Title

Plasma 25-hydroxyvitamin D_3 and bladder cancer risk according to tumor stage and *FGFR3* status

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Key words: Bladder cancer; 25-hydroxyvitamin D_3; 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,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 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$(OH)D_3$ plasma levels and the risk of this disease. While in the ATBC 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 it was not,(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 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 $25(OH)D_3$ 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 $1\alpha,25(OH)_2D_3$ 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 $25(OH)D_3$ 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 $25(OH)D_3$, cell proliferation and *FGFR3* expression assays using UBC cell lines, and *FGFR3* expression and mutational status analyses in tumoral tissue are specified in Supplementary methods.

Statistical analysis

Mann-Whitney U test was used to assess differences between cases and controls 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 (OR) and their 95% confidence intervals (95%CI), comparing each category of low plasma $25(OH)D_3$ 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 D,(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 $25(OH)D_3$ 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 $25(OH)D_3$ was assessed by including an interaction term as the product of the median of each category of plasma $25(OH)D_3$ 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 $25(OH)D_3$ 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 $25(OH)D_3$ 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 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 $1\alpha,25(OH)_2D_3$ 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 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 $1\alpha,25(OH)_2D_3$ induced a more epithelioid phenotype and formation of more compact colonies, consistent with findings reported in other cell types (**Figure 1A**). In the four UBC cell lines studied, $1\alpha,25(OH)_2D_3$ (10-100 nM) induced a growth arrest (**Figure 1B**). This was associated with the up-regulation of the CDK inhibitors $p21^{CIP1}$ and $p27^{KIP1}$ at the protein level (**Figure 1C**). As shown in **Figure 1D-E**, $1\alpha,25(OH)_2D_3$ 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 $25(OH)D_3$ 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 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 $25(OH)D_3$ 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 $25(OH)D_3$ 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 $25(OH)D_3$ were found to be associated with increased risk of UBC (*P*-trend=0.004; **Table 2**). Individuals slightly (OR_{adj}=1.63, 95%CI 1.06-2.51, *P*=0.025), moderately (OR_{adj}=1.67, 95%CI 1.09-

2.56, *P*=0.019), and severely deficient (OR_{adj}=1.83, 95%CI 1.19-2.82, *P*=0.006) in vitamin D presented a >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*=$2x10^{-18}$). 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)D_3 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)D_3, 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 $25(OH)D_3$ 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.

References

1. Garland CF, Gorham ED, Mohr SB*, et al.* Vitamin D for cancer prevention: global perspective. Ann Epidemiol. 2009;19(7):468-83.

2. Giovannucci E. The epidemiology of vitamin D and cancer incidence and mortality: a review (United States). Cancer Causes Control. 2005;16(2):83-95.

3. Holick MF. Vitamin D deficiency. N Engl J Med. 2007;357(3):266-81.

4. Fedirko V, Bostick RM, Long Q*, et al.* Effects of supplemental vitamin D and calcium on oxidative DNA damage marker in normal colorectal mucosa: a randomized clinical trial. Cancer Epidemiol Biomarkers Prev. 2010;19(1):280-91.

5. Touvier M, Chan DSM, Lau R*, et al.* Meta-analyses of vitamin D intake, 25-hydroxyvitamin D status, vitamin D receptor polymorphisms and colorectal cancer risk. Cancer Epidemiol Biomarkers Prev. 2011;20(5):1003-16.

6. Yin L, Grandi N, Raum E*, et al.* Meta-analysis: Serum vitamin D and breast cancer risk. Eur J Cancer. 2010;46(12):2196-2205.

7. Helzlsouer KJ. Overview of the Cohort Consortium Vitamin D Pooling Project of Rarer Cancers. Am J Epidemiol. 2010;172(1):4-9.

8. Albanes D, Mondul AM, Yu K*, et al.* Serum 25-hydroxy vitamin D and prostate cancer risk in a large nested case-control study. Cancer Epidemiol Biomarkers Prev. 2011;20(9):1850-60.

