(30.5)	
T2b	17(50.0)	47(28.6)	
Biopsy Gleason score, n (%)			0.028
6	14(41.2)	101(61.6)	
7	20(58.8)	63(38.4)	
Sema 3A expression, n (%)			<0.001
Negative	29(85.3)	75(45.7)	
Positive	5(14.7)	89(54.3)	
Sema 3B expression, n (%)			0.020
Negative	25(73.5)	73(44.5)	
Positive	9(26.5)	91(54.5)	
Sema 3C expression, n (%)			0.045
Negative	11(32.4)	84(51.2)	
Positive	23(67.6)	80(48.8)	
Sema 3D expression, n (%)			0.871
Negative	20(58.8)	94(57.3)	
Positive	14(41.2)	70(42.7)	
Sema 3E expression, n (%)			0.007
Negative	24(70.6)	74(45.1)	
Positive	10(29.4)	90(54.9)	
Sema 3F expression, n (%)			0.241
Negative	13(38.2)	75(45.7)	
Positive	21(61.8)	89(54.3)	

Abbreviations: BCR, biochemical recurrence; PSA, prostate-specific antigen.

histologic grading is one of the critical predictors of BCR, its use is limited because most patients have moderately-differentiated tumors (Gleason scores of 6 or 7) [19]. The use

of appropriate tumor biomarkers, as supplements to clinical parameters, might improve the predictive value of biochemical and histological analyses [20, 21]. However, there is still a lack of reliable biomarkers that can specifically and accurately reflect the likelihood of BCR after curative treatment. In the present study, we evaluated the value of Sema3s expression by IHC on preoperative biopsy samples for the prediction of BCR after radical prostatectomy. Our results suggest that patients with Sema3C-positive and Sema3A-negative, 3B-negative, 3E-negative tumors had significantly more chance to experience BCR.

Prior studies demonstrated that Sema3s not only played a critical role in axon guidance, but also could regulate the response of cancer cells and endothelial cells to either promote or inhibit tumor angiogenesis and progression through various mechanisms [5, 6]. Yacoub et al. [22] evaluated 120 patients with localized prostate cancer who were treated with radical prostatectomy and found that expression of Sema3A was associated with lower preoperative PSA and pathological stage. Moreover, their analysis revealed that dysregulation of Sema3A plays a key role in the progression of prostate cancer and suggested a loss of the inhibitory Sema3A autocrine loop in hormone-refractory prostate cancer [22]. Similar results were described by Herman et al. [23], who suggested that overexpression of Sema3A decreased prostate cancer invasion and adhesion *in vitro*. In contrast, tumors in prostate cancer patients with high Sema3C expression possessed higher invasive and metastatic characteristics [23]. Consistent with these studies, we found that Sema3C-positive and Sema3A-negative expressions were associated with a higher probability of BCR. Furthermore, the multivariate analysis showed that Sema3A-negative and Sema3C-positive expressions were independent risk factors for predicting relapse.

Sema3B and 3F were initially characterized as tumor suppressors whose functions are lost in carcinoma cells by allelic loss or tumor-acquired promoter methylation. Sema3B can induce apoptosis and its pro-apoptotic effect is inhibited by VEGF [24]. In addition, Sema3F can affect tumor cell behavior

Table 2. Univariable and multivariable analyses for biochemical recurrence

Variable	Univariable analysis		Multivariable analysis	
	OR (95%CI)	P	OR (95%CI)	P
Age: <65yr, ≥65yr	0.98(0.96-1.02)	0.39	-	-
Preoperative PSA (ng/ml): <10, 10-20	1.47(0.80-2.73)	0.21	-	-
Biopsy Gleason score: 6, 7	2.49(1.15-3.93)	0.016	1.96(1.06-3.62)	0.033
Clinical T stage: T1c-T2a, T2b	2.31(1.26-4.21)	0.007	1.24(1.02-2.93)	0.091
Sema 3A: positive, negative	0.20(0.09-0.44)	<0.001	0.19(0.089-0.42)	<0.001
Sema 3B: positive, negative	0.48(0.26-0.91)	0.023	0.38(0.19-0.72)	0.003
Sema 3C: positive, negative	2.71(1.41-5.21)	0.003	2.31(1.19-4.49)	0.014
Sema 3D: positive, negative	0.91(0.49-1.68)	0.77	-	-
Sema 3E: positive, negative	0.34(0.18-0.66)	0.02	0.39(0.20-0.78)	0.007
Sema 3F: positive, negative	0.93(0.51-1.69)	0.81	-	-

Abbreviations: OR, odds ratio; CI, confidence interval; PSA, prostate-specific antigen.

