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Research Article

Cancer Prevention Research

Prognostic Significance of VEGF after Twenty-Year Follow-up in a Randomized Trial of Fenretinide in Non–Muscle-Invasive Bladder Cancer

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Abstract

Non–muscle-invasive bladder cancer (NMIBC) may progress to muscle-invasive disease, but no effective preventive treatments are available. In addition, no reliable prognostic biomarkers have been identified. We assessed the long-term effect of the oral retinoid fenretinide and the prognostic value of circulating VEGF levels. We updated through the Tumor Registry the vital status of 99 patients with resected Ta/T1 bladder tumors who were recruited in a randomized trial of 2 years of fenretinide or no treatment in 1993–1994. Serum VEGF levels measured at baseline and 12 months were available in a subgroup of 62 patients. After a median of 20.5 years, 54 subjects died, 35 of any cancer and 14 of bladder cancer. Neither overall survival (OS), nor cancer survival (CS) or bladder cancer survival (BCS) was affected by fenretinide (log-rank $P \geq 0.2$). DNA aneuploidy in bladder washing was associated with shorter OS ($P = 0.02$), CS ($P = 0.05$), and BCS ($P = 0.09$). Subjects with baseline VEGF levels in the top quintile (≥350 pg/mL) had a significantly shorter OS ($P = 0.01$), CS ($P = 0.02$), and BCS ($P = 0.008$). The trend across quintiles of VEGF was significant for BCS ($P = 0.007$). Multivariate analyses showed that, in addition to smoking status, VEGF level in the top quintile was an independent prognostic factor for OS (HR = 2.7; 95% CI, 1.1–6.5), CS (HR = 3.3; 95% CI, 1.1–9.4) and BCS (HR = 8.9; 95% CI,1.3–61). Fenretinide did not affect the long-term outcome of patients with NMIBC. High serum VEGF level was a significant predictor of overall and cancer death and may help to identify high-risk subjects who may benefit from a preventive therapy. *Cancer Prev Res; 9(6); 437–44. ©2016 AACR.*

Introduction

Bladder cancer is the fourth most common tumor in men and the twelfth most common cancer in women in the United States, with approximately 74,000 new cases being expected in 2015 (1).

Non–muscle-invasive bladder cancer (NMIBC) represents about 70% to 80% of bladder neoplasms and includes low-grade papillary forms (stage Ta), tumors invading the lamina propria (T1) and carcinoma in situ (Tis) (2, 3). Roughly between 50% and 70% of non–muscle-invasive tumors do recur, and approximately 10% to 20% of them progress to muscle-invasive disease (4–6). Bacillus Calmette–Guerin (BCG) intravesical immunotherapy is the recommended treatment for high-risk, early-stage bladder cancer, but high recurrence and progression rates (15–20%) to muscle-invasive cancer still remain (5). Furthermore, no reliable quantitative biomarkers that predict progression and death from the affected target organ have been identified (7, 8).

NMIBC is an important target for the accelerated development of new agents designed to reduce cancer incidence and mortality (9). There is increasing need to identify risk biomarkers that are measurable and repeatable in order to allow selection of high-risk individuals to allow efficient drug testing in chemoprevention clinical trials (10–12).

Based on encouraging findings from a phase IIa trial (10), in 1993 we conducted a randomized trial of the synthetic retinoid fenretinide given orally for 2 years in patients with resected stage T1 or Ta bladder cancer, with negative results on the primary outcome measure, which was DNA flow cytometry in bladder washings (13). More recently, we showed the prognostic effect of DNA aneuploidy (i.e., DNA index > 1.1) from bladder washed cells on disease progression and death after a median follow-up time of 11.5 years (14).

Angiogenesis is critical for tumor growth by providing oxygen, nutrients, and growth factors to the cancer cells (15). VEGF, a homodimeric glycoprotein with a molecular weight

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of approximately 45 kDa, is considered to be a crucial regulator of both normal and pathologic angiogenesis (16, 17). The expression of VEGF, either in tissue and serum, has been extensively investigated in patients with NMIBC and bladder cancer with different findings (18–25). Retinoids have been shown to modulate angiogenesis and to inhibit carcinogenesis through different mechanisms, including an angiopreventive effect (26, 27).