9. Yin L, Raum E, Haug U*, et al.* Meta-analysis of longitudinal studies: Serum vitamin D and prostate cancer risk. Cancer Epidemiol. 2009;33(6):435-45.

10. Wolpin BM, Ng K, Bao Y*, et al.* Plasma 25-hydroxyvitamin D and risk of pancreatic cancer. Cancer Epidemiol Biomarkers Prev. 2012;21(1):82-91.

11. Shui IM, Mucci LA, Kraft P*, et al.* Vitamin D-related genetic variation, plasma vitamin D, and risk of lethal prostate cancer: a prospective nested case-control study. J Natl Cancer Inst. 2012;104(9):690-9.

12. Silverman D, Devesa S, Moore L*, et al.* Bladder cancer. In: Schottenfeld D, Fraumeni Jr J, (eds). *Cancer epidemiology and prevention*. 3rd ed. New York, NY: Oxford University Press; 2006, 1101–1127.

13. Samanic C, Kogevinas M, Dosemeci M*, et al.* Smoking and Bladder Cancer in Spain: Effects of Tobacco Type, Timing, Environmental Tobacco Smoke, and Gender. Cancer Epidemiol Biomarkers Prev. 2006;15(7):1348-1354.

14. Mondul AM, Weinstein SJ, Mannisto S*, et al.* Serum Vitamin D and Risk of Bladder Cancer. Cancer Res. 2010;70(22):9218-23.

15. Mondul AM, Weinstein SJ, Horst RL*, et al.* Serum vitamin D and risk of bladder cancer in the Prostate, Lung, Colorectal, and Ovarian (PLCO) Cancer Screening Trial. Cancer Epidemiol Biomarkers Prev. 2012;21(7):1222-1225.

16. Konety BR, Lavelle JP, Pirtskalaishvili G*, et al.* Effects of vitamin D (calcitriol) on transitional cell carcinoma of the bladder in vitro and in vivo. J Urol. 2001;165(1):253-8.

17. Sahin MO, Canda AE, Yorukoglu K*, et al.* 1,25 Dihydroxyvitamin D(3) receptor expression in superficial transitional cell carcinoma of the bladder: a possible prognostic factor? Eur Urol. 2005;47(1):52-7.

18. Luis NM, Lopez-Knowles E, Real FX. Molecular biology of bladder cancer. Clin Transl Oncol. 2007;9(1):5-12.

19. Hernandez S, Lopez-Knowles E, Lloreta J*, et al.* Prospective study of FGFR3 mutations as a prognostic factor in nonmuscle invasive urothelial bladder carcinomas. J Clin Oncol. 2006;24(22):3664-71.

20. Garcia-Closas M, Malats N, Silverman D*, et al.* NAT2 slow acetylation, GSTM1 null genotype, and risk of bladder cancer: results from the Spanish Bladder Cancer Study and meta-analyses. Lancet. 2005;366(9486):649-59.

21. van Dam RM, Snijder MB, Dekker JM*, et al.* Potentially modifiable determinants of vitamin D status in an older population in the Netherlands: the Hoorn Study. Am J Clin Nutr. 2007;85(3):755-61.

22. Jaaskelainen T, Knekt P, Marniemi J*, et al.* Vitamin D status is associated with sociodemographic factors, lifestyle and metabolic health. Eur J Nutr. 2012;in press.

23. Kinyamu HK, Gallagher JC, Rafferty KA*, et al.* Dietary calcium and vitamin D intake in elderly women: effect on serum parathyroid hormone and vitamin D metabolites. Am J Clin Nutr. 1998;67(2):342-8.

24. Looker AC, Pfeiffer CM, Lacher DA*, et al.* Serum 25-hydroxyvitamin D status of the US population: 1988-1994 compared with 2000-2004. Am J Clin Nutr. 2008;88(6):1519-27.