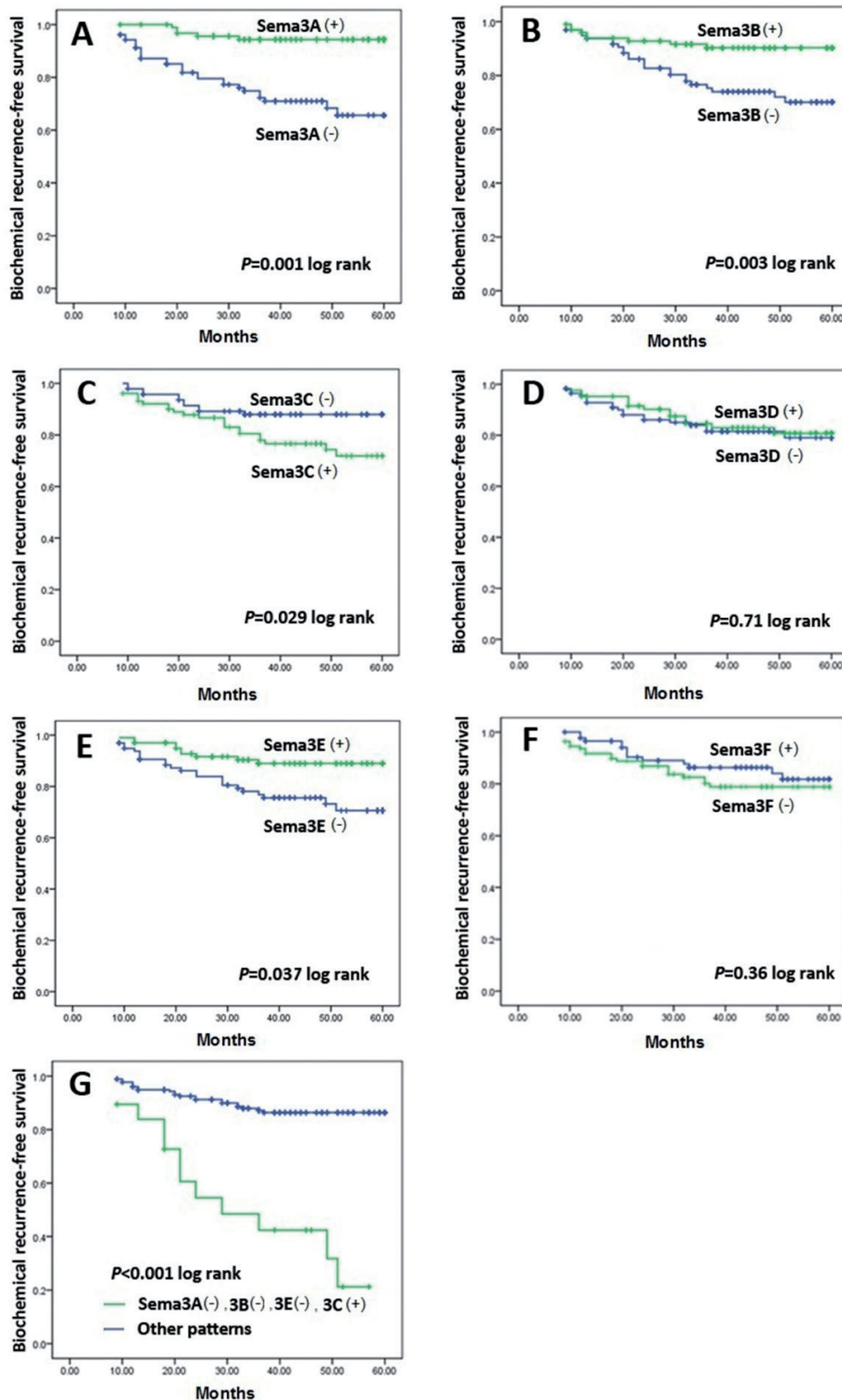


Figure 2. Association between the Sema3s protein expression and biochemical recurrence: Kaplan-Meier analysis showed that patients with Sema3A-negative, Sema3B-negative, Sema3E-negative and Sema3C-positive tumors (each alone or four markers combined) were significantly more likely to experience biochemical recurrence (A,B,C,E,G), while Sem3D or Sema3F expression alone could not predict biochemical recurrence with statistical significance(D,F).

