A simple and sensitive method to detect vitamin D receptor expression in various disease models using stool samples

Yong-guo Zhang\textsuperscript{a}, Yinglin Xia\textsuperscript{a}, Jun Sun\textsuperscript{a,b,c,*}

\textsuperscript{a} Division of Gastroenterology and Hepatology, Department of Medicine, University of Illinois at Chicago, Chicago, IL 60612, USA
\textsuperscript{b} UIC Cancer Center, University of Illinois at Chicago, Chicago, IL 60612, USA
\textsuperscript{c} Department of Microbiology and Immunology, University of Illinois at Chicago, Chicago, IL 60612, USA

Received 8 January 2020; received in revised form 24 February 2020; accepted 6 March 2020
Available online 17 March 2020

\textbf{KEYWORDS}
Aging; Biomarker; Correlation; Infection; Inflammation; Microbiome; \textit{Salmonella}; Vitamin D deficiency

\textbf{Abstract}
Vitamin D receptor (VDR) executes the main biological functions of its ligand vitamin D. VDR/vitamin D plays critical roles in regulating host immunity, maintaining barrier functions, and shaping gut microbiome. Reduction of intestinal VDR has been reported in various diseases, including inflammatory diseases and colon cancer. However, it is always challenging to get biopsies to test the pathologic changes of VDR in intestine. In the current study, we reported a simple and sensitive quantitative PCR (qPCR) method to detect reduction of intestinal VDR using fecal samples. We validated this method in several experimental models, such as colitis, bacterial infection, and aging. We further correlated the qPCR data of VDR with the protein level of VDR in colon or serum 25 (OH)D3 in mice with different VDR status (VDR\textsuperscript{+/+}, VDR\textsuperscript{+/-}, and VDR\textsuperscript{-/-}). Our data indicate that the qPCR method to test VDR using fecal samples could detect the expression level of intestinal VDR in various diseases. Our study highlights the feasibility, sensitivity, and simplicity of a molecular method to study the status of VDR as a biomarker.

Copyright \textcopyright{} 2020, Chongqing Medical University. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
Background

Clinically, the serum 25(OH)D$_3$ is used as a biomarker for the vitamin D status of human body. However, the biological most active vitamin D is 1, 25(OH)$_2$D$_3$, which binds to vitamin D receptor (VDR). The biological function of 1, 25(OH)$_2$D$_3$ is executed via VDR, a nuclear receptor. Vitamin D/VDR plays critical roles regulating host immunity, maintaining barrier functions, and shaping gut microbiome. A fundamental relationship among vitamin D/VDR, gut microbiota, and genetic factors is essential not only for intestinal homeostasis, but also for the development of chronic inflammation, such as inflammatory bowel diseases (IBD). This knowledge could be used to develop intestinal VDR as a clinical biomarker for identifying patients who might benefit from currently available interventions, as well as for the eventual development of novel strategies for the prevention and treatment of human IBD and other chronic diseases. Reduction of intestinal VDR has been also reported in other diseases, such as colon cancer. However, it is always challenging to get biopsies to test the pathologic changes of VDR in intestine. We hypothesize that the fecal samples contain sufficient shed epithelial cells expressing VDR, which allow the detection by qPCR. In the current study, we reported a simple qPCR method to measure the mRNA level of VDR, using fecal samples. We validated this method in several experimental models, such as colitis, chronic infection, and aging.

Materials and methods

Animals

VDR$^{-/-}$ mice on a C57BL/6 background were obtained by breeding heterozygous VDR$^{+/+}$ mice. VDR$^{loxP}$ mice were originally reported by Dr. Geert Carmeliet. VDR$^{loxP}$ mice were obtained by crossing the VDR$^{loxP}$ mice with villin-cre mice (Jackson Laboratory, 004586), as we previously reported. Mice were provided with water ad libitum and maintained in a 12-h dark/light cycle. The animal work was approved by the UIC Office of Animal Care.