25. Jebar AH, Hurst CD, Tomlinson DC*, et al.* FGFR3 and Ras gene mutations are mutually exclusive genetic events in urothelial cell carcinoma. Oncogene. 2005;24(33):5218-25.

26. Tomlinson DC, Baldo O, Harnden P*, et al.* FGFR3 protein expression and its relationship to mutation status and prognostic variables in bladder cancer. J Pathol. 2007;213(1):91-8.

27. Palmer HG, Larriba MJ, Garcia JM*, et al.* The transcription factor SNAIL represses vitamin D receptor expression and responsiveness in human colon cancer. Nat Med. 2004;10(9):917-9.

28. Palmer HG, Gonzalez-Sancho JM, Espada J*, et al.* Vitamin D(3) promotes the differentiation of colon carcinoma cells by the induction of E-cadherin and the inhibition of beta-catenin signaling. J Cell Biol. 2001;154(2):369-87.

29. Pereira F, Barbachano A, Silva J*, et al.* KDM6B/JMJD3 histone demethylase is induced by vitamin D and modulates its effects in colon cancer cells. Hum Mol Genet. 2011;20(23):4655-65.

30. Gonzalez-Padilla E, Soria Lopez A, Gonzalez-Rodriguez E*, et al.* High prevalence of hypovitaminosis D in Medical Students in Gran Canaria. Canary Islands (Spain). Endocrinol Nutr. 2011;58(6):267-73.

31. Gonzalez-Molero I, Morcillo S, Valdes S*, et al.* Vitamin D deficiency in Spain: a population-based cohort study. Eur J Clin Nutr. 2011;65(3):321-8.

32. Lips P. Vitamin D deficiency and secondary hyperparathyroidism in the elderly: consequences for bone loss and fractures and therapeutic implications. Endocr Rev. 2001;22(4):477-501.

33. Melhus H, Snellman G, Gedeborg R*, et al.* Plasma 25-hydroxyvitamin D levels and fracture risk in a community-based cohort of elderly men in Sweden. J Clin Endocrinol Metab. 2010;95(6):2637-45.

34. Looker AC, Johnson CL, Lacher DA*, et al.* Vitamin D status: United States, 2001-2006. NCHS Data Brief. 2011(59):1-8.

35. Hofmann JN, Yu K, Horst RL*, et al.* Long-term variation in serum 25-hydroxyvitamin D concentration among participants in the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial. Cancer Epidemiol Biomarkers Prev. 2010;19(4):927-31.

36. Zeleniuch-Jacquotte A, Gallicchio L, Hartmuller V*, et al.* Circulating 25-hydroxyvitamin D and risk of endometrial cancer: Cohort Consortium Vitamin D Pooling Project of Rarer Cancers. Am J Epidemiol. 2010;172(1):36-46.

37. Meng JE, Hovey KM, Wactawski-Wende J*, et al.* Intraindividual Variation in Plasma 25-Hydroxyvitamin D Measures 5 Years Apart among Postmenopausal Women. Cancer Epidemiol Biomarkers Prev. 2012;21(6):916-924.

38. Saliba W, Barnett O, Stein N*, et al.* The longitudinal variability of serum 25(OH)D levels. Eur J Intern Med. 2012;23(4):e106-11.

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 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).

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$(OH)_2D_3$ (100 nM) or vehicle for 72 h. (**B**) 1α,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 1α,25$(OH)_2D_3$ 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$(OH)_2D_3$ 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$(OH)_2D_3$ (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 D_3 [25(OH)D_3] 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 D_3 [25(OH)D_3] 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.

	Controls (N = 1028)	%	Cases (N = 1125)	%	*P**
Age, median (range) (yrs)	66 (20-81)		68 (22-81)		$1.4x10^{-4}$
Gender					
Males	909	88	986	88	0.578
Females	119	12	139	12	
Region					
Barcelona	205	20	197	18	0.409
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	$1.9x10^{-23}$
Occasional smoker	82	8	45	4	
Former smoker	383	37	439	39	
Current smoker	273	27	484	43	
BMI (kg/m2)†					
<25	415	53	499	58	0.127
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)		$1.1x10^{-3}$

*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 yeld polymerase chain reaction (PCR) product.

