Journal of Steroid Biochemistry & Molecular Biology xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry & Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Colonic transcriptional response to 1α,25(OH)$_2$ vitamin D$_3$ in African- and European-Americans

Dereck Alleyne[a,1], David B. Witonsky[b,1], Brandon Mapes[a], Shigeki Nakagome[b], Meredith Sommars[a], Ellie Hong[a], Katy A. Muckala[a], Anna Di Rienzo[b], Sonia S. Kupfer[a,*]

[a] University of Chicago, Department of Medicine, Section of Gastroenterology, Chicago, IL, United States
[b] University of Chicago, Department of Human Genetics, Chicago, IL, United States

ARTICLE INFO

Article history:
Received 9 December 2016
Received in revised form 30 January 2017
Accepted 2 February 2017
Available online xxx

Keywords:
Vitamin D
Gene transcription
Expression quantitative trait loci
Colorectal cancer
Health disparities

ABSTRACT

Colorectal cancer (CRC) is a significant health burden especially among African Americans (AA). Epidemiological studies have correlated low serum vitamin D with CRC risk, and, while hypovitaminosis D is more common and more severe in AA, the mechanisms by which vitamin D modulates CRC risk and how these differ by race are not well understood. Active vitamin D (1α,25(OH)$_2$D$_3$) has chemoprotective effects primarily through transcriptional regulation of target genes in the colon. We hypothesized that transcriptional response to 1α,25(OH)$_2$D$_3$ differs between AA and European Americans (EA) irrespective of serum vitamin D and that regulatory variants could impact transcriptional response. We treated *ex vivo* colon cultures from 34 healthy subjects (16 AA and 18 EA) with 0.1 μM 1α,25(OH)$_2$D$_3$ or vehicle control for 6 h and performed genome-wide transcriptional profiling. We found 8 genes with significant differences in transcriptional response to 1α,25(OH)$_2$D$_3$ between AA and EA with definitive replication of inter-ethnic differences for uridine phosphorylase 1 (*UPP1*) and zinc finger-SWIM containing 4 (*ZSWIM4*). We performed expression quantitative trait loci (eQTL) mapping and identified response *cis*-eQTLs for *ZSWIM4* as well as for histone deacetylase 3 (*HDAC3*), the latter of which showed a trend toward significant inter-ethnic differences in transcriptional response. Allele frequency differences of eQTLs for *ZSWIM4* and *HDAC3* accounted for observed transcriptional differences between populations. Taken together, our results demonstrate that transcriptional response to 1α,25(OH)$_2$D$_3$ differs between AA and EA independent of serum 25(OH)D levels. We provide evidence in support of a genetic regulatory mechanism underlying transcriptional differences between populations for *ZSWIM4* and *HDAC3*. Further work is needed to elucidate how response eQTLs modify vitamin D response and whether genotype and/or transcriptional response correlate with chemopreventive effects. Relevant biomarkers, such as tissue-specific 1α,25(OH)$_2$D$_3$ transcriptional response, could identify individuals likely to benefit from vitamin D for CRC prevention as well as elucidate basic mechanisms underlying CRC disparities.

1. Introduction

Colorectal cancer (CRC) is a significant health burden worldwide but especially among African Americans (AA) who have the highest CRC incidence and mortality of all US populations [1–3]. Multiple lines of evidence show that vitamin D protects against CRC [4–6]. Active vitamin D (1α,25(OH)$_2$D$_3$) is a steroid hormone with direct transcriptional effects mediated through the vitamin D receptor (VDR) [7]. Transcriptome-wide studies have identified thousands of differentially expressed (DE) genes of which many are primary targets of the VDR and show cell type-specificity [7,8]. 1α,25(OH)$_2$D$_3$ is thought to exert its chemoprotective effects in the colon through inhibition of proliferation and induction of differentiation and apoptosis [9–13], although additional mechanisms are likely and remain understudied. Inactive 25-hydroxyvitamin D (25(OH)D), which is measured in the serum, is converted to 1α,25(OH)$_2$D$_3$ locally in the colonic epithelium [7,8], and also likely contributes to anti-tumor effects. Epidemiological studies have found an inverse correlation between serum hypovitaminosis D with CRC risk [6,14], and this effect was attenuated in AA due to lower 25(OH)D levels due to dark skin pigmentation, which inhibits the local synthesis of vitamin D in the

* Corresponding author at: 900 East 57th Street, MB#9, Chicago, IL 60637, United States.
E-mail address: skupfer@medicine.bsd.uchicago.edu (S.S. Kupfer).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.jsbmb.2017.02.001
0960-0760/

skin [15,16]. Higher 25(OH)D levels have not only been associated with reduced risk of developing CRC, but was also shown to improve survival in patients with CRC [17]. While hypovitaminosis D is more common and more severe in AA [18], the mechanisms by which vitamin D modulates CRC risk and how these differ by race are not well understood. Answering these questions is likely to have an important impact on understanding and addressing CRC disparities.

Despite strong epidemiological data of an inverse relationship between vitamin D status and CRC, reanalysis of the Women's Health Initiative of calcium and vitamin D supplementation [19] and a recent intervention trial did not support a protective role for vitamin D supplementation on CRC incidence or recurrence of colorectal neoplasia (adenomas or CRC), respectively [20]. In the intervention trial, even when stratifying by serum 25(OH)D levels before and after treatment, there was no benefit of vitamin D supplementation. There are several potential explanations for these unexpected findings [21]. The dose administered was low (1000 IU) and higher doses are likely required for chemoprevention in the colon [14,22]. Subjects participating in this study had a mean baseline 25(OH)D levels of 24 ng/ml that could have been too high to observe a beneficial effect for supplementation [23]. There were relatively few AA included in the study, so it is not possible to generalize the results for this high-risk population. Another explanation is that there could be inter-individual and inter-ethnic differences in responses to the effects of active vitamin D irrespective of vitamin D serum levels which could impact treatment response. Identification of more biologically relevant endpoints of treatment response, such as tissue-specific transcriptional response, could help personalize chemoprevention and identify individuals most likely to benefit from treatment.

