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Epigenetic regulation of vitamin D converting enzymes☆

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ABSTRACT

While 25-OH-D3 serum levels in humans undergo a large seasonal variation, 1,25-(OH)2-D3 is regulated within a narrow range. We speculate that in addition to the known genomic and nongenomic regulation there could be further epigenetic mechanisms involved.

We annotated the human CYP27B1 (α-1-hydroxylase) and CYP24A1 (24-hydroxylase) genes for CpG islands and sequenced these in bisulfite treated DNA extracted of peripheral blood lymphocytes from 384 individuals. 40 CpG sites could be analyzed, of these 15 in CYP27B1 and 25 in CYP24A1. The average methylation ratio (MR) in CYP27B1 was 11% (s.d. 5%) with the highest ratio observed in exon 1 (38%). CYP24A1 showed only a 6.5% MR (s.d. 5%). Neither CYP24A1 nor CYP27B1 MR correlated with season of examination date nor with current 25-OH-D3 and 1,25-(OH)2-D3 serum levels except of a weak association of three consecutive CYP27B1 CpG sites and 25-OH-D3 levels.

In summary, human PBLs showed only weak methylation in the upstream region of CYP27B1 and none in CYP24A1. As PBLs represent an heterogeneous pool of cells, a further analysis of the seasonal methylation pattern in B or T cell subsets (or other tissues like liver or kidney) is warranted including an extended coverage of the CYP27B1 promotor.

1. Introduction

Epigenetics is usually described as the heritable change in gene function that occurs without a change in the nuclear DNA sequence [1]. In addition to RNA-associated silencing and histone modification, the main epigenetic mechanism in eukaryotes is DNA methylation at cytosine residues, especially of CpG nucleotides (where p denotes a phosphate group) that are enriched in DNA regions of less than 0.5 kb. Such CpG islands are usually clustered around the 5′ regulatory region of genes and may affect the transcriptional regulation of these genes. Typically, CpG islands are unmethylated while methylation is frequently associated with gene inactivation [1].

Vitamin D is activated and degraded through the actions of cytochrome P450 hydroxylase enzymes [2]. While the synthesis to the active hormone 1,25-dihydroxyvitamin D3 is catalyzed by α-1 hydroxylase (CYP27B1), inactivation of 1,25-dihydroxyvitamin D3 occurs by C23/C24 oxidation pathways that are catalyzed by CYP24A1. These hydroxylases are regulated by two mechanisms either a rapid response involving protein kinase C and mitogen-activated protein kinase (MAPK) pathway [2], or by binding of 1,25-dihydroxyvitamin D3 to the vitamin D receptor (VDR) and the subsequent interaction of the VDR-1,25-D3 complex with its heterodimer partner retinoid-X-receptor and associated coactivators. Methylation as a third active mechanisms has also been reported [3–7] and could operate on a more intermediate time scale [8].

As vitamin D supplements have been associated with allergy in earlier studies [9,10] we were interested in any genetic abnormalities of the vitamin D metabolisms. As genomic variants, however, showed only weak or no association so far with serum levels of 25-OH-D3 and 1,25-(OH)2-D3 [11,12] we determined the methylation rate (MR) of the main vitamin D converting enzymes and examined its dependency of environmental conditions.

2. Methods

The German Asthma Family Study collected between 1995 and 2001 affected sib pairs in 26 paediatric centres in Germany and Sweden for a two-stage genome-wide linkage scan. In these families at least two children were required with confirmed clinical asthma. Unaffected siblings were also sampled if they were at least 6 years old and eligible for pulmonary function testing. Families being tested year-round with a slight preponderance of January

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and a deficit of July due to logistic reasons. Each study participant including the children signed a consent form. All study methods were approved by the ethics commission of "Ärztekammer Nordrhein-Westfalen".

25-OH-D3 was determined with an enzymatic immunoassay (OCTEIA 25-Hydroxy Vitamin D kit, Immunodiagnostic Systems IDS, Frankfurt, Germany). 1,25-OH2-D3 was determined by immunoextraction followed by an enzyme-immunoassay (OCTEIA 1,25-Hydroxy Vitamin D kit, Immunodiagnostic Systems IDS, Frankfurt, Germany). 25-OH-D3 values reported are the mean of a duplicate analysis while due to the limited serum availability only single assays have been performed for 1,25-OH2-D3.

DNA was isolated from peripheral white blood cells using Qiamp (Qiagen, Germany) or Puregene isolation kits (Gentra Systems, Minneapolis, MN, USA) before being stored at −20 °C. For the present study DNA was thawed and bisulfite treated with the Epi-Tect Bisulfite Kit (Qiagen, Hilden) according to the manufacture's instruction. This converted cytosine residues to uracil, but left 5-methylcytosine residues unaffected.

In the mouse, two CpG islands have already been identified in CYP24A1 [3]. A bioinformatics analysis using EMBOSS CpGPlot of the human sequence extended by 1 kb to both sides showed four islands while the UCSC Genome Browser highlighted only one in the 5′ upstream promotor. In CYP27B1 2 islands were identified covering exons 2–4 while the UCSC genome browser version again combined both regions into one. This location was slightly different from the older literature. Shi tested only exon 1 sequence [5] but extended the analysis later towards exon 2 [13].