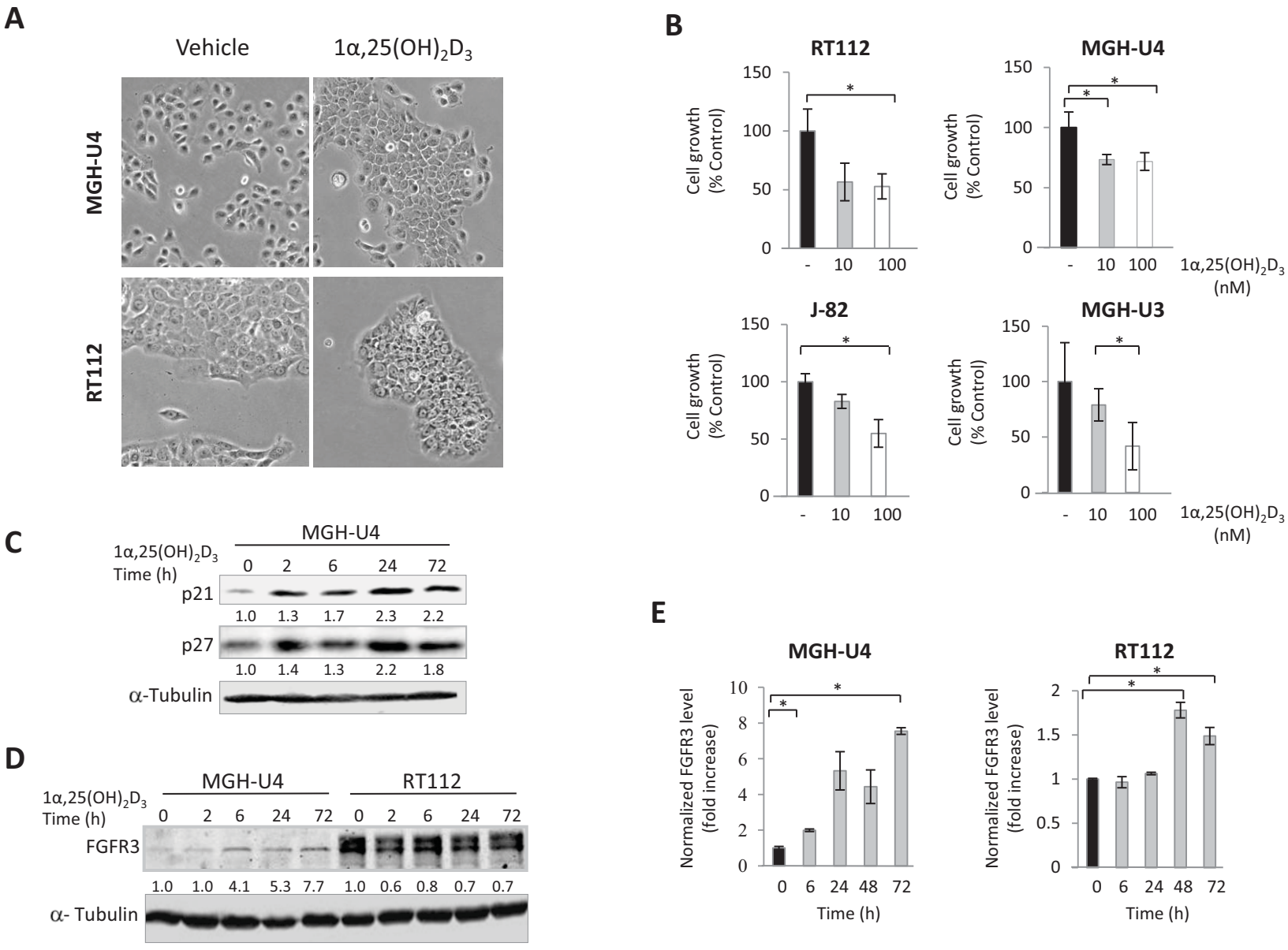
‖Four hundred fifty-eight cases did not have immunohistochemistry staining for FGFR3.

