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ARTICLE

Plasma 25-Hydroxyvitamin D_3 and Bladder Cancer Risk According to Tumor Stage and *FGFR3* Status: A Mechanism-Based Epidemiological Study

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Background Previous evidence suggests that 25-hydroxyvitamin D_3 [25(OH)D_3] protects against several cancers. However, little is known regarding urothelial bladder cancer (UBC). We analyzed the association between plasma 25(OH)D_3 and overall risk of UBC, as well as according to stage and *FGFR3* molecular subphenotypes.

Methods Plasma concentrations of 25(OH)D_3 in 1125 cases with UBC and 1028 control subjects were determined by a chemiluminescence immunoassay. *FGFR3* mutational status and expression in tumor tissue were assessed. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated by logistic regression adjusting for potential confounders. Analyses were further stratified by tumor invasiveness and grade, FGFR3 expression, and smoking status. Cell proliferation was measured in human UBC cell lines cultured with 1α,25-dihydroxyvitamin D_3.

Results A statistically significantly increased risk of UBC was observed among subjects presenting the lowest concentrations of 25(OH)D_3 (OR_{adj} = 1.83; 95% CI = 1.19 to 2.82; P = .006), showing a dose–response effect (P_{trend} = .004). The association was stronger for patients with muscle-invasive tumors, especially among low-FGFR3 expressers (OR_{adj} = 5.94; 95% CI = 1.72 to 20.45; P = .005). The biological plausibility of these associations is supported by the fact that, in vitro, 1α,25-dihydroxyvitamin D_3 upregulates FGFR3 expression in UBC cell lines with low levels of wild-type FGFR3.

Conclusion These findings support a role of vitamin D in the pathogenesis of UBC and show that 25(OH)D_3 levels are associated with FGFR3 expression in the tumor. Because *FGFR3* mutation and overexpression are markers of better outcome, our findings suggest that individuals with low levels of plasma 25(OH)D_3 may be at high risk of more aggressive forms of UBC.

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Vitamin D, or cholecalciferol, is a prohormone involved in bone biology that may also protect against a variety of cancers (1–3). The most active vitamin D metabolite, 1α,25dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$, calcitriol], can regulate proliferation, apoptosis, and cell adhesion at the tumor cell level and it can also affect tumor interaction with the microenvironment through modulation of angiogenesis, invasion, and metastasis [reviewed in (1)]. In addition, it decreases oxidative DNA damage (4). Epidemiologic evidence shows that vitamin D insufficiency, as defined by low levels of 25-hydroxyvitamin D_3 [25(OH)D_3, calcidiol], which is the major circulating and most stable form of vitamin D, is associated with an increased risk of colorectal and breast cancers (5,6). As for other cancers, including pancreatic and prostate cancers, evidence of association with 25(OH)D_3 is null or controversial (7–10). A recent paper on prostate cancer reported a statistically significant association with lethal disease only (11).

Urothelial bladder cancer (UBC) is an important public health issue because of its high incidence in most developed countries and the high costs to society. Spain presents one of the highest UBC incidence rates worldwide, with a male-to-female ratio of seven (12,13). The main established risk factors for UBC are smoking, occupational exposure to aromatic amines, and high levels of arsenic intake (12). Smoking accounts for a large proportion of the etiology of UBC, whereas the other factors contribute only to specific risk groups; yet, an important fraction of the disease remains unexplained. Little is known about the contribution of vitamin D to UBC, and only two studies have examined the association between 25(OH)D_3 plasma levels and the risk of this disease. Whereas, in the Alpha-Tocopherol, Beta-Carotene Cancer Prevention–nested case–control study of male smokers, low levels of 25(OH)D_3 were associated with increased risk of UBC (14), in the PLCO nested case–control study, no association was found (15). 1α,25$(OH)_2D_3$ has been shown to

inhibit proliferation and induce apoptosis of human bladder tumor cells in vitro and to reduce tumorigenesis in an N-methylnitrosourea-induced model of bladder cancer in rats (16). Expression of vitamin D receptor has also been detected in human urothelium (17).

UBC is a heterogeneous disease at the clinical, pathological, and genetic levels and at least two major progression pathways have been identified: papillary, low-grade, non-muscle-invasive bladder cancer (NMIBC) harbors *FGFR3* mutations in approximately 60% of cases and displays low levels of genomic instability; high-grade NMIBC and muscle-invasive bladder cancers (MIBC) display a low prevalence of *FGFR3* mutations and frequent alterations in the p53 and Rb pathways (18). Overall, *FGFR3* mutations and FGFR3 protein overexpression characterize a large subgroup of NMIBC with good prognosis (19), but the molecular mechanisms underlying this association are not well established.