directly and has ability to suppress the metastatic spread of cells from tumors [25]. Overexpression of both *Sema3B* and *3F* is negatively associated with tumor progression. Beuten et al. [26] performed a case-controlled study of 789 prostate cancer cases and found that polymorphisms in *Sema3B* and *3F* were associated with a high Gleason score (OR 1.44, $P = 0.021$) and correlated with increased prostate cancer risk and poor prognosis (OR 2.72, $P = 0.016$ and OR 3.32, $P = 0.02$, respectively). However, our present findings showed only *Sema3B* was independently predictive of recurrence-free survival in patients with low- and intermediate-risk prostate cancer. The role of *Sema3F* in predicting BCR need to be evaluated in further prospective studies.

Sema3E is considered unique among *Sema3s* in that it binds directly to the plexin-D1 receptor, but not to neuropilins [27]. Plexin-D1 can transduce anti-angiogenic signals and is up-regulated in tumor-associated blood vessels [28]. It also forms complexes with neuropilins. Whether the *Sema3E* secreted by tumor cells promotes or inhibits cancer growth may depend on the constitution of the receptor complex to which it binds on the tumor cells. Prostate tumors express high levels of neuropilin-1, and this overexpression correlates with advanced disease and a high Gleason score [29]. Blanc et al. [30] showed that strong staining for *Sema3E* was observed in 52% of prostate cancer tumor tissue samples, compared with 11% in non-malignant tissue samples ($P = 0.0001$). The fact that 43% of tumor samples had a greater intensity of *Sema3E* staining, compared to non-malignant tissues from the same patient, suggests that *Sema3E* is overexpressed in prostate cancer. Our present data corroborated this association and demonstrated that *Sema3E* was negatively correlated with the risk of BCR after radical prostatectomy. Meanwhile, Kigel et al. [11] showed that *Sema3D* can function as a potent anti-tumorigenic agent, and could inhibit tumor development and attenuate angiogenesis. Interestingly, although 58.8% of cases with BCR in our study had *Sema3D*-negative staining, *Sema3D* expression alone was not an independent predictive factor for BCR.

In this study, recurrent-free survival plot analysis confirmed that positive staining of *Sema3A*, *3B*, and *3E* and negative *Sema3C* staining were associated with a favorable prognosis for patients with low- and intermediate-risk prostate cancer who underwent radical prostatectomy. In multivariate analysis, *Sema3A*, *3B*, *3C*, and *3E* staining were shown to be independent risk factors for predicting BCR. We propose that the expression status of *Sema3A*, *3B*, *3C*, and *3E* in biopsy samples may supplement the current prognostic factors, i.e. clinical or pathological stage, Gleason score and PSA, as predictors of BCR. We note that even with relatively short follow up in our study, the BCR rate (17.2%) is higher than those reported by some authors [15]. In our opinion, this may be due to the high proportion of patients with intermediate-risk prostate cancer (about 60%) included in our cohort.

Some limitations of this study should be noted. Firstly, our study was in a retrospective fashion; and secondly, the number

of evaluated patients was not particularly large, which might limit the statistical power of the predictive value of some markers. Consequently, further prospective studies in larger patient populations are needed to validate the current observations.

In summary, we showed that *Sema3A*, *3B*, *3C*, and *3E* immunostaining in prostate biopsies, as supplements to clinicopathological parameters, could be used for predicting BCR in low- and intermediate-risk prostate cancer patients after radical prostatectomy. Specially, concurrent *Sema3C*-positive and *Sema3A*-negative, *3B*-negative, *3E*-negative staining is associated with an adverse prognosis.

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