In the present paper, we report the results of fenretinide on survival endpoints after a median of 20.5 years and assess the prognostic value of circulating VEGF, which was measured in a subgroup of patients ($n = 62$).

Materials and Methods

Study design, subjects, and treatment

A phase IIb chemoprevention trial with fenretinide in NMIBC started at the National Cancer Institute of Genoa in 1993. A detailed description of the trial has been published elsewhere (13). Briefly, 99 subjects, ages < 80, with NMIBC (stage Ta or T1, any grade) resected within the previous 3 years, were randomized to receive fenretinide 200 mg/day or no treatment for 2 years. An additional year of follow-up was included to evaluate any potential rebound effect. The primary outcome measure was the flow cytometric DNA content in epithelial cells obtained after 12 months. A detailed description of the original trial design, study procedures, and main results has previously been described (10, 13, 28). Years later, we decided to assess the prognostic significance of circulating VEGF and its potential surrogate effect on survival by fenretinide modulation. Available samples from 63 subjects who had completed the first year of study and had serum aliquots at both time points were identified; data from one subject was further excluded because of a second malignancy at the end of the first year of treatment. Analysis was thus performed on samples from 62 subjects, 29 allocated in the fenretinide and 33 in the control arm, respectively.

Assay methods

Serum concentrations of VEGF were measured on morning fasting blood samples. Blood were drawn at baseline and after 12-month treatment and serum aliquots were stored at −80°C until assayed in a single experiment at the end of the trial to minimize analytical variability and blinded as to treatment allocation.

Serum VEGF was measured by means of a quantitative sandwich enzyme immunoassay kit (SVE00) produced by R&D Systems. A monoclonal antibody specific for VEGF was precoated onto a 96-well microplate. Standards and samples were dispensed into the wells in duplicate. Any VEGF present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for VEGF was added to the wells. Following washing to remove any unbound antibody–enzyme reagent, a substrate solution was added to the wells and color developed in proportion to the amount of VEGF bound in the initial step. The color development was stopped and the optical density was determined on a microliter plate reader at 450 nm. A standard curve was created by plotting the logarithm of the mean absorbance of each standard versus the logarithm of the cytokine concentration. The sensitivity of the assay was 9.0 pg/mL. The intra-assay and inter-assay coefficients of variation were less than 7% and 9%, respectively.

Cohort follow-up. Information on patients' health status was ascertained from the Urology Units who participated in the trial or, when the patient was lost to follow-up, through computer linkage with the local Mortality Registry or by phone. Causes of death were ascertained through death certificates or by review of the local Mortality Registry.

Statistical analysis. The main descriptive statistics were mean and standard deviation, median, interquartile range (IQR), and quintiles for continuous factors, absolute and relative frequency for categorical factors. The Mann–Whitney rank test was used to compare continuous factors and Pearson χ^2 or Fisher exact test were used to compare categorical factors between treatment arms. Kaplan–Meier estimates of the cumulative probability for overall survival (OS), cancer survival (CS), bladder cancer–specific survival (BCS), defined as the time from randomization to death, death from cancer, death from bladder cancer, respectively, were obtained for all the baseline factors, including allocated treatment (fenretinide versus control), age, sex, smoking habit, tumor stage, tumor grade, recurrence history, previous intravesical treatment, DNA aneuploidy (DNA index < 1.1 vs. ≥ 1.1) and VEGF baseline levels. To assess the prognostic role of VEGF and in the absence of any validated threshold, we considered quintiles of VEGF distribution and evaluated the clinical outcome in terms of OS, CS, and BCS.

Multivariate Cox modeling was used to assess the independent prognostic effect of all factors under investigation. All covariates were tested for the proportional hazards assumption. Starting from a full model (i.e., including all baseline factors cited above), we adopted a backward stepwise selection approach with a significance cutoff level for removal from the model equal to 0.20 (Wald P value). P values were two-sided and considered significant if <0.05; all calculations were performed using STATA version 13 software.

Results

Patient characteristics at baseline according to the treatment arm had been described extensively in previous publications (10, 16) and are shown in Supplementary Table S1. Briefly, the two arms were well balanced for patient and tumor characteristics except for a trend toward an excess of former smokers in the fenretinide treatment arm.