We aimed to assess the association between plasma 25(OH)D_3 levels and risk of UBC in the Spanish Bladder Cancer/EPICURO Study (SBC/EPICURO Study) and explore the molecular mechanisms involved therein. We found that treatment with 1α,25(OH)$_2$$D_3$ leads to the upregulation of FGFR3 messenger RNA and protein in cultured UBC cells with low basal expression levels; these observations led to an analysis of the association of plasma 25(OH)D_3 levels with UBC subphenotypes defined according to tumor invasiveness and grade and *FGFR3* mutational and expression status.

Methods

Study Participants

Subjects came from the SBC/EPICURO Study, a hospital-based, case–control study conducted in 18 hospitals from five areas in Spain (20). Briefly, cases were patients newly diagnosed with histologically confirmed UBC in 1998 to 2001. A panel of expert pathologists classified homogeneously all cases according to the invasiveness and grade (21,22). Control subjects were selected from patients admitted to participating hospitals for diagnoses believed to be unrelated to the exposures of interest and were individually matched to the cases on age, sex, ethnic origin, and region. Written informed consent was obtained from all subjects, and the study was approved by the local institutional review boards and the US National Cancer Institute. Information on known or potential cancer risk factors and blood samples were obtained during the inpatient hospital stay for both cases and control subjects. A total of 1219 cases (84% eligible) and 1271 control subjects (88% eligible) agreed to participate in the study and were interviewed. Plasma from blood samples collected at diagnostic time was available from 1130 cases and 1038 control subjects.

Experimental Procedures

Details on the quantification of 25(OH)D_3, cell proliferation, and *FGFR3* expression assays using UBC cell lines and FGFR3 expression and mutational status analyses in tumoral tissue are specified in the Supplementary Methods (available online).

Statistical Analysis

Student's *t* test was applied to analyze the effects of vitamin D on proliferation and expression of p21, p27, and FGFR3 in UBC cells. Mann–Whitney *U* test was used to assess differences between cases and control subjects regarding median plasma concentrations of 25(OH)D_3. For the analysis of association between 25(OH)D_3 and UBC, logistic regression was applied to estimate odds ratios (ORs) and their 95% confidence intervals (CIs), comparing each category of low plasma 25(OH)D_3 concentration (20–29.99, 15–19.99, 10–14.99, and <10 ng/mL) with the reference category (≥30 ng/mL). A basic model was adjusted for age at interview, sex, region, smoking status, and season of blood draw. Further adjustments were made for body mass index, alcohol and calcium intake as previously associated with vitamin D (23–26), and occupational exposure to aromatic amines and toenail arsenic. Tests for linear trend were computed with the median of each category of the plasma 25(OH)D_3 concentration treated as a continuous variable. Because almost a quarter of the control subjects were admitted to the hospital with bone fractures, a sensitivity analysis was carried out excluding these control subjects. The risk of UBC was further evaluated according to smoking status, with the "ever smoker" category created by collapsing the categories of occasional, former, and current smokers. Statistical interaction between smoking and 25(OH)D_3 was assessed by including an interaction term as the product of the median of each category of plasma 25(OH)D_3 and the never smoker or ever smoker categories. This was repeated by adjusting for duration of cigarette smoking, cigarettes per day, and pack-years among the ever smokers. The association with 25(OH)D_3 was also examined by type of tobacco (blond or black) among the ever smokers. The analysis was also stratified by season of blood draw, and the interaction between season and plasma 25(OH)D_3 was assessed.

Polytomous logistic regression models were applied to analyze the association between 25(OH)D_3 and risk of low-grade NMIBC, high-grade NMIBC, and MIBC. Adjustment was performed for the same variables included in the logistic regression models. Difference in odds ratios between case groups was tested using a likelihood ratio test comparing models with and without the odds ratio constrained to be equal for the corresponding case groups. The polytomous logistic regression models were also applied to the stratified analysis for low and high FGFR3 expression and the presence or absence of mutations in *FGFR3* in tumor tissue, adjusting for the same variables as before. All statistical tests were two-sided, and results were considered significant when *P* was less than or equal to .05. Statistical analyses were performed using STATA/SE version 10.1. This study conforms to the guidelines of Strengthening the Reporting of Observational Studies in Epidemiology Statement for observational studies.

Results

Effects of 1α,25(OH)$_2$$D_3$ on Cultured UBC

For this study, four UBC cell lines with distinct features were selected. Under basal conditions, two of them show an epithelial adhesive phenotype and form compact colonies: RT112 displays high levels of constitutively active wild-type FGFR3, whereas MGH-U3 harbors constitutively active mutant FGFR3. In contrast, the other two lines show a less epithelial phenotype: J82 cells have a mutated *FGFR3* but lack expression both at the RNA and protein levels, whereas MGH-U4 cells express low levels of wild-type FGFR3 (27).

