Title

Plasma 25-hydroxyvitamin D₃ and bladder cancer risk according to tumor stage and FGFR3 status

Authors

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Key words: Bladder cancer; 25-hydroxyvitamin D₃; case-control study; FGFR3; cancer sub-phenotypes
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Abstract

**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 sub-phenotypes.

**Methods:** Plasma concentrations of 25(OH)D$_3$ in 1,125 cases with UBC and 1,028 controls 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/grade, FGFR3 expression, and smoking status. Cell proliferation was measured in human UBC cell lines cultured with 1α,25-dihydroxyvitamin D$_3$.

**Results:** A significantly increased risk of UBC was observed among subjects presenting the lowest concentrations of 25(OH)D$_3$ [OR$_{adj}$=1.83, 95%CI 1.19-2.82, \( P=0.006 \)], showing a dose-response effect (\( P\)-trend=0.004). The association was stronger for patients with muscle-invasive tumors, especially among low-FGFR3 expressers [OR$_{adj}$=5.94, 95%CI 1.72-20.45, \( P=0.005 \)]. The biological plausibility of these associations is supported by the fact that, in vitro, 1α,25-dihydroxyvitamin D$_3$ up-regulates 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.
Vitamin D, or cholecalciferol, is a pro-hormone involved in bone biology that may also protect against a variety of cancers.(1-3). The most active vitamin D metabolite - 1α,25-dihydroxyvitamin D₃ [1α,25(OH)₂D₃, 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 D₃ [25(OH)D₃, 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)D₃ is null or controversial,(7-10). A recent paper on prostate cancer reported a significant association with lethal disease only,(11).

Urothelial bladder cancer (UBC) is an important public health issue due to 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)D₃ plasma levels and the risk of this disease. While in the ATBC nested case-control study of male smokers low levels of 25(OH)D₃ were associated with increased risk of UBC,(14), in the PLCO nested case-control study it was not,(15). 1α,25(OH)₂D₃ has been shown to inhibit proliferation and induce apoptosis of human bladder tumor cells in vitro and to reduce tumorigenesis in a N-methylnitrosourea-induced model of bladder cancer in rats,(16). Expression of vitamin D receptor (VDR) 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) harbor FGFR3 mutations in approximately 60% of cases and display 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)D₃ levels and risk of UBC in the Spanish Bladder Cancer/EPICURO Study (SBCS/EPICURO) and explore the molecular mechanisms involved therein. We found that treatment with 1α,25(OH)₂D₃ leads to the up-regulation of FGFR3 mRNA and protein in cultured UBC cells with low basal expression levels; these observations led to an analysis of the association of plasma 25(OH)D₃ levels with UBC sub-phenotypes defined according to tumor invasiveness/grade and FGFR3 mutational and expression status.
Methods

Study participants
Subjects came from the Spanish Bladder Cancer/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 1998-2001. A panel of expert pathologists classified homogeneously all cases according to the invasiveness (T) and grade (G). Controls 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, gender, 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 controls. A total of 1,219 cases (84% eligible) and 1,271 controls (88% eligible) agreed to participate in the study and were interviewed. Plasma from blood samples collected at diagnostic time was available from 1,130 cases and 1,038 controls.

Experimental procedures
Details on the quantification of 25(OH)D₃, cell proliferation and FGFR3 expression assays using UBC cell lines, and FGFR3 expression and mutational status analyses in tumoral tissue are specified in Supplementary methods.

Statistical analysis
Mann-Whitney U test was used to assess differences between cases and controls regarding median plasma concentrations of 25(OH)D₃. For the analysis of association between 25(OH)D₃ and UBC, logistic regression was applied to estimate odds ratios (OR) and their 95% confidence intervals (95%CI), comparing each category of low plasma 25(OH)D₃ concentration (20-29.99, 15-19.99, 10-14.99, <10 ng/ml) with the reference category (≥30 ng/ml). A basic model was adjusted for age at interview, gender, region, smoking status, and season of blood draw. Further adjustments were made for body mass index (BMI), alcohol and calcium intake as previously associated with vitamin D₃,(21-24), 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)D₃ concentration treated as a continuous variable. Because almost a quarter of the controls were admitted to the hospital with bone fractures, a sensitivity analysis was carried out excluding these controls. The risk of UBC was further evaluated according to smoking status, ever-smokers category being created by collapsing the categories of occasional, former and current smokers. Statistical interaction between smoking and 25(OH)D₃ was assessed by including an interaction term as the product of the median of each category of plasma 25(OH)D₃ by the never 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)D₃ was examined by type of tobacco (blond or black) among the ever smokers, as well. The analysis was also stratified by season of blood draw and the interaction between season and plasma 25(OH)D₃ was assessed.