Table 1 illustrates VEGF levels by the treatment arm, in the subgroup of 62 patients who had measured this biomarker at baseline and after 12 months from randomization. VEGF levels were not significantly different between fenretinide-treated and control subjects either at baseline or after 12 months. Overall, a significant decrease of VEGF levels was observed after 12 months ($P = 0.007$), regardless of the treatment arm.

A comparison of subjects' characteristics according to VEGF levels determination is shown in Table 2. Except for age, where subjects without VEGF determination were on average 5 years older, there was no statistically significant difference between groups in any patients' characteristic. However, as illustrated in Fig. 1, patients with VEGF determinations showed a trend to an increased survival (HR 0.59 and 0.42 for OS and BCS, respectively), suggesting that VEGF analysis was not performed in a random subgroup.

After a median follow-up of 20.5 years (interquartile range, 20.3–20.8), a total of 54 subjects died, 35 of any cancer and 14 of

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Table 1. VEGF levels at baseline and after 12 months by the treatment arm ($n = 62$)

	Control ($n = 33$)	Fenretinide ($n = 29$)	P^a
Baseline, pg/mL			
Mean ± SD	233.3 ± 133.4	263.1 ± 162.1	0.4
Median (IQR)	201.3 (132.8–277.3)	245.8 (135.2–313.3)	
Bottom quintile	72.2–123.1	88.2–124.6	
2nd quintile	127.0–187.5	129.6–176.9	
3rd quintile	189.3–229.3	217.4–254.4	
4th quintile	230.3–351.8	278.2–347.6	
Top quintile	371.6–608.0	423.2–741.7	
After 1 year, pg/mL			
Mean ± SD	219.2 ± 132.0	224.0 ± 133.4	0.7
Median (IQR)	166.8 (136.1–268.5)	201.5 (137.5–275.3)	
Bottom quintile	57.1–124.5	52.5–119.5	
2nd quintile	125.0–157.6	131.8–178.6	
3rd quintile	161.5–191.3	183.2–211.3	
4th quintile	206.6–310.0	212.3–302.2	
Top quintile	370.8–641.1	323.3–605.7	

[a]Mann–Whitney test. Difference in VEGF levels (independently from treatment arm) between baseline and 24 months: Wilcoxon signed-rank test $P = 0.007$.

bladder cancer. The distribution of VEGF levels at baseline (quintiles of distribution) according to subsequent deaths is summarized in Supplementary Table S2. Within the subgroup of 62 in whom VEGF levels were measured, all 6 bladder cancer deaths had baseline VEGF levels higher than or equal to the median level (216 pg/mL), and 3 had VEGF levels in the top quintile (>350 pg/mL).

Table 2. Main subject characteristics by serum availability for VEGF level determination

	VEGF level determined ($n = 62$)	VEGF level undetermined ($n = 37$)	P^a
Age, years[b]	60 ± 9.5	65 ± 7.9	0.03[c]
Sex			
Male	48 (77.0)[d]	32 (86.5)	0.3
Female	14 (23.0)	5 (13.5)	
Smoking habit			
Non smoker	16 (25.8)	7 (18.9)	0.5
Former smoker	21 (33.9)	17 (46.0)	
Current smoker	25 (40.3)	13 (35.1)	
Tumor stage			
pT_a	37 (59.7)	21 (56.8)	0.8
pT_1	25 (40.3)	16 (43.2)	
Tumor grade			
G1	25 (40.3)	16 (43.2)	0.6
G2	30 (48.4)	19 (51.4)	
G3	7 (11.3)	2 (5.4)	
Tumor history			
First event	24 (38.7)	12 (32.4)	0.5
Recurrent tumor	38 (61.3)	25 (67.6)	
DNA index[e]			
DI < 1.1 (Diploid)	25 (42.4)	10 (29.4)	0.2
DI ≥ 1.1 (Aneuploid)	34 (57.6)	24 (70.6)	
Previous intravesical treatment			
None	5 (8.0)	5 (13.5)	0.1
BCG	37 (59.7)	14 (37.8)	
Chemotherapy	20 (32.3)	18 (48.7)	
Treatment arm			
Control	33 (53.2)	17 (45.9)	0.5
Fenretinide	29 (46.8)	20 (54.1)	

[a]Pearson χ^2 or Fisher exact test.
[b]Mean ± standard deviation.
[c]Mann–Whitney rank test.
[d]Number in parentheses are percentages.
[e]Three patients missed DNA index evaluation in the control group.