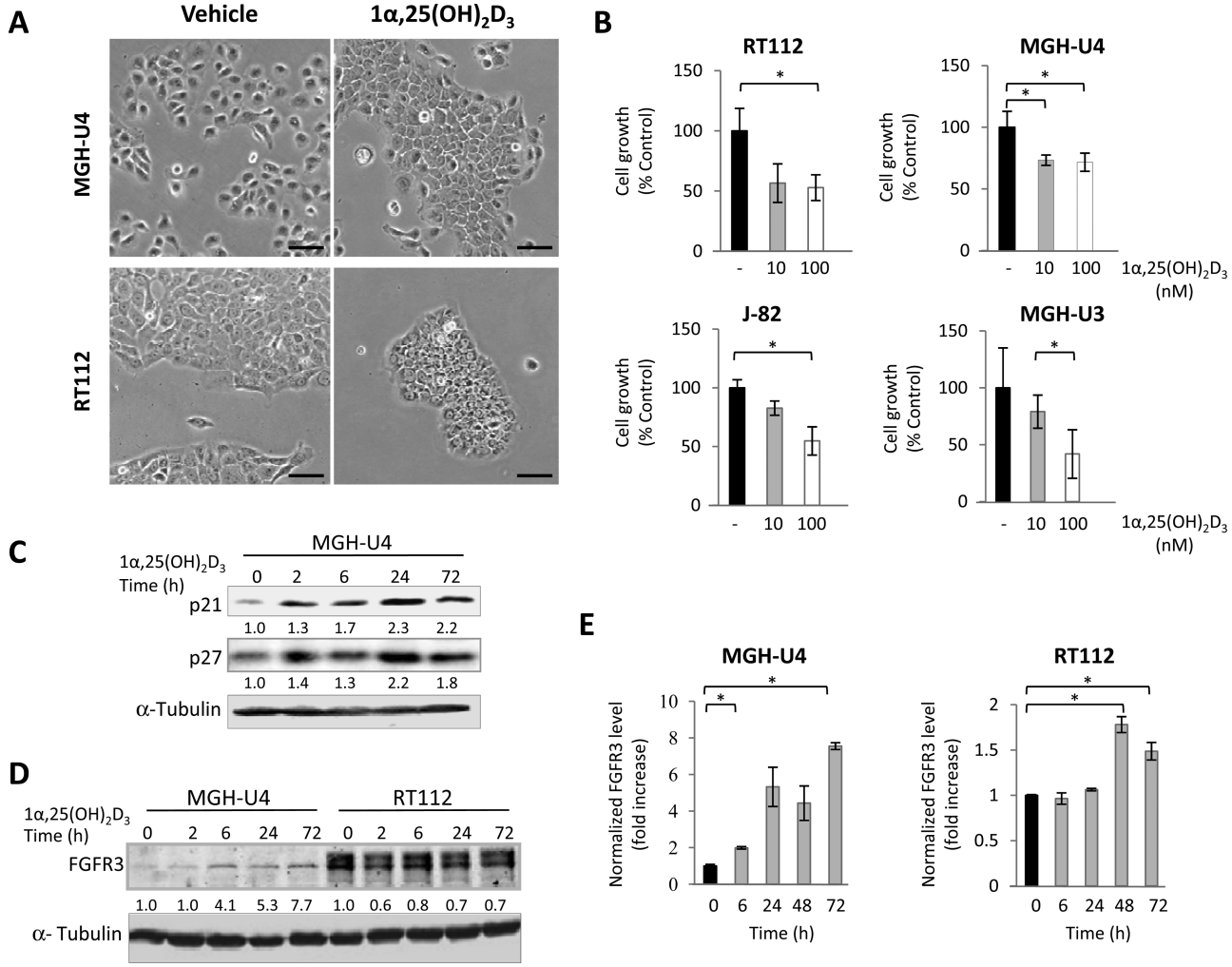

Figure 1. Effects of vitamin D on proliferation and expression of p21, p27, and FGFR3 in urothelial bladder cancer (UBC) cells. Vitamin D induces growth arrest and an upregulation of p21, p27, and FGFR3 in UBC cells. **A**) Phase contrast microscopy of MGH-U4 and RT112 cells treated with 1α,25-dihydroxyvitamin D_3 [1α,25 $(OH)_2D_3$] (100 nM) or vehicle for 72 hours (scale bar = 100 μm). **B**) $1\alpha,25(OH)_2D_3$ treatment inhibits proliferation of UBC cells in vitro. **C**) Western blot analysis showing induction of the CDK inhibitors p21 and p27 in MGH-U4 cells treated with $1\alpha,25(OH)_2D_3$ at different time points. Tubulin was used as a loading control. Values indicate fold-change referred to zero time point. **D**) Western blot analysis showing changes in FGFR3 in MGH-U4 and RT112 cells treated with $1\alpha,25(OH)_2D_3$ at the indicated time points. **E**) Quantitative reverse-transcription polymerase chain reaction analysis showing the upregulation of FGFR3 messenger RNA levels in MGH-U4 and RT112 cells treated with 10 nM $1\alpha,25(OH)_2D_3$ **(gray bars)**. Values were normalized to *HPRT* and referred to expression at time zero **(black bars)**. Comparisons with *P* less than .05 are indicated with an asterisk. Error bars represent standard deviation.

Treatment with $1\alpha,25(OH)_2D_3$ induced a more epithelioid phenotype and formation of more compact colonies, consistent with findings reported in other cell types (Figure 1, A). In the four UBC cell lines studied, $1\alpha,25(OH)_2D_3$ (10–100 nM) induced a growth arrest (Figure 1, B). This was associated with the upregulation of the CDK inhibitors $p21^{CIP1}$ and $p27^{KIP1}$ at the protein level (Figure 1, C). As shown in Figure 1, D and E, $1\alpha,25(OH)_2D_3$ also induced an upregulation of FGFR3 at the messenger RNA level in MGH-U4 and RT112 cells and at the protein level in MGH-U4 cells. Altogether, these findings indicate that vitamin D treatment is associated with phenotypic changes and growth inhibition; in addition, it leads to higher FGFR3 levels in cells with low basal expression.

Plasma $25(OH)D_3$ and Risk of UBC