Colon tissues and fecal samples

The intestinal tissues and fecal samples were obtained from specific-pathogen-free C57BL/6 mice or mouse models used in previous studies, including a chronic Salmonella infection model, a dextran sulfate sodium (DSS) treated colitis model. Samples of aging mice were from 20 to 24 week-old C57BL/6 mice, and the control mice were 7–8 week-old.

Immunohistochemistry (IHC)

The immunohistochemistry was performed on paraffin-embedded tissue sections (4 μm). After preparation of the slides, as described previously, antigen retrieval was achieved by incubation of the slides for 15 min in the hot preheating sodium citrate (pH 6.0) buffer, and 30 min of cooling at room temperature. Endogenous peroxidases were quenched by incubating the slides in 3% hydrogen peroxide for 10 min, followed by three rinses with HBSS, and incubation for 1 h in 3% BSA +1% goat serum in HBSS to reduce nonspecific background. Primary antibodies VDR (1:300, Santa Cruz, Cat.No.SC-13133) was applied for overnight in a cold room. After three rinses with HBSS, the slides were incubated in secondary antibody (1:100, Jackson ImmunoResearch Laboratories, Cat.No.115-065-174) for 1 h at room temperature. After washing with HBSS for 10 min, the slides were incubated with vectastain ABC reagent (Vector Laboratories, Cat.No. PK-6100) for 1 h. After washing with HBSS for 5 min, color development was achieved by applying peroxidase substrate kit (Vector Laboratories, Cat.No. SK-4800) for 2–5 min, depending on the primary antibody. The duration of peroxidase substrate incubation was determined through pilot experiments and was then held constant for all of the slides. After washing in distilled water, the sections were counterstained with hematoxylin (Leica, Cat.No.3801570), dehydrated through ethanol and xylene, and cover-slipped using a permount (Fisher Scientific, Cat.No.SP15-100).

Fecal sample collection

The tubes for fecal sample collection were autoclaved and then irradiated with ultraviolet light to destroy the contaminating environmental bacterial DNA. Fresh fecal pellets were collected by holding the mouse in one hand, during which the mouse can defecate directly in 1.5 mL tube held in the other hand. About 3–4 fresh fecal pellets were collected from each mouse. Alternatively, individual mice were put in sterile cages, and fresh fecal pellets were collected from the cages. Tubes containing fecal pellets were stored in −80 °C freezer.

RNA extraction from fecal sample

Approximately 100 mg of frozen fecal pellet was added to a sterile 1.5 mL tube containing 0.5 mL TRIzol Reagent (Thermo Fisher Scientific, Cat.No.15596018), and homogenized with a pestle for 2–3 min. After homogenization, the slurry was poured into 1.5 mL tubes, which were centrifuged at 12,000×g for 5 min at 4 °C. The supernatant from each tube was transferred carefully to new sterile 1.5 mL tubes. To each tube, 0.3 mL of TRIzol Reagent and 0.3 mL chloroform were added, the tubes were shaken vigorously for 30 s, incubated for 5 min at 4 °C, and centrifuged at 12,000×g for 15 min at 4 °C. The aqueous phase from each tube was removed carefully without contamination from the interface and transferred to a fresh 1.5 mL tube. An equal volume of 70% ethanol was added, and the tubes were vortexed vigorously for 30 s. The mixed solution (700 μL) was added to an RNeasy minispin column (Qiagen, Cat.No.217004), and the columns were centrifuged at 10,000×g for 15 s at 4 °C. The remaining steps were performed according to the manufacturer’s instructions. Total RNA concentrations were determined by ultraviolet spectrophotometry, and the RNA samples were stored at −80 °C.
Mouse colonic epithelial cells were collected by scraping the tissue from the colon of the mouse, including the proximal and distal regions. Total RNA was extracted from mouse epithelial cells using TRIzol reagent (Invitrogen, Grand Island, NY, USA) by following company’s instructions. Per 5–10 × 10^6 of cells were lysed with 1 mL of TRIzol Reagent by repetitive pipetting, and then followed by 5-min incubation at room temperature to permit the complete dissociation of nucleoprotein complexes. Then 0.2 mL of chloroform per 1 mL of TRIzol Reagent was used. Samples were vortexed vigorously for 15 s followed by incubation of 2–3 min at room temperature. Samples were then subjected to centrifugation at no more than 12,000×g at 4 °C for 15 min. Upper aqueous phase was carefully transferred without disturbing the interphase into a new Eppendorf tube, and then precipitated using 0.5 mL of isopropl alcohol per 1 mL of TRIzol Reagent for 10 min at room temperature. Pellet after 10-min centrifugation at 12,000×g at 4 °C was washed with at least 1 mL of 75% ethanol per 1 mL of TRIzol Reagent used for the initial homogenization. After the centrifugation of 7500×g for 5 min at 4 °C, RNA pellet was air-dried for 5–10 min, and 40 ul of RNase-free water was added for resuspension by pipetting. The solution can be incubated at 55–60 °C for 10 min if the pellet is hard to dissolve. Total RNA concentrations were determined by ultraviolet spectrophotometry, and the RNA samples were stored at –80 °C.