In univariate analyses, we tested the effect of fenretinide treatment, aneuploidy in bladder washings (DNA index < 1.1 vs. ≥ 1.1) and VEGF levels (< and ≥ the 5th quintile, 350 pg/mL) on OS, CS, and BCS. There was no effect of the retinoid on any of the three outcome measures (Fig. 2), nor was there any interaction between retinoid treatment and smoking status (data not shown). As regards DNA index (Supplementary Fig. S1), the cumulative probability of death at 20 years from any cause, any cancer and bladder cancer was 28%, 53%, and 77% for DNA diploid washings and 60%, 71% and 88% for DNA aneuploid washings, respectively (log-rank test $P = 0.02$, 0.05, and 0.1, respectively). Finally, compared with subjects in quintiles 1–4 of VEGF levels pooled together, subjects in the top VEGF quintile had a significantly shorter OS (cumulative probability of death at 20 years: 62% vs. 33%, respectively, $P = 0.01$), CS (74% vs. 44%, $P = 0.02$) and BCS (92% vs. 69%, $P = 0.008$; Fig. 3). More importantly, a statistically significant trend across ordered quintiles of VEGF was found for BCS ($P = 0.007$), but not for OS and CS ($P = 0.13$ and 0.2, respectively).

Table 3 shows the results of Cox multivariate analysis. The final models showed that the top VEGF quintile at baseline were the only independent predictor for all three outcomes, namely, all-cause mortality (HR = 2.65, 95% CI, 1.09–6.47; $P = 0.03$), cancer mortality (HR = 3.27, 95% CI, 1.14–9.40; $P = 0.03$), and bladder cancer mortality (HR = 8.87, 95% CI, 1.29–61.2; $P = 0.03$). Smoking and T1 stage had prognostic effect on OS and CS, respectively, whereas there was a trend toward a higher risk of death from bladder cancer in subjects in the fenretinide arm ($P = 0.09$). Adjusting for other variables, DNA index was not independently associated with any of the mortality outcomes ($P > 0.7$).

Discussion

Despite recent advances in screening and multimodality therapy, the outcome for advanced bladder cancer remains generally poor, thus emphasizing the need for early detection and effective prevention strategies as well as better prognostic and predictive markers and tailored treatment approaches. Currently, the most widely studied prognostic factors are related to pathologic characteristics of the neoplasm, including tumor size, grade, stage, and vascular invasion (29–32).

Angiogenesis is a multistep process and there is evidence that the angiogenic switch, defined as the point at which a tumor is able to induce the formation of new vessels, occurs very early during the carcinogenesis process with VEGF being a pivotal regulator of this event (15, 16). It thus appears plausible that chemoprevention may include inhibition of angiogenesis among its potential targets (33, 34).

Our results after a median follow-up time of 20.5 years show that 55% of subjects with NMIBC who participated in a chemoprevention trial of fenretinide have died, 35% of any cancer and 15% of invasive bladder cancer, supporting the notion that this is a high-risk group in whom effective preventive interventions are needed. Conventional clinical factors such as tumor stage and smoking habit were associated with a greater risk of death, in line with literature findings (35). Interestingly, we showed an intriguing trend to an association between circulating VEGF levels at baseline and death for bladder cancer inasmuch as all of the 6 subjects who died of bladder cancer had VEGF levels above the median value, and the half of them were in the top VEGF quintile. We showed a significant trend across ordered quintiles of VEGF