Based on previous epidemiological evidence and on the above in vitro findings, we analyzed the association between vitamin D levels and UBC risk in the SBC/EPICURO Study, both overall and according to molecular subphenotypes. Cases and control subjects were mostly men, with a high frequency of cigarette smokers (Table 1). Median concentrations of $25(OH)D_3$ were lower in cases than in control subjects (13.9 vs 15.0 ng/mL; $P = .001$) (Table 1); 73% of all individuals (75% of cases and 71% of control subjects) had less than 20 ng/mL (Supplementary Table 1, available online). The distribution of cases by $25(OH)D_3$ status was similar in the three tumor subphenotypes examined ($P = .70$) (Supplementary Table 1, available online).

After adjusting for age, sex, region, smoking status, and season of blood draw, decreasing concentrations of plasma $25(OH)D_3$ were found to be associated with increased risk of UBC ($P_{trend} = .004$) (Table 2). Individuals slightly ($OR_{adj} = 1.63$; 95% CI = 1.06 to 2.51; $P = .03$), moderately ($OR_{adj} = 1.67$; 95% CI = 1.09 to 2.56; $P = .02$), and severely deficient ($OR_{adj} = 1.83$; 95% CI = 1.19 to 2.82; $P = .006$) in vitamin D presented a greater than 50% increased risk of UBC when compared with individuals with sufficient levels. The results were not

Table 1. Characteristics of study participants*

Characteristics	Control subjects (N = 1028)	%	Cases (N = 1125)	%	*P*†
Age, median (range), y	66 (20–81)		68 (22–81)		1.4×10^{-4}
Sex					
Males	909	88	986	88	.58
Females	119	12	139	12	
Region					
Barcelona	205	20	197	18	.41
Valles	159	15	180	16	
Elche	81	8	88	8	
Tenerife	153	15	196	17	
Asturias	430	42	464	41	
Smoking status					
Never smoker	290	28	157	14	1.9×10^{-23}
Occasional smoker	82	8	45	4	
Former smoker	383	37	439	39	
Current smoker	273	27	484	43	
BMI (kg/m^2)‡					
<25	415	53	499	58	.13
25–26.99	169	22	172	20	
27–29.99	136	17	123	14	
>30	65	8	60	7	
Tumor type§					
Low-grade NMIBC (TaG1/G2)	—	—	579	56	
High-grade NMIBC (TaG3/T1)	—	—	205	20	
MIBC (≥T2)	—	—	246	24	
FGFR3‖	—	—			
Wild-type	—	—	496	59	
Mutated	—	—	340	41	
FGFR3 expression¶	—	—			
Low	—	—	396	59	
High	—	—	271	41	
Plasma 25-hydroxyvitamin D_3, median (interquartile range), ng/mL	15.0 (10.0–21.2)		13.9 (9.0–19.9)		1.1×10^{-3}

* BMI = body mass index; MIBC = muscle-invasive bladder cancer; NMIBC = non-muscle-invasive bladder cancer †For age and plasma 25-hydroxyvitamin D_3, the *P* value is from the Mann–Whitney *U* test. For all the other variables, the *P* value is from the χ^2 test.