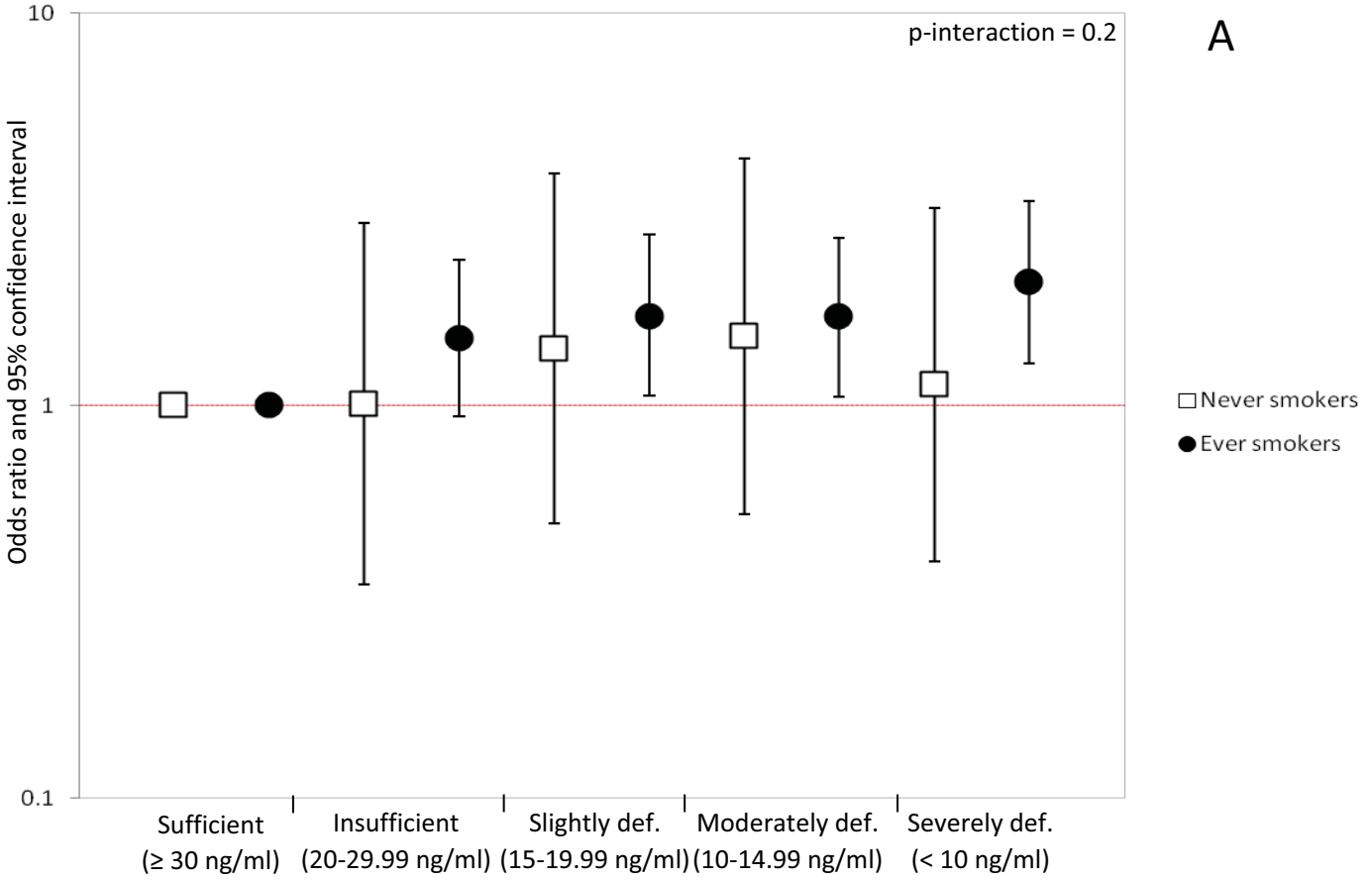
Table 2. Odds ratios (OR), 95% confidence intervals (95% CI), and *P* for the association between plasma 25-hydroxyvitamin D_3 [25(OH)D_3] and overall bladder cancer risk, risk of low- and high-grade non-muscle invasive (NMIBC), and muscle-invasive bladder cancer (MIBC).