**qPCR analysis**

RNA reverse transcription was performed using the iScript cDNA synthesis kit (Bio-Rad, Cat.No.170889) according to the manufacturer’s protocol. The RT cDNA reaction products were subjected to qPCR using Bio-Rad CFX 96 Real-time system and SYBR green supermix (Bio-Rad, Cat.No.170088). The optimal primer sequences were designed using Primer-BLAST or obtained from PrimerBank. The primer pairs used in this study were listed as follows: mouse β-actin, Forward 5′-TGTATACCACTGGGACGACA-3′ and Reverse 5′-CTGGGTCATCTTTTCACGGT-3′; mVDRF, Forward 5′-GAATGTGCGATCGTG-3′ and Reverse 5′-ATGCAGCAATCTCCATGAG-3′ (Table 1).

### Table 1 Real-time PCR primers.

| Primers name | Sequence          |
|--------------|------------------|
| mβ-actinF    | 5′-TGTATACCACTGGGACGACA-3′ |
| mβ-actinR    | 5′-CTGGGTCATCTTTTCACGGT-3′ |
| mVDRF        | 5′-GAATGTGCGATCGTG-3′   |
| mVDRR        | 5′-ATGCAGCAATCTCCATGAG-3′ |

**Western blot**

Mouse colonic epithelial cells were collected by scraping the proximal and distal regions of colon tissues. The harvested cells were sonicated in lysis buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, pH 8.0, 1% Triton X-100) with 0.2 mM sodium ortho-vanadate, and protease inhibitor cocktail. Cultured cells were rinsed twice with ice-cold HBSS, lysed in protein loading buffer (50 mM Tris, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol). After sonication, protein concentration was measured using the BioRad Reagent (BioRad, Cat.No. 500-0006). Equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and immunoblotted with primary antibodies: anti-Villin (Santa Cruz Biotechnology, SC-58897), and anti-VDR (Santa Cruz Biotechnology, SC-1313). Membranes probed with more than one antibody were stripped before re-probing. Visualization was performed by using ECL (Thermo Fisher Scientific, Cat.No. 32106).

**Serum 25-hydroxyvitamin D3**

25-Hydroxyvitamin D3 in mouse serum was measured using ELISA kit (Biomatik, Cat.No. EKL54383) according to the manufacturer’s protocol. Mouse serum (50 μL per well, 1:10 dilution) was added to each well, then followed by 50 μL of detection reagent A and 2-h incubation at 37 °C. After wash, 100 μL of detection reagent B working solution was added to each well and incubated for 1 h at 37 °C. Following complete washing, 90 μL of Substrate solution was added to each well. After 15–25 min incubation at 37 °C, 50 μL of Stop Solution was added. Then the samples were ready for absorbance measurement at 450 nm.