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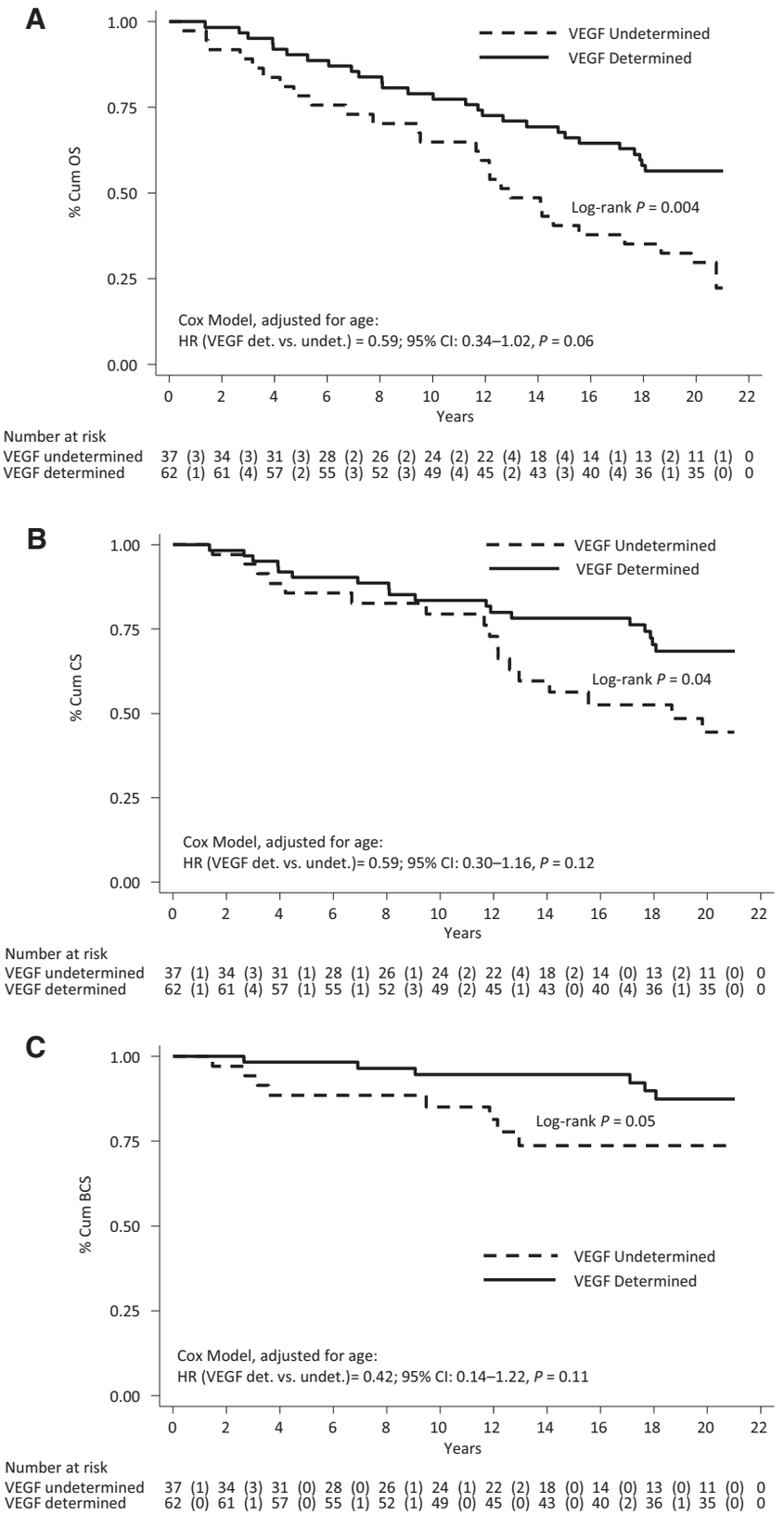

Figure 1.
Kaplan–Meier OS (A), CS (B), and BCS (C) according to VEGF determination (undetermined, dotted line; determined, continuous line). HRs from a Cox proportional hazards model adjusted for age are provided.