Polytomous logistic regression models were applied to analyze the association between 25(OH)D₃ 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 ORs between case groups was tested using a likelihood ratio test comparing models with and without the OR constrained to be equal for the corresponding case groups. The polytomous logistic regression models were also applied to the stratified analysis for low/high FGFR3 expression and the presence/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 \( P \leq 0.05 \). Statistical analyses were performed using STATA/SE v.10.1. This study conforms to the guidelines of STROBE for observational studies.
Results

Effects of 1α,25(OH)2D3 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 while 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.(25).

Treatment with 1α,25(OH)2D3 induced a more epithelioid phenotype and formation of more compact colonies, consistent with findings reported in other cell types (Figure 1A). In the four UBC cell lines studied, 1α,25(OH)2D3 (10-100 nM) induced a growth arrest (Figure 1B). This was associated with the up-regulation of the CDK inhibitors p21CIP1 and p27KIP1 at the protein level (Figure 1C). As shown in Figure 1D-E, 1α,25(OH)2D3 also induced an upregulation of FGFR3 at the mRNA 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, growth inhibition; in addition, it leads to higher FGFR3 levels in cells with low basal expression.

Plasma 25(OH)D3 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 SBCS/EPICURO, both overall and according to molecular sub-phenotypes. Cases and controls were mostly men, with a high frequency of cigarette smokers (Table 1). Median concentrations of 25(OH)D3 were lower in cases than in controls (13.9 vs. 15.0 ng/ml; P=0.001) (Table 1); 73% of all individuals (75% of cases and 71% of controls) had less than 20 ng/ml (Supplementary Table 1). The distribution of cases by 25(OH)D3 status was similar in the three tumor sub-phenotypes examined (P=0.7; Supplementary Table 1).

After adjusting for age, gender, region, smoking status, and season of blood draw, decreasing concentrations of plasma 25(OH)D3 were found to be associated with increased risk of UBC (P-trend=0.004; Table 2). Individuals slightly (ORadj=1.63, 95%CI 1.06-2.51, P=0.025), moderately (ORadj=1.67, 95%CI 1.09-
and severely deficient (OR$_{adj}$=1.83, 95%CI 1.19-2.82, $P=0.006$) in vitamin D presented a $\geq 50\%$ increased risk of UBC when compared to individuals with sufficient levels. The results were not substantially changed after excluding the controls with bone fractures, adjusting for BMI (Supplementary Table 2), 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 ($P=0.003$) (Figure 2A). 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 2B).

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 1.29-6.13, $P=0.009$) was 1.7 times higher than the risk of low-grade NMIBC (OR$_{adj}$=1.64, 95%CI 0.97-2.76, $P=0.065$) (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 ($P=0.7$; Supplementary Table 1). Risk of UBC among subjects with deficient 25(OH)D$_3$ concentrations was slightly higher among FGFR3 mutated than wild-type tumors, though the differences were not statistically significant (Supplementary Table 3). 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) but this difference did not reach statistical significance ($P=0.1$).
A more detailed analysis revealed that low plasma concentrations of 25(OH)D₃ were associated with an increased risk of developing low FGFR3-expressing UBC but not of high FGFR3-expressing UBC. This association was more notable among those severely deficient in vitamin D (OR_{adj}=3.03, 95% CI 1.55-5.94, \( P=0.001; \) \( P\)-trend=0.0002) (Supplementary Table 3). Furthermore, in individuals with low levels of 25(OH)D₃, the risk of MIBC expressing low levels of FGFR3 was almost 6-fold higher than in those with sufficient levels (OR_{adj}=5.94, 95% CI 1.72-20.45, \( P=0.005\)). This association was not significantly different from that of low-(\( P=0.3\)) and high-grade NMIBC (\( P=0.1\)) (Figure 3; Supplementary Table 4), 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 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 an almost 2-fold higher risk than individuals with concentrations greater or equal than 30 ng/ml (sufficient status). This risk pattern was mainly observed for MIBC. Furthermore, while 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 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,\(^{(19,26)}\). 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,\(^{(27,28)}\). 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,\(^{(29)}\). 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 extends 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. While we cannot discard that the relationship with tobacco smoking could be due to chance because of the small sample size of the non-smoker group, it is in agreement with those of Mondul et al., 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,(30,31), where the incidence rates of UBC are among the highest worldwide,(12,13), and in many other Western world countries. The concentrations of 25(OH)D$_3$ found in the present study were similar to those of same-age individuals from other Southern European countries, though lower than those from the U.S.A. and Sweden. A potential explanation is that, in the latter countries, several food items are fortified with vitamin D,(32-34).