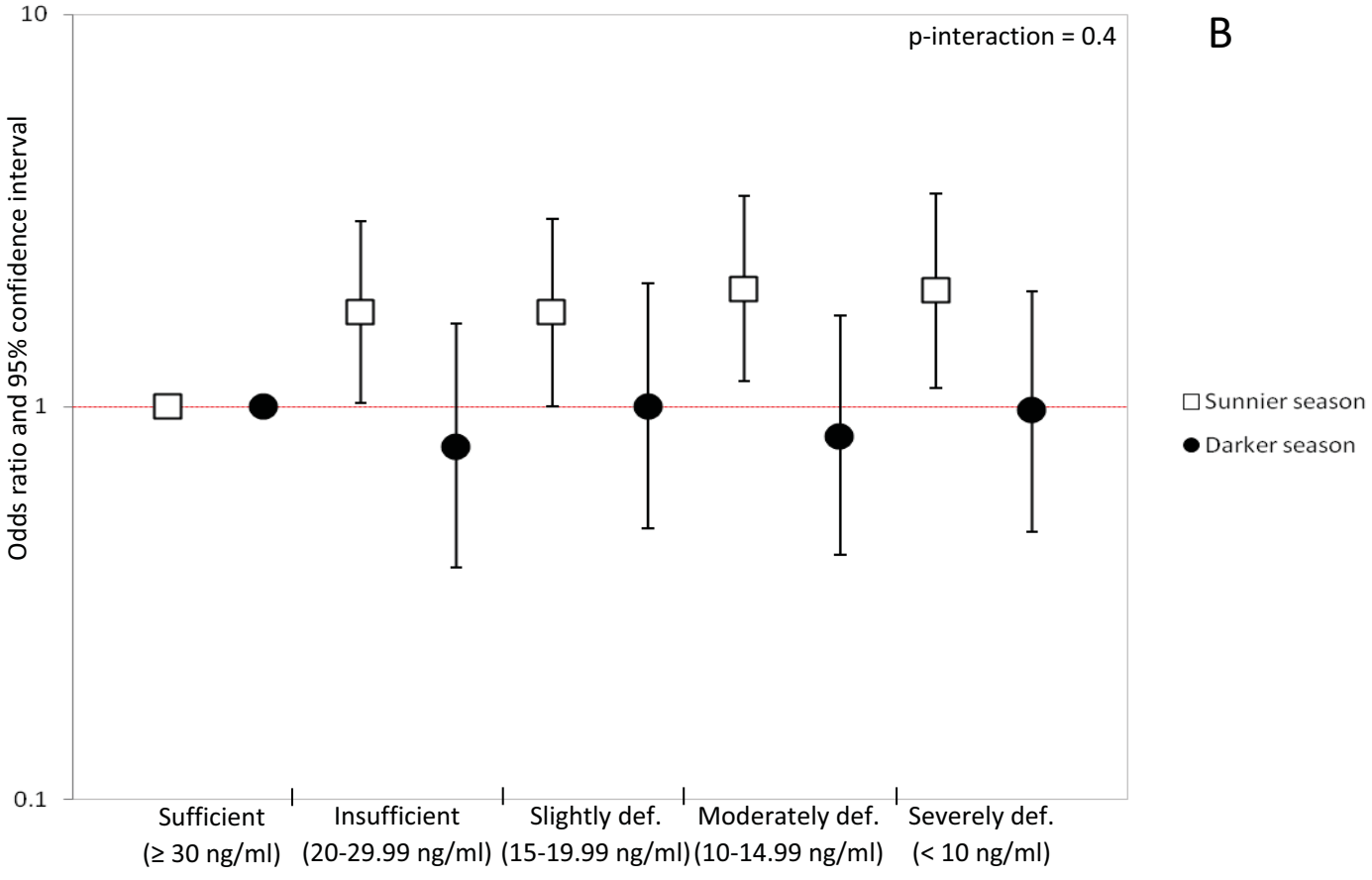
		Overall				Low-grade NMIBC			High-grade NMIBC			MIBC		
Vitamin D status	**25(OH)D_3, ng/ml**	**Co**	**Ca**	**OR (95%CI)***	***P***	**Ca**	**OR (95% CI)***	***P***	**Ca**	**OR (95% CI)***	***P***	**Ca**	**OR (95% CI)***	***P***
Sufficient	≥ 30.00	74	51	1.00 (referent)		27	1.00 (referent)		10	1.00 (referent)		9	1.00 (referent)	
Insufficient	20.00-29.99	227	229	1.40 (0.92-2.14)	0.118	120	1.35 (0.81-2.26)	0.245	38	1.14 (0.53-2.45)	0.730	44	1.44 (0.66-3.14)	0.365
Slightly deficient	15.00-19.99	212	219	1.63 (1.06-2.51)	0.025	116	1.58 (0.94-2.66)	0.084	46	1.68 (0.79-3.57)	0.180	43	1.83 (0.83-4.02)	0.135
Moderately deficient	10.00-14.99	255	280	1.67 (1.09-2.56)	0.019	146	1.55 (0.92-2.59)	0.096	49	1.40 (0.66-2.99)	0.384	61	2.13 (0.98-4.65)	0.057
Severely deficient	< 10.00	260	346	1.83 (1.19-2.82)	0.006	170	1.64 (0.97-2.76)	0.065	62	1.55 (0.72-3.32)	0.264	89	2.81 (1.29-6.13)	0.009
Trend					0.004			0.066			0.176			0.0005