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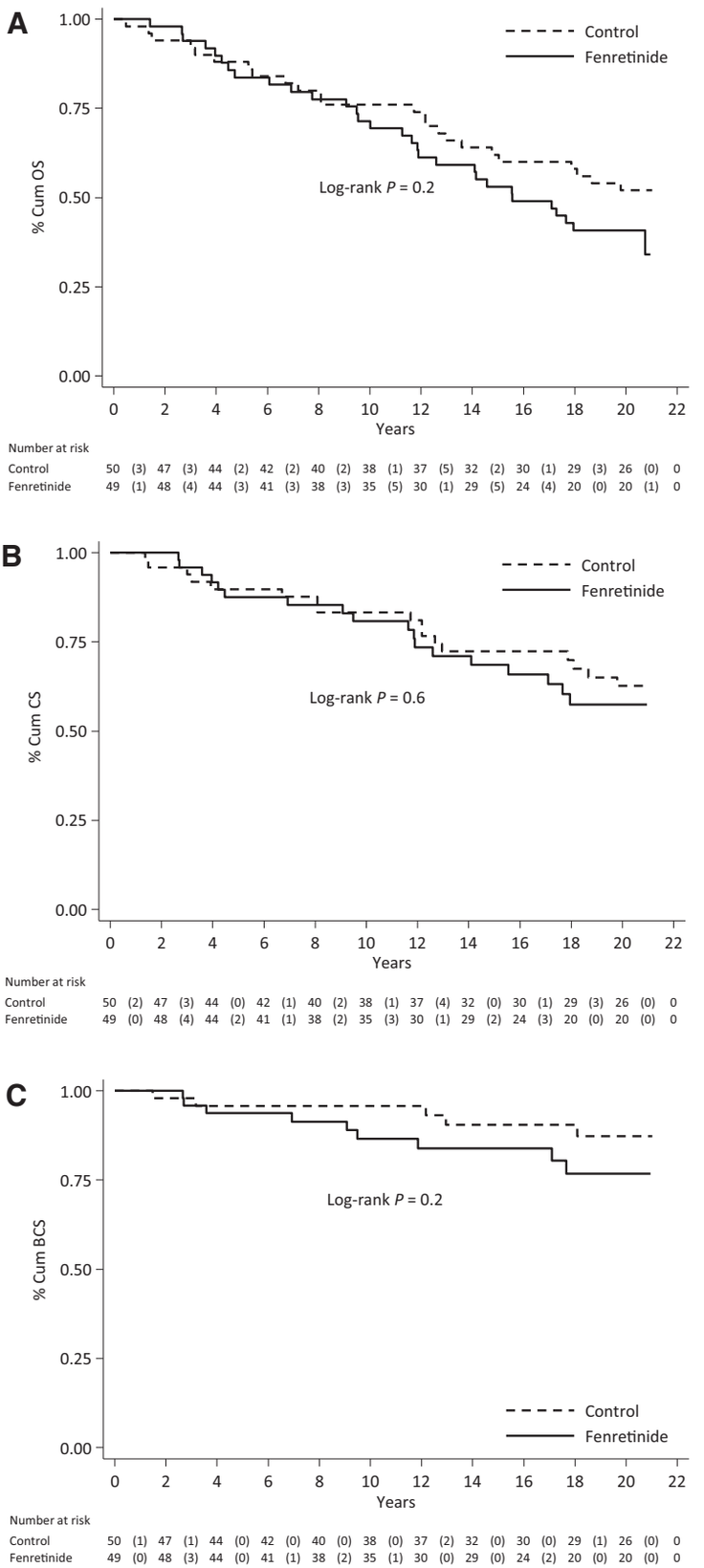

Figure 2.
Kaplan–Meier OS (A), CS (B), and BCS (C) according treatment (fenretinide, dotted line; no treatment, continuous line).