**Statistical analysis**

All the data are expressed as the mean ± SD. All statistical tests were 2-sided. The P values < 0.05 were considered statistically significant. Student’s t-test was used to compare the differences between two-groups samples. Pairwise correlation analyses and scatter plots were conducted among fold changes of VDR protein, serum vitamin D3, and VDR mRNA, using SAS version 9.4 (SAS Institute, Inc., Cary, NC, USA). Other statistical analyses were performed using GraphPad Prism 6 (GraphPad, Inc., San Diego, CA., USA).

**Results and discussion**

Establish a qPCR method to measure the VDR expression in feces

To establish a method to test VDR expression in feces, we first examined the total RNA extracted from mouse feces. Agarose gel electrophoresis in Fig. 1A showed that the RNA extracted from the fecal samples had high quality with the fragment length between 285 and 185 bands, suggesting the amount and quality of mammal RNAs were sufficient for the studies.
We then tested the mRNA expression of VDR in fecal samples from wild-type and VDR knockout (VDR\(^{-/-}\)) mice. No VDR mRNA expression was detected in fecal samples from VDR\(^{-/-}\) mice, whereas \(\beta\)-catenin expression was detectable in both VDR\(^{+/+}\) and VDR\(^{-/-}\) mice, suggesting the PCR method was specific to differentiate the VDR level in these mice (Fig. 1B).

We further examined the expression levels of VDR and \(\beta\)-catenin, using fecal samples from the intestinal epithelial VDR conditional knockout (VDR\(^{DIEC}\)) mice. Fecal samples from the VDR\(^{loxp}\) mice were used as a control group. We found significantly reduced VDR mRNA expression in VDR\(^{DIEC}\) mice, compared with the VDR\(^{loxp}\) mice (Fig. 1C). Because VDR\(^{DIEC}\) mice had specific VDR deletion in intestinal epithelial cells, feces had mixture of shed cells from other cell types. We could still detect some mRNA of VDR in feces of VDR\(^{DIEC}\) mice. However, we did not find the significant changes of \(\beta\)-catenin expression in feces of VDR\(^{DIEC}\) mice, compared with the VDR\(^{loxp}\) mice (Fig. 1C). Our data suggest that this established method is specific and sensitive.

### Reduced VDR expression in a colitis mouse model

VDR expression is known to be down-regulated in colons of patients with IBD, in both UC and CD, compared to normal colons\(^5,21\). In a DSS-induced colitis model, VDR was also down-regulated in colon (Fig. 2A). To validate the new method for VDR expression, we compared the expression profiles of Vdr gene from paired DSS-treated and control fecal samples. We were able to test the down-regulated VDR expression at the mRNA level in feces from the DSS-treated mice (Fig. 2B). We further found that VDR expression was also significantly down-regulated at the mRNA level in colon samples from DSS-treated mice (Fig. 2C). These observations in colon were consistent with the data using the fecal samples.
Using qPCR method to test the level of VDR in a Salmonella infection model

Chronic Salmonella infection led to the reduction of VDR in colon. Here, we showed less VDR expression in the Salmonella treated colon 4 days post infection (Fig. 3A). Comparing qPCR expression profiles of Vdr gene from paired Salmonella-treated and control feces, we found that Vdr expression was significantly down-regulated in feces from Salmonella infected mice (Fig. 3B) (\( n = 6 \), Student’s \( t \)-test, \( P < 0.05 \)). VDR mRNA level was also down-regulated in intestinal tissue samples from Salmonella infected mice (Fig. 3C) (Data are expressed as mean ± SD. \( n = 6 \), Student’s \( t \)-test, \( P < 0.05 \)). Again, these observations in colon tissue were consistent with the data using the fecal samples.