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 to the epidemiological findings. Other relevant strengths of this study are the high participation rates of cases and controls as well as their match for area of residence and similar age distribution. In addition, detailed information on several potential confounders (e.g., smoking habits, BMI) were considered too. 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 as several studies have shown moderate to very high intraclass correlation coefficients ($\geq$0.59) indicating a good concordance in 25(OH)D3 across time points,(35-37). However, our study also has some limitations. Despite its large sample size, the assessment of associations in subgroups is limited by the smaller numbers of subjects in each sub-phenotype, especially when considering the association with tumor FGFR3 mutation/expression. The association between 25(OH)D$_3$ levels and FGFR3 expression and tumor subtype needs to be confirmed in adequately sized independent series. Temporality should
also be taken into account since the study is inherently retrospective and we cannot discard a reverse causality due to the carcinogenesis process. While we hypothesized, according to the mechanistic evidence provided here, that the protective effect of 25(OH)D3 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)D3 concentrations. Nevertheless, all present patients with UBC were incident cases, most of who 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)D3 are considered reasonably consistent over time,(35,37,38). 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 in other cancer sites,(5,6,14).

In summary, low plasma 25(OH)D3 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.
References


Conflicts of interest

The authors declare no conflict of interest.

Notes

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Table 1. Characteristics of study participants.

Table 2. Odds ratios (OR), 95% confidence intervals (95% CI), and P for the association between plasma 25-hydroxyvitamin D₃ [25(OH)D₃] and overall bladder cancer risk, risk of low- and high-grade non-muscle invasive (NMIBC), and muscle-invasive bladder cancer (MIBC).

Figure 1. Vitamin D induces growth arrest and an up-regulation of p21, p27, and FGFR3 in UBC cells. (A) Phase contrast microscopy of MGH-U4 and RT112 cells treated with 1α,25(OH)₂D₃ (100 nM) or vehicle for 72 h. (B) 1α,25(OH)₂D₃ 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 1α,25(OH)₂D₃ at different time points. Tubulin was used as a loading control. (D) Western blot analysis showing changes in FGFR3 in MGH-U4 and RT112 cells treated with 1α,25(OH)₂D₃ at the indicated time points. (E) Quantitative RT-PCR analysis showing the up-regulation of FGFR3 mRNA levels in MGH-U4 and RT112 cells treated with 10 nM 1α,25(OH)₂D₃ (grey bars). Values were normalized to HPRT and referred to expression at time 0 (black bars). Comparisons with P < 0.05 are indicated with an asterisk.

Figure 2. Odds ratios (OR) and 95% confidence intervals (95%CI) for the association between plasma 25-hydroxyvitamin D₃ [25(OH)D₃] and bladder cancer risk (A) by smoking status and (B) by season of blood draw. Estimates are adjusted for age, gender, region, smoking status, and season of blood draw, when appropriate.

Figure 3. Odds ratios (OR) and 95% confidence intervals (95%CI) for the association between plasma 25-hydroxyvitamin D₃ [25(OH)D₃] levels and risk of low- and high-grade non-muscle invasive (NMIBC) and muscle invasive (MIBC) in relationship with tumor FGFR3 expression levels. Estimates are adjusted for age, gender, region, and smoking status.
**Supplementary Table 1.** Characteristics of study participants by plasma 25-hydroxyvitamin D$_3$ [25(OH)D$_3$] status.

**Supplementary Table 2.** Odds ratios (OR), 95% confidence intervals (95% CI), and $P$ for the association between plasma 25-hydroxyvitamin D$_3$ [25(OH)D$_3$] and bladder cancer risk.

**Supplementary Table 3.** Odds ratios (OR), 95% confidence intervals (95% CI), and $P$ for the association between plasma 25-hydroxyvitamin D$_3$ [25(OH)D$_3$] and risk of bladder cancer with FGFR3 mutational status and FGFR3 expression in tumoral tissue.

**Supplementary Table 4.** Odds ratios (OR), 95% confidence intervals (95% CI), and $P$ for the association between plasma 25-hydroxyvitamin D$_3$ [25(OH)D$_3$] and bladder cancer risk for NMIBC (low-grade; high-grade) and MIBC low-FGFR3 expressing tumors.
Table 1. Characteristics of study participants.