‡ Two hundred forty-three control subjects and 271 cases had no information on height or weight or both.

§ Ninety-five cases could not be assigned to any tumor/grade group because the paraffin block could not be retrieved.

‖ Two hundred eighty-nine cases did not yield polymerase chain reaction product.

¶ Four hundred fifty-eight cases did not have immunohistochemistry staining for FGFR3.

substantially changed after excluding the control subjects with bone fractures or adjusting for body mass index (Supplementary Table 2, available online), intake of alcohol and calcium, occupational exposure to aromatic amines, or toenail arsenic levels (data not shown).

The association of plasma 25(OH)D_3 levels and risk of UBC was restricted to smokers, showing a dose–response effect (P = .003) (Figure 2, A). However, no statistical interaction was observed between tobacco and 25(OH)D_3 levels. Adjusting for duration of cigarette smoking, cigarettes per day, and pack-years among ever smokers did not change these results (data not shown). Stratifying by type of tobacco among ever smokers did not substantially change the results either (data not shown).

The association of plasma 25(OH)D_3 concentration with risk of UBC was stronger and showed a dose–response pattern among individuals whose blood was drawn in spring and summer seasons, but a statistically significant interaction was not observed (Figure 2, B).

Low concentrations of plasma 25(OH)D_3 were more strongly associated with risk of MIBC. Among individuals severely deficient in vitamin D, the adjusted risk of MIBC (OR_{adj} = 2.81; 95% CI = 1.29 to 6.13; P = .009) was 1.7 times higher than the risk of low-grade NMIBC (OR_{adj} = 1.64; 95% CI = 0.97 to 2.76; P = .07) (Table 2). However, the differences in risk between both tumor types were not statistically significant, possibly due to low sample size.

Plasma 25(OH)D_3 and *FGFR3* Mutation and Protein Expression

Tumors with *FGFR3* mutations were more likely to show high FGFR3 expression than those without mutations ($P = 2 \times 10^{-18}$). Plasma 25(OH)D_3 levels were not associated with somatic *FGFR3* mutations (P = .70) (Supplementary Table 1, available online). Risk of UBC among subjects with deficient 25(OH)D_3 concentrations was slightly higher among *FGFR3*-mutated than wild-type tumors, although the differences were not statistically significant (Supplementary Table 3, available online). The percentage of cases with high FGFR3-expressing tumors was slightly higher in the 25(OH)D_3-sufficient group than in the 25(OH)D_3-deficient group (Supplementary Table 1, available online), but this difference did not reach statistical significance (P = .10).

A more detailed analysis revealed that low plasma concentrations of 25(OH)D_3 were associated with an increased risk of