VDR level in the young and aging mice

VDR expression was significantly reduced over aging process. We further examined the expression levels of VDR in young (C57BL/6, 7–8 weeks) and aged (C57BL/6, 20–24 weeks) mice. Compared with younger mice, feces of aged mice significantly reduced the expression level of VDR mRNA. However, we did not find distinct changes of \( \beta \)-catenin expression between the young and aging mice (Fig. 4A) (Data are expressed as mean ± SD. \( n = 5 \), Student’s \( t \)-test, \( P < 0.05 \)).

Positive correlation differentiated by the VDR status and serum 25-hydroxyvitamin D3

Expression of colonic VDR protein was dramatically reduced in the VDR\(^{+/+}\) and VDR\(^{−/−}\) mice. Compared with wild-type VDR\(^{+/+}\) mice, our Western blot data showed the reduced
VDR protein in colon of VDR+/− mice and no-VDR protein in the whole-body VDR −/− mice (Fig. 4B Western blots of VDR protein). In the meantime, 25-hydroxyvitamin D3 levels in serum of VDR+/− mice and VDR −/− mice were significantly reduced, respectively, compared to the VDR+/+ mice (Fig. 4B ELISA data of 25-hydroxyvitamin D3). Thus, VDR expression in colon correlates with the serum level of 25-hydroxyvitamin D3 (Fig. 4C). The results showed that serum 25-hydroxyvitamin D3 and intestinal VDR were positively associated with Pearson correlation coefficient (P-values) (0.86, P = 0.0734). In Fig. 4D, correlation analysis also showed a positive correlation of intestinal VDR and fecal VDR (0.79, P = 0.0032). Serum 25-hydroxyvitamin D3 and fecal VDR were also positively correlated (0.62, P = 0.0110), as shown in Fig. 4E.

In summary, we have established a simple qPCR method to detect reduction of VDR using fecal samples from different experimental models. The qPCR data from feces are consistent with the pathological IHC data and PCR data of VDR in colon. A positive correlation was differentiated by VDR status among serum 25-hydroxyvitamin D3, intestinal VDR protein, and fecal VDR mRNA.

We recognize the limit of current study. In the future study, we plan to examine the samples from patients with vitamin D deficiency and chronic diseases to further validate our method.

Conclusion

Our data indicate that the qPCR method to test VDR using fecal samples could detect the reduction of VDR at the mRNA level. We highlight the feasibility, sensitivity, and simplicity of a molecular method to determine the status of VDR as a biomarker in various diseases, such as colitis, bacterial infection, and aging.

Ethics approval and consent to participate

All animal studies were performed following ACC guidelines at the University of Illinois at Chicago (UIC), IL, USA.

Consent for publication

Not applicable.

Availability of data and material

Data and material will be available by request.

Authors contribution

Yong-guo Zhang performed qPCR, IHC, and detailed analysis, prepared for figures and draft, and analyzed the data. Yinglin Xia directed the project for the statistical analysis of data. Jun Sun obtained funds, designed the study, and directed the project. All authors contributed to the writing of the manuscript.

Conflict of Interests

The authors declare no conflict of interest.

Funding

We would like to acknowledge the support from UIC Cancer Center, the NIDDK grant R01DK105118 and R01DK114126 to Jun Sun.

Acknowledgements

We would like to thank Jason Xia for helping with proofreading.