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<tr>
<td>High-grade NMIBC (TaG3/T1)</td>
<td>—</td>
<td>—</td>
<td>205</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>MIBC (≥T2)</td>
<td>—</td>
<td>—</td>
<td>246</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>FGFR3§</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>—</td>
<td>—</td>
<td>496</td>
<td>59</td>
<td></td>
</tr>
<tr>
<td>Mutated</td>
<td>—</td>
<td>—</td>
<td>340</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td>FGFR3 expression</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>—</td>
<td>—</td>
<td>396</td>
<td>59</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>—</td>
<td>—</td>
<td>271</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td>Plasma 25-hydroxyvitamin D3,</td>
<td>15.0 (10.0-21.2)</td>
<td></td>
<td>13.9 (9.0-19.9)</td>
<td></td>
<td>1.1x10^-3</td>
</tr>
<tr>
<td>median (interquartile range) (ng/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*For age and plasma 25-hydroxyvitamin D3, the P is from the Mann-Whitney U test. For all the other variables, the P is from the Chi-square test.

†BMI, body mass index. Two hundred forty-three controls and two hundred seventy-one cases had no information on height or weight or both.

‡Ninety-five cases could not be assigned to any T-G group because the paraffin block could not be retrieved.

NMIBC, non-muscle invasive bladder cancer. MIBC, muscle invasive bladder cancer.

§Two hundred eighty-nine cases did not yield polymerase chain reaction (PCR) product.

||Four hundred fifty-eight cases did not have immunohistochemistry staining for FGFR3.
Table 2. Odds ratios (OR), 95% confidence intervals (95% CI), and $P$ 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).

<table>
<thead>
<tr>
<th>Vitamin D status</th>
<th>25(OH)D$_3$, ng/ml</th>
<th>Overall</th>
<th>Low-grade NMIBC</th>
<th>High-grade NMIBC</th>
<th>MIBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sufficient</td>
<td>$\geq$ 30.00</td>
<td>27</td>
<td>1.00 (referent)</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>Insufficient</td>
<td>20.00-29.99</td>
<td>120</td>
<td>1.35 (0.81-2.26)</td>
<td>38</td>
<td>44</td>
</tr>
<tr>
<td>Slightly deficient</td>
<td>15.00-19.99</td>
<td>116</td>
<td>1.58 (0.94-2.66)</td>
<td>46</td>
<td>43</td>
</tr>
<tr>
<td>Moderately deficient</td>
<td>10.00-14.99</td>
<td>146</td>
<td>1.55 (0.92-2.59)</td>
<td>49</td>
<td>61</td>
</tr>
<tr>
<td>Severely deficient</td>
<td>&lt; 10.00</td>
<td>170</td>
<td>1.64 (0.97-2.76)</td>
<td>62</td>
<td>89</td>
</tr>
<tr>
<td>Trend</td>
<td></td>
<td>0.004</td>
<td>0.066</td>
<td>0.176</td>
<td>0.0005</td>
</tr>
</tbody>
</table>

*Adjusted for age, gender, region, smoking status, and season of blood draw.

Note: Ninety-five cases could not be assigned to any T-G group because the paraffin block could not be retrieved. Likelihood-ratio test $P$ for the pairwise comparisons between OR of low-grade and high-grade NMIBC and MIBC among the severely deficient (vs. sufficient) were: 0.891, low-grade vs. high-grade NMIBC; 0.191, low-grade NMIBC vs. MIBC; 0.242, high-grade NMIBC vs. MIBC.
Low-\textit{FGFR3} expression

<table>
<thead>
<tr>
<th>Group</th>
<th>OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low-risk NMIBC</td>
<td>2.83</td>
<td>[1.09; 7.33]</td>
</tr>
<tr>
<td>High-risk NMIBC</td>
<td>1.64</td>
<td>[0.51; 5.23]</td>
</tr>
<tr>
<td>MIBC</td>
<td>5.94</td>
<td>[1.73; 20.45]</td>
</tr>
</tbody>
</table>

High-\textit{FGFR3} expression

<table>
<thead>
<tr>
<th>Group</th>
<th>OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low-risk NMIBC</td>
<td>1.75</td>
<td>[0.74; 4.12]</td>
</tr>
<tr>
<td>High-risk NMIBC</td>
<td>0.59</td>
<td>[0.10; 3.35]</td>
</tr>
<tr>
<td>MIBC</td>
<td>0.85</td>
<td>[0.24; 2.99]</td>
</tr>
</tbody>
</table>

*Likelihood-ratio test \( p \) for the comparison between the odds ratio of MIBC in the low- and high-\textit{FGFR3} expression groups = 0.26