Previous studies of responses to glucocorticoids and 1α,25$(OH)_2D_3$ in peripheral blood [19,20] and monocytes [21] have demonstrated inter-individual and inter-ethnic differences in transcriptional and cellular responses and have characterized the genetic architecture of treatment-specific effect [20–26]. Taken together, these findings provide rationale for testing the hypothesis that there are inter-individual and inter-ethnic differences in responses to 1α,25$(OH)_2D_3$ in human colon and that genetic variants contribute to these differences. To do this, we utilized an *ex vivo* system in which colon biopsies were maintained in short-term culture and treated with 1α,25$(OH)_2D_3$ or vehicle in parallel [27]. Use of primary tissue is advantageous because it is not transformed, comes from normal colon, the target tissue for chemoprevention, and can be obtained from diverse subjects. In addition, treatment of biopsies from the same individual with vitamin D and vehicle in parallel controls for confounding variables that could impact transcriptional response. In this study, we identified genes with differences in transcriptional response to 1α,25$(OH)_2D_3$ between AA and EA in normal colon. eQTL mapping of transcriptional response yielded insights into the contribution of genetic variants to inter-individual and inter-ethnic response to active vitamin D treatment.

2. Materials and methods

2.1. Human subjects

Healthy individuals undergoing outpatient screening colonoscopy at the University of Chicago Medical Center were recruited. Two cohorts were recruited (hereafter referred to as "discovery" and "validation" cohorts). For the discovery cohort, a total of 34 subjects (16 AA and 18 EA) were recruited between May 2012 and February 2013. For the validation cohort, a total of 27 subjects (12 AA and 15 EA) were recruited between September 2014 and January 2015. Demographic data including self-identification as AA or EA, age and gender were collected. Peripheral blood was obtained at consent and serum 25$(OH)D_3$ levels were measured in the Clinical Chemistry laboratory at the University of Chicago. During colonoscopy, 4 biopsies were obtained using standard forceps (Boston Scientific; Natick, MA) in the recto-sigmoid colon approximately 20 cm from the anal verge and immediately placed in transport media containing antibiotics. Biopsies were transported to the laboratory for further processing as previously described [27]. This study was approved by the Institutional Review Board at the University of Chicago, and all subjects signed informed consent prior to data and sample collection.

2.2. Organ culture and treatment

We used an *ex vivo* organ culture protocol previously described [27]. Briefly, colon biopsies were washed and cut into 1–2 mm pieces in cold phosphate-buffered saline (PBS). The pieces from each biopsy were placed on a cell strainer and placed into a 6-well culture dish, containing culture media that partially submerged the tissue. Two biopsies each were treated in parallel with 0.1 μM 1α,25$(OH)_2D_3$ or vehicle (ethanol) for 6 h. This dose was selected based on a number of previous studies of vitamin D response [8,27,28]. The 6 h treatment point had the greatest number of DE genes in response to vitamin D treatment in a pilot study. The dish was incubated at 37 °C in 5% CO_2 and 95% air. After treatment, biopsies pieces were washed in cold PBS and immediately submerged in RNAlater (Ambion Inc; Austin, TX) and placed at 4 °C.

2.3. Transcriptional response profiling

Genome-wide transcriptional profiling was performed on samples from the discovery cohort after 6 h of treatment with vitamin D or vehicle. Profiling was done in 2 batches from 24 and 12 subjects, respectively. The second batch included 2 subjects from batch 1 and concordance in gene expression across batches was high (**Supplementary** Fig. 1). Total RNA was extracted from each sample using the QIAgen RNeasy Plus mini kit (Qiagen; Cat. no 74134), and RNA from replicate treatments was pooled. Total RNA was reverse transcribed into cDNA, labeled, hybridized to Human HT-12 v3 Expression Beadchips (Illumina; San Diego, CA) and scanned at the University of Chicago Functional Genomics Core facility. Low-level microarray analyses were performed using the Bioconductor R package, LUMI [29]. Probes were annotated by mapping RNA sequences to RefSeq (GRCh37) using BLAT. Probes mapped to multiple genes or containing one or more SNPs identified by the 1000 Genomes Project were discarded. Further, we performed variance stabilizing transformations and probes indistinguishable from background fluorescence levels were also discarded and quantile normalization was done across all arrays. 12,175 probes remained after quality control for downstream analysis.

The Bioconductor R package LIMMA [30] was used to perform linear regression at each gene, with vitamin D treatment as the variable of interest. Covariates including ancestry, age, gender, serum 25$(OH)D_3$ levels and principal components (PCs) 1&2 of the expression data (to account for unmeasured variation in the expression data) were included in the regression model. FDR was estimated using the Q-value function in R [31]. Gene set enrichment of all DE genes was performed using DAVID [32]. To assess the difference in genome-wide transcriptional response between populations, we used LIMMA to fit a linear regression model at each gene with LFC regressed on race including the covariates age, gender, serum 25$(OH)D_3$ levels and 2 PCs of the expression data. An adjusted p-value from LIMMA was used to assess significance after correction for multiple testing.

Please cite this article in press as: D. Alleyne, et al., Colonic transcriptional response to 1α,25$(OH)_2$ vitamin D_3 in African- and European-Americans, J. Steroid Biochem. Mol. Biol. (2017), http://dx.doi.org/10.1016/j.jsbmb.2017.02.001