References

1. Sun J. Dietary vitamin D, vitamin D receptor, and microbiome. *Curr Opin Clin Nutr Metab Care*. 2018;21(6):471–474.
2. Shang M, Sun J. Vitamin D/VDR, probiotics, and gastrointestinal diseases. *Curr Med Chem*. 2017;24(9):876–887.
3. Clark A, Mach N. Role of vitamin D in the hygiene hypothesis: the interplay between vitamin D, vitamin D receptors, gut microbiota, and immune response. *Front Immunol*. 2016;7:e627.
4. Nielsen OH, Hansen TI, Gubatan JM, Jensen KB, Rejnmark L. Managing vitamin D deficiency in inflammatory bowel disease. *Frontline Gastroenterol*. 2019;10(4):394–400.
5. Wu S, Zhang YG, Lu R, et al. Intestinal epithelial vitamin D receptor deletion leads to defective autophagy in colitis. *Gut*. 2015;64(7):1082–1094.
6. Bakke D, Sun J. Ancient nuclear receptor VDR with new functions: microbiome and inflammation. *Inflamm Bowel Dis*. 2018;24(6):1149–1154.
7. Bakke D, Chatterjee I, Agrawal A, Dai Y, Sun J. Regulation of microbiota by vitamin D receptor: a nuclear weapon in metabolic diseases. *Nucl Receptor Res*. 2018;5,e101377.
8. Schulten HJ, Bangash M, Karim S, et al. Comprehensive molecular biomarker identification in breast cancer brain metastases. *J Transl Med*. 2017;15(1),e269.
9. Meerkerk S, Seamons A, Maggio-Price L, Paik J. Protective links between vitamin D, inflammatory bowel disease and colon cancer. *World J Gastroenterol*. 2016;22(3):933–948.
10. Jeon SM, Shin EA. Exploring vitamin D metabolism and function in cancer. *Exp Mol Med*. 2018;50(4):1–14.
11. Sun J. The role of vitamin D and vitamin D receptors in colon cancer. *Clin Transl Gastroenterol*. 2017;8(6),e103.
12. Zhang YG, Wu S, Lu R, et al. Tight junction CLDN2 gene is a direct target of the vitamin D receptor. *Sci Rep*. 2015;5,e10642.
13. Van Cromphaut SJ, Dewerchin M, Hoenderop JG, et al. Duodenal calcium absorption in vitamin D receptor-knockout mice: functional and molecular aspects. *Proc Natl Acad Sci U S A*. 2001;98(23):13324–13329.
14. Lin Z, Zhang YG, Xia Y, Xu X, Jiao X, Sun J. *Salmonella enteritidis* effector AvrA stabilizes intestinal tight junctions via the JNK pathway. *J Biol Chem*. 2016;291(52):26837–26849.
15. Lu R, Bosland M, Xia YL, Zhang YG, Kato I, Sun J. Presence of Salmonella AvrA in colorectal tumor and its precursor lesions in mouse intestine and human specimens. Oncotarget. 2017;8(33):55104–55115.

16. Wu S, Yoon S, Zhang YG, et al. Vitamin D receptor pathway is required for probiotic protection in colitis. Am J Physiol Gastrointest Liver Physiol. 2015;309(5):G341–G349.

17. Zhang YG, Lu R, Xia YL, et al. Lack of vitamin D receptor leads to hyperfunction of claudin-2 in intestinal inflammatory responses. Inflamm Bowel Dis. 2019;25(1):97–110.

18. Wu S, Lu R, Zhang YG, Sun J. Chronic Salmonella infected mouse model. J Vis Exp. 2010;39:e1947.

19. Wu S, Liao AP, Xia Y, et al. Vitamin D receptor negatively regulates bacterial-stimulated NF-kappaB activity in intestine. Am J Pathol. 2010;177(2):686–697.

20. Zhang YG, Zhu X, Lu R, et al. Intestinal epithelial HMGB1 inhibits bacterial infection via STAT3 regulation of autophagy. Autophagy. 2019;15(11):1935–1953.

21. Liu W, Chen Y, Golan MA, et al. Intestinal epithelial vitamin D receptor signaling inhibits experimental colitis. J Clin Invest. 2013;123(9):3983–3996.

22. Tong T, Liu ZH, Zhang H, et al. Age-dependent expression of the vitamin D receptor and the protective effect of vitamin D receptor activation on H2O2-induced apoptosis in rat intervertebral disc cells. J Steroid Biochem Mol Biol. 2019;190:126–138.

23. Campolina-Silva GH, Maria BT, Mahecha GAB, Oliveira CA. Reduced vitamin D receptor (VDR) expression and plasma vitamin D levels are associated with aging-related prostate lesions. Prostate. 2018;78(7):532–546.