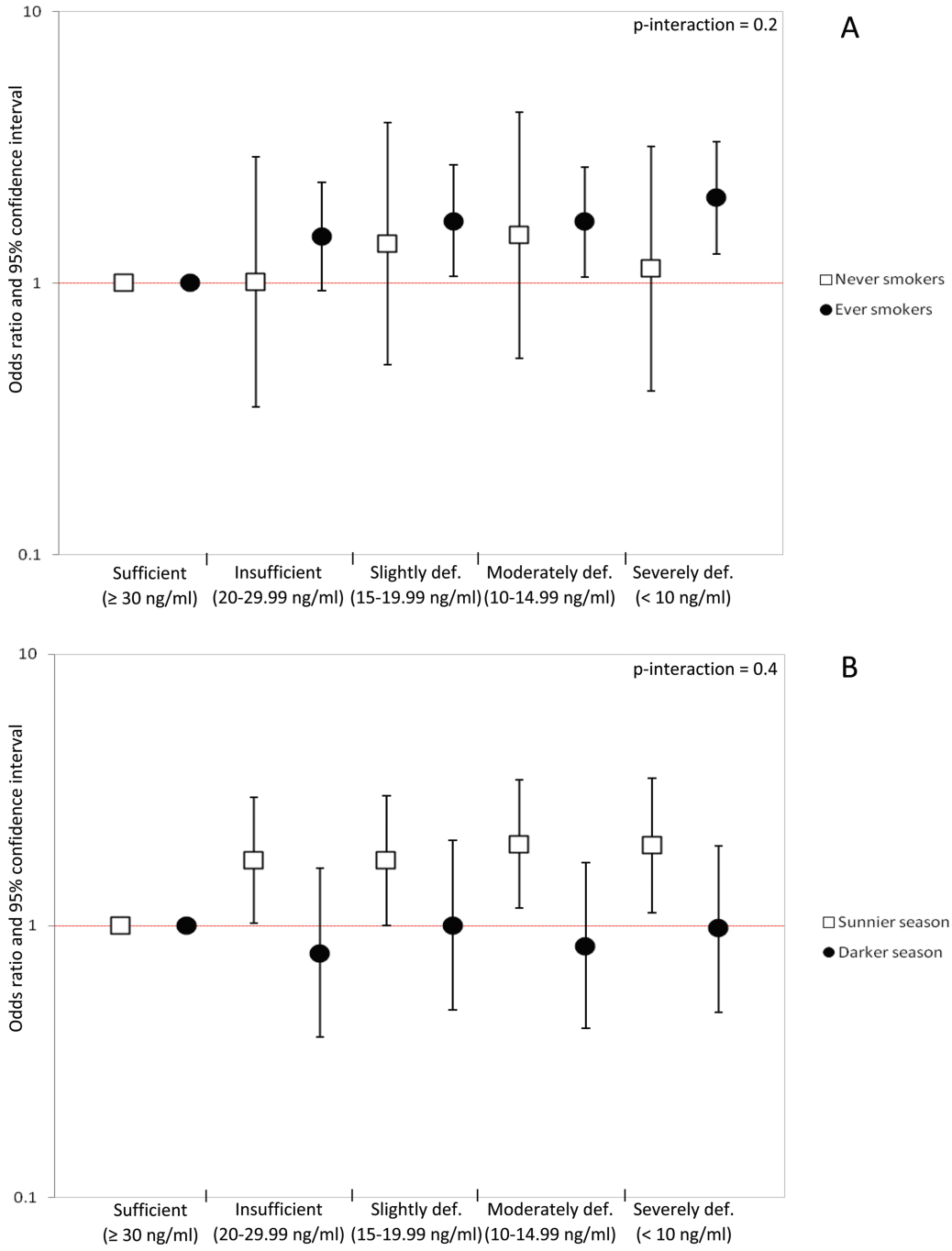

Figure 2. Odds ratios (OR) and 95% confidence intervals for the association between plasma 25-hydroxyvitamin D_3 [25(OH)D_3] and bladder cancer risk by smoking status (**A**) and by season of blood collection (**B**). Estimates are adjusted for age, sex, region, smoking status, and season of blood collection, when appropriate.

developing low FGFR3-expressing UBC but not high FGFR3-expressing UBC. This association was more notable among those severely deficient in vitamin D (OR_{adj} = 3.03; 95% CI = 1.55 to 5.94; P = .001; P_{trend} = .0002) (Supplementary Table 3, available online). Furthermore, in individuals with low levels of 25(OH)D_3, the odds of MIBC expressing low levels of FGFR3 were almost 6-fold higher than in those with sufficient levels (OR_{adj} = 5.94; 95% CI = 1.72 to 20.45; P = .005). This association was not statistically significantly different from that of low-grade (P = .30) and high-grade NMIBC (P = .10) (Figure 3; Supplementary Table 4, available online), also possibly due to small sample size in the subgroups.

Discussion

In the present study, we analyzed the association of plasma 25(OH)D_3 with risk of UBC in the largest and most representative patient series tested so far. For the first time, we placed the findings in the context of the molecular taxonomy of this tumor, namely according