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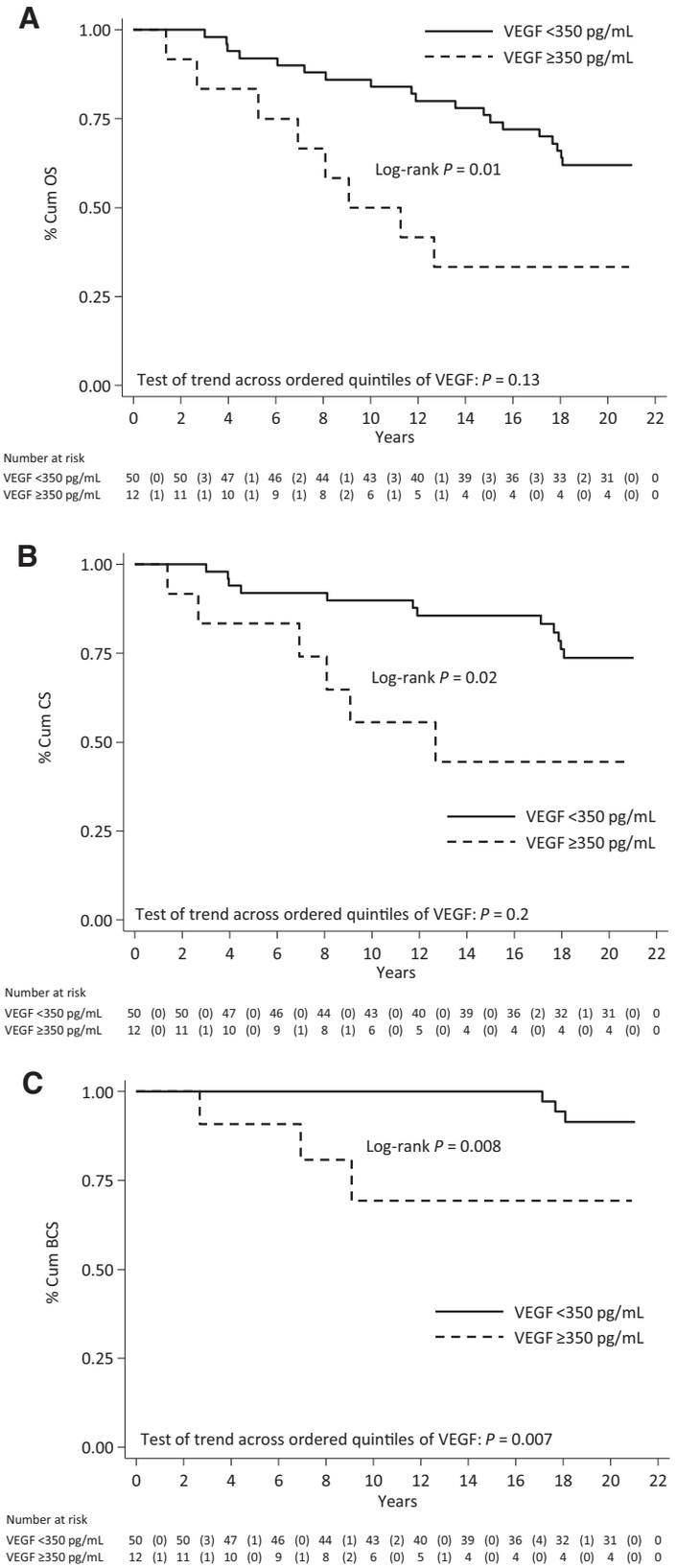

Figure 3.
Kaplan–Meier OS (A), CS (B), and BCS (C) curves according to VEGF values at baseline (below vs. above or equal the top quintile, i.e., 350 pg/mL). *P* values for test for trend across ordered quintiles of VEGF are provided.

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Table 3. Multivariate Cox proportional hazard model OS, CS, and BCS ($n = 59$)[a]

		OS		CS		BCS	
	n	HR (95% CI)	*P*[b]	HR (95% CI)	*P*[b]	HR (95% CI)	*P*[b]
Tumor stage							
pTa	36	1.0		1.0		1.0	
pT1	23	1.81 (0.80–4.11)	0.16	2.75 (1.03–7.37)	0.04	4.10 (0.67–25.08)	0.13
Smoking habit							
Never	15	1.0		1.0		—	
Ever (current/former)	44	4.34 (1.25–15.07)	0.02	5.02 (1.10–23.02)	0.04	—	—
Treatment							
Control	30	—		—		1.0	
Fenretinide	29	—	—	—	—	6.85 (0.74–63.79)	0.09
VEGF (at baseline)							
<350 pg/mL	47	1.0		1.0		1.0	
≥350 pg/mL (5th quintile)	12	2.65 (1.09–6.47)	0.03	3.27 (1.14–9.40)	0.03	8.87 (1.29–61.22)	0.03

[a]The risk estimates are age-adjusted; we adopted a backward stepwise selection approach with a significance level equal to 0.20 for removal from the model; total $n = 59$ due to missing data for $n = 3$ DNA index values.
[b]Two-sided Wald test.

with BCS only ($P = 0.007$), supporting a biologic link between angiogenesis and bladder carcinogenesis progression. More importantly, high circulating levels of VEGF also had an independent, statistically significant, prognostic effect on all mortality endpoints after adjustment for age, stage, and smoking habit. Specifically, VEGF level ≥350 pg/mL was the strongest predictor of BCS, although the small sample size precludes any firm conclusion, as reflected by the wide confidence interval of the estimate.