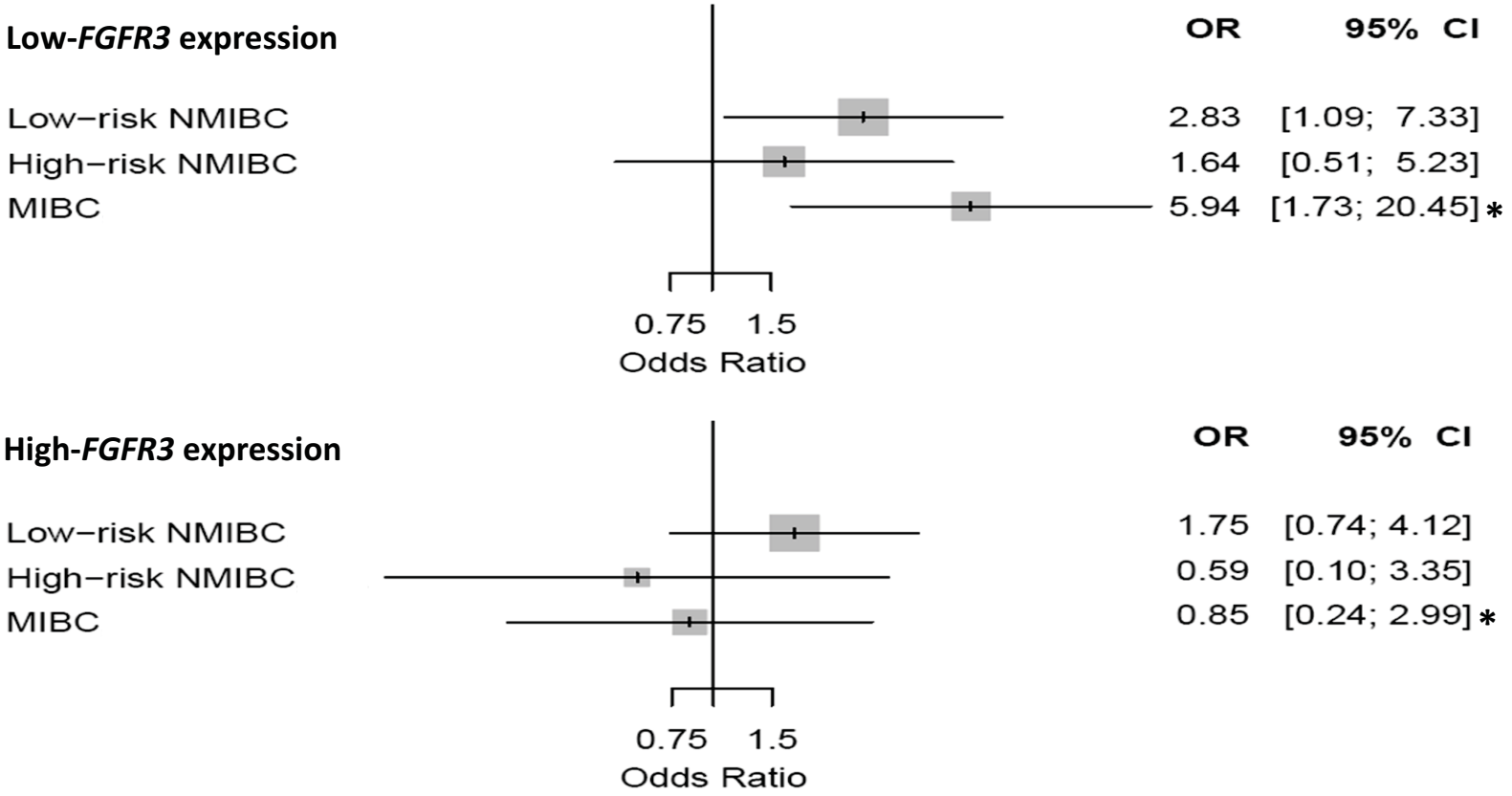
*Adjusted for age, gender, region, smoking status, and season of blood draw.