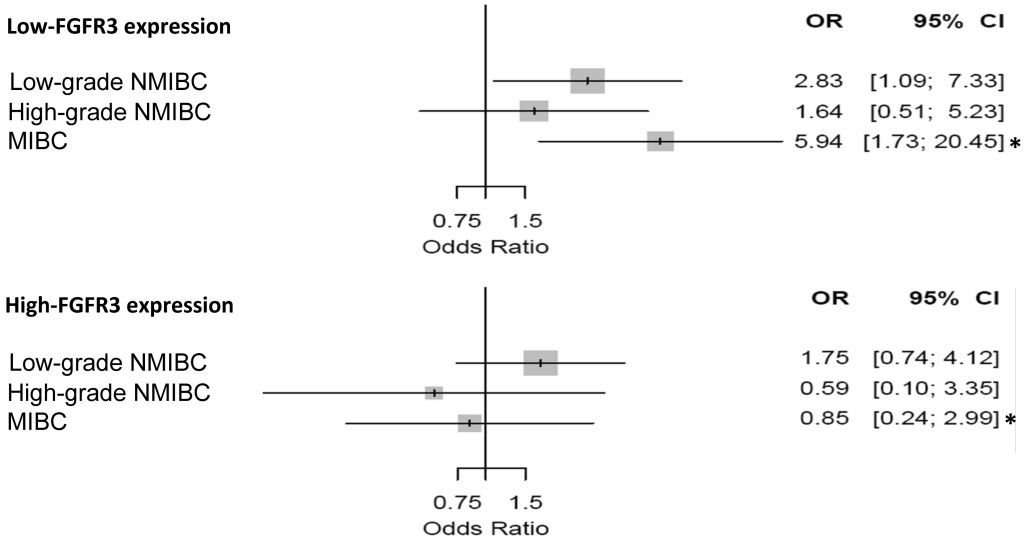

Figure 3. Odds ratios (ORs) and 95% confidence intervals (CIs) for the association between plasma 25-hydroxyvitamin D_3 [25(OH)D_3] levels and risk of low- and high-grade non-muscle-invasive bladder cancer (NMIBC) and muscle-invasive bladder cancer (MIBC) in relationship with tumor FGFR3 expression levels. Estimates are adjusted for age, sex, region, and smoking status. *Likelihood-ratio test *P* for the comparison between the odds ratio of MIBC in the low- and high-FGFR3-expression groups = .26.

to alterations in *FGFR3*, which is the most commonly mutated oncogene in UBC (18). We observed an inverse, statistically significant association between plasma 25(OH)D_3 levels and risk of UBC with a dose–response effect: individuals with the lowest concentrations of plasma 25(OH)D_3 presented almost twofold higher odds than individuals with concentrations greater to or equal than 30 ng/mL (sufficient status). This risk pattern was mainly observed for MIBC. Furthermore, although the increased risk of low-grade NMIBC was independent of FGFR3 expression in the tumor, that of MIBC was not: individuals with deficient levels of vitamin D showed the highest risk for developing low-FGFR3-expressing MIBC. These findings suggest that vitamin D modulates tumor phenotype in specific tumor subtypes.

Tumors that express low FGFR3 protein levels or that are *FGFR3* wild-type are more likely to invade muscle and display an aggressive behavior, whereas tumors that are *FGFR3* mutant and express high FGFR3 levels have a lower tendency to progress (19,28). The in vitro data indicate that vitamin D regulates FGFR3 mainly in cells expressing low FGFR3 levels, and a large body of evidence indicates that tumor cells that respond to vitamin D display more differentiated properties and less aggressive in vitro behavior (29,30). Therefore, our findings are consistent with the notion that vitamin D sufficiency may support higher levels of expression of FGFR3, particularly in wild-type tumors, thus favoring a less-aggressive tumor phenotype at presentation. The recent observation that vitamin D can affect gene expression through the epigenetic modulation of histone marks suggests a possible mechanism of this effect (31). Our results support the notion that in other tumor types, such as colon and breast cancers, similar analyses should be performed to determine whether the effects of vitamin D are restricted to specific tumor subphenotypes and/or molecular pathways.

The study findings build upon data reported by the few previous epidemiologic and molecular studies suggesting that vitamin D may act as a protective factor against UBC (14,16) and extend our understanding by exploring interactions and the association of 25(OH)D_3 plasma levels with UBC FGFR3 subphenotypes. None of the interactions tested were statistically significant. However, the inverse association between 25(OH)D_3 and risk of UBC appeared stronger among ever smokers and among those individuals whose blood was collected during spring or summer months. Although we cannot discard that the relationship with tobacco smoking could be due to chance because of the small sample size of the nonsmoker group, it is in agreement with the findings of Mondul and colleagues, who reported an increased risk of bladder cancer in male smokers associated with low 25(OH)D_3 serum concentrations (14). The stronger effects found among subjects whose blood was drawn during spring and summer confirm prior findings and suggest that assessing plasma levels of 25(OH)D_3 during sunnier months provides a more sensitive biomarker of a constitutive deficiency of vitamin D and thus better discriminates those individuals with higher susceptibility to UBC (14).

These results are of relevance given that vitamin D deficiency and insufficiency are highly prevalent in Spain (32,33), where the incidence rates of UBC are among the highest worldwide (12,13), and in many other Western countries. The concentrations of 25(OH)D_3 found in this study were similar to those of same-age individuals from other Southern European countries, although lower than those of individuals from the United States and Sweden. A potential explanation is that, in the latter countries, several food items are fortified with vitamin D (34–36).