To our knowledge this is the first report showing a prognostic value of circulating VEGF levels in NMIBC, with not only OS but also cancer-specific survival and BCS as endpoints, thus linking a strong and validated angiogenesis biomarker to death for invasive bladder cancer.

The role of VEGF in bladder carcinogenesis has extensively been studied, but results remain inconclusive. Higher urinary and serum VEGF levels have been associated with a greater risk of recurrence and progression to invasive cancer in NMIBC (18, 19). Furthermore, the degree of invasiveness of NMIBC have recently been linked to high levels of VEGF (20, 21), suggesting a relationship between VEGF and prognosis of NMIBC (22, 23), and a recent meta-analysis (36) of over 1,200 patients showed that elevated VEGF expression was significantly associated with poor prognosis, both in terms of OS (HR = 1.84; 95% CI, 1.23–2.76) and disease-free survival (HR = 1.50; 95% CI, 1.26–1.79).

A strength of our observation is the long-term follow-up and the source of data derived from a randomized clinical trial. Moreover, in contrast to other reports, in our study, circulating VEGF was determined in patients after TURB and therefore possibly unrelated to the papillary tumor burden. Thus, we hypothesize that high serum levels reflect the degree of field cancerization in the bladder and high serum VEGF may represent a biomarker of imbalance between proangiogenic and antiangiogenic factors, which may ultimately result in an angiogenic switch toward proliferation of endothelial cells and the development of a neoplastic phenotype. Additional studies are necessary to confirm our exploratory finding. As for the significant decrease of VEGF levels 1 year apart, we have no ready explanation for this, except for a regression to the mean phenomenon rather than an a real biologic effect associated with the prolonged interval from initial TURB or an analytical artifact.

An important weakness of our analysis is the lack of VEGF measurements in all subjects as only a subgroup of 62 had available serum aliquots. These subjects were on average 5 years younger and, consequently, had increased survival compared with the 37 subjects without VEGF determination. However, age difference does not appear to fully explain the increased survival of this group since the association still persists after adjusting for age, although not significantly so. The reason for this selection bias, which clearly limits the generalizability of our findings, is still unclear but has likely to do with the possibility that older patients could be less likely to remain on the fenretinide trial for a full year.

As in a previous report (14), we showed no effect of the treatment with fenretinide (200 mg day for 2 years) on survival endpoint. This finding is consistent with previous results suggesting a lack of efficacy (13, 37) or even a detrimental effect by beta carotene and retinoids when used to inhibit tobacco-related carcinogenesis in large clinical trials (38, 39). We have no strong evidence to support a detrimental effect, although a slight trend to an increase in overall mortality was noted in the fenretinide arm, in line with preclinical evidence of a detrimental effect of beta-carotene in smokers (40).

In conclusion, our 20-year survival data show that fenretinide does not affect disease outcome in subjects with NMIBC. However, we detected an inverse association between baseline VEGF levels and BCS in our cohort of patients with resected NMIBC, even if generalizability of our results may not be fully applicable due to the apparent selection bias in the cohort we examined. Because angiogenesis represents a key step in tumor progression, and VEGF is implied in early stages of angiogenesis, its circulating levels may represent a putative biomarker to select high-risk patients as well as a candidate surrogate endpoint for future chemoprevention studies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: M. Puntoni, R. Torrisi, A. DeCensi
Development of methodology: M. Puntoni, R. Torrisi, H. Johansson, A. Curotto, A. DeCensi
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Campora, E. Garrone, C. Defferrari, R. Torrisi, H. Johansson, S. Bruno, A. Curotto
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Puntoni, A. DeCensi

Writing, review, and/or revision of the manuscript: M. Puntoni, M. Petrera, R. Torrisi, H. Johansson, A. DeCensi
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Campora, E. Garrone, R. Torrisi
Study supervision: R. Torrisi, A. DeCensi

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