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.


A
Vehicle
1α,25(OH)2D3
MGH-U4
RT112
B
RT112
MGH-U4
J-82
MGH-U3
Cell growth (% Control)
150
100
50
0
-
10
100
1α,25(OH)2D3 (nM)
C
MGH-U4
1α,25(OH)2D3
Time (h)
0 2 6 24 72
p21
1.0 1.3 1.7 2.3 2.2
p27
1.0 1.4 1.3 2.2 1.8
α-Tubulin
D
MGH-U4
RT112
1α,25(OH)2D3
Time (h)
0 2 6 24 72 0 2 6 24 72
FGFR3
1.0 1.0 4.1 5.3 7.7 1.0 0.6 0.8 0.7 0.7
α- Tubulin
E
MGH-U4
RT112
Normalized FGFR3 level (fold increase)
10
8
6
4
2
0
2
1.5
1
0.5
0 6 24 48 72
Time (h)


A
p-interaction = 0.2
10
1
0.1
Odds ratio and 95% confidence interval
Never smokers
Ever smokers
Sufficient (≥ 30 ng/ml)
Insufficient (20-29.99 ng/ml)
Slightly def. (15-19.99 ng/ml)
Moderately def. (10-14.99 ng/ml)
Severely def. (< 10 ng/ml)


B
p-interaction = 0.4
10
1
0.1
Odds ratio and 95% confidence interval
Sunnier season
Darker season
Sufficient (≥ 30 ng/ml)
Insufficient (20-29.99 ng/ml)
Slightly def. (15-19.99 ng/ml)
Moderately def. (10-14.99 ng/ml)
Severely def. (< 10 ng/ml)



*Likelihood-ratio test *P* for the comparison between the odds ratio of MIBC in the low- and high-*FGFR3* expression groups = 0.26