This is the largest study assessing the risk of UBC in relation to 25(OH)D_3 levels and the first one analyzing this association in the context of the molecular features of the tumor and the biological effects of vitamin D, supported by parallel experimental in vitro evidence providing mechanistic explanations for the epidemiological findings. Other relevant strengths of this study are the high

Table 2. Odds ratios (ORs), 95% confidence intervals (CIs), and *P* values for the association between plasma 25-hydroxyvitamin D_3 [25(OH)D_3] and overall bladder cancer risk, risk of low- and high-grade non-muscle-invasive (NMIBC) and muscle-invasive bladder cancer (MIBC)*

		Overall				Low-grade NMIBC			High-grade NMIBC			MIBC		
Vitamin D status	25(OH) D_3, ng/mL	Controls	Cases	OR (95% CI)†	*P*	Cases	OR (95% CI)†	*P*	Cases	OR (95% CI)†	*P*	Cases	OR (95% CI)†	*P*
Sufficient	≥30.00	74	51	1.00 (referent)		27	1.00 (referent)		10	1.00 (referent)		9	1.00 (referent)	
Insufficient	20.00–29.99	227	229	1.40 (0.92 to 2.14)	.12	120	1.35 (0.81 to 2.26)	.25	38	1.14 (0.53 to 2.45)	.73	44	1.44 (0.66 to 3.14)	.37
Slightly deficient	15.00–19.99	212	219	1.63 (1.06 to 2.51)	.03	116	1.58 (0.94 to 2.66)	.08	46	1.68 (0.79 to 3.57)	.18	43	1.83 (0.83 to 4.02)	.14
Moderately deficient	10.00–14.99	255	280	1.67 (1.09 to 2.56)	.02	146	1.55 (0.92 to 2.59)	.096	49	1.40 (0.66 to 2.99)	.38	61	2.13 (0.98 to 4.65)	.06
Severely deficient	<10.00	260	346	1.83 (1.19 to 2.82)	.006	170	1.64 (0.97 to 2.76)	.07	62	1.55 (0.72 to 3.32)	.26	89	2.81 (1.29 to 6.13)	.009
P_{trend}					.004			.07			.18			.0005

* Ninety-five cases could not be assigned to any tumor/grade group because the paraffin block could not be retrieved. Likelihood-ratio test *P* for the pairwise comparisons between odds ratio of low-grade and high-grade NMIBC and MIBC among the severely deficient (vs sufficient) were .89, low-grade vs high-grade NMIBC; .19, low-grade NMIBC vs MIBC; and .24, high-grade NMIBC vs MIBC.

† Adjusted for age, sex, region, smoking status, and season of blood collection.

participation rates of cases and control subjects as well as their match for area of residence and similar age distribution. In addition, detailed information on several potential confounders (eg, smoking habits, body mass index) were considered. Even though the results of the present study are based on the concentration of plasma 25(OH)D_3 at a single time point, this measurement is considered a good biomarker of long-term vitamin D status because several studies have shown moderate to very high intraclass correlation coefficients (≥0.59), indicating a good concordance in 25(OH)D_3 across time points (37–39).

However, our study also has some limitations. Despite its large sample size, the assessment of associations in subgroups is limited by the smaller numbers of subjects in each subphenotype, especially when considering the association with tumor *FGFR3* mutation and expression. The association between 25(OH)D_3 levels and FGFR3 expression and tumor subtype needs to be confirmed in adequately sized independent series. Temporality should also be taken into account because the study is inherently retrospective and we cannot discard a reverse causality due to the carcinogenesis process. Although we hypothesized that, according to the mechanistic evidence provided here, the protective effect of 25(OH)D_3 should be more pronounced among patients with MIBC low-FGFR3 expressers, we cannot exclude that a cancer diagnosis may lead to a change in diet and outdoor habits, potentially influencing 25(OH)D_3 concentrations. Nevertheless, all present patients with UBC were incident cases, most of whom were in good general health at the time of diagnosis and were not malnourished or cachetic. Also, blood was drawn at time of diagnosis, and plasma levels of 25(OH)D_3 are considered reasonably consistent over time (37,39,40). Furthermore, the fact the association is more evident in a group molecularly defined would exclude the possibility of reverse causality. Importantly, our results are in line with previous evidence from both case–control and cohort studies at other cancer sites (5,6,14).

In summary, low plasma 25(OH)D_3 concentrations were found associated with an increased risk of UBC, and the effects of vitamin D may be stronger among smokers. Our data suggest that this risk is higher among those individuals with MIBC expressing low FGFR3 levels. The in vitro findings reported here lend support to the biological plausibility of this association. Our results need to be replicated in independent populations, and the benefits of vitamin D intake have to be conclusively assessed through a clinical trial.

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Notes

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