CpG sites in the present analysis were denoted according to the following scheme. Forward primer was constructed by exchanging C → T and reverse primer by exchanging G → A as in the following sequence.

```
CYP27B1 hg18,chr12:56,446,824-56,447,243
GGGTGCTAATCCCCAGCACAGACCACTCAGGAGGAGGGATTGGCTGAGGAGCTTGGAGAG  5′ UTR
    1
GGGGCGTCATCACCTCACCCAAAGGTTAAATAGGGGTTGAGATATGATGCTCAGGAGAAG  primer forward
2          3 4                                    5
CGCTTTCTTTCGCGAGCACCCTGAACCAGACCATGACCCAGACCCTCAAGTACGCCTCCA  exon 1
             6 7    8        9    10         11              12
GAGTGTTCCATCGCGTCCGCTGGGCGCCCGAGTTGGGCGCCTCCCTAGGCTACCGAGAGT
               13 14                              15
ACCACTCAGCACGCCGGAGCTTGGCAGACATCCCAGGCCCCTCTACGCCCAGCTTTCTGG
 16                          17          18                  19
CCGAACTTTTCTGCAAGGGGGGGCTGTCGAGGCTACACGAGCTGCAGGTAGGAAGGGACG
         20
CCTTTCCCGAGACAGAGTGCTGGGGAAACTGGTTTTGACAGCGTCAGAAAGGACTGACTA  primer reverse

CYP24A1 hg18,chr20:52,223,249-52,224,448
                                                          45
AAGGCAAAAAAAAAAAAAAAAATCACTTCAGTCCAGGCTGGGGGTATCTGGCTCCCCGGGA primer reverse
  44  43        42              41 40                  39    38
GGCGCCCGGGGCTCCCCGGGGCCCTGGCAGACGCCGGCAGCTTTTCTGGGCCCGCACTCGG
      37  36  35    3433          32 31  30        29  28 27
GGACCTCGCCCGCCCGGCATCGCGATTGTGCAAGCGCCGGGCGGCAACCACGGCCGCCGC
   26          25 24         23       2221  20          19
TGCCGGCTCCTGCCCGCCGGGGGAGGGCGGGGAGGCGCGTTCGAAGCACACCCGGTGAAC
  18     17
TCCGGGCTTCGCATGACTTCCTGGGGGTTATCTCCGGGGTGGAGTCTGCCGCCCCCACCC primer forward
CACCTCCCGCGCCCAGCGAACATAGCCCCGGTCACCCCAGGCCCGGACGCCCTCGCTCAC
CTCGCTGACTCCATCCTCCTTCCACCCCCCCTCCCCTGGGTCCCGCGTCCCTCGGAGTCT
GGCCAGCCGGGGGCCACTCCGCCCTCCTCTGCGTGCTCATTGGCCACCCAGGGCATGCTC
TGTCTCCATAAATGCATGGTCCCTGGGCATAGGAACATGGAGAGGGACAGGAGGAAACGC 5′ UTR
AGCGCCAGCAGCATCTCATCTACCCTCCTTGACACCTCCCCGTGGCTCCAGCCAGACCCT
AGAGGTCAGCCTTGCGCACCAACAGGAGGACTCCCAGCTTTCCCTTTTCAAGAGGTCCCC
AGACACCGGCCACCCTCTTCCAGCCCCTGCGGCCAGTGCAAGGAGGCACCAATGCTCTGA
GGCTGTCGCGTGGTGCAGCGTCGAGCATCCTCGCCGAGGTCCTTTCTGCTGCCTGTCCCG
CCTCACCCCGCTCCATCACACCAGCTGGCCCTCTTTGCTTCCTTTTCCCAGAATCGTTAA
GCCCCGACTCCCACTAGCACCTCGTACCAACCTCGCCCCACCCCATCCTCCTGCCTTCCC
GCGCTCCGGTGTCCCCCGCTGCCATGAGCTCCCCCATCAGCAAGAGCCGCTCGCTTGCCG exon1
CCTTCCTGCAGCAGCTGCGCAGTCCGAGGCAGCCCCCGAGACTGGTGACATCTACGGCGT
ACACGTCCCCTCAGCCGCGAGAGGTGCCAGTCTGCCCGCTGACAGCTGGTGGCGAGACTC
AGAACGCCGGCCGCCCTGCCGGGCCCCACCAGCTGGCCACTGCTGGGCAGCCTGCTGCAGA
TTCTCTGGAAAGGGGGTCTCAAGAAACAGCACGACACCCTGGTAAACCCCTTTTCGCGCC
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All sequencing was done at Qiagen, Hildesheim. Reactions were pretested with methylated and unmethylated control DNA, as well as with mixed DNA. Only samples passing quality control measures were included in the current analysis excluding CYP27B1 site #1–4 and CYP24A1 #43–45.

3. Results

Of 384 DNA samples randomly selected from the study population [11], 383 could be analyzed for MR (Fig. 1). The average MR in the CYP27B1 promotor was 11% (s.d. 5%) with the highest MR observed at exon 1 at site #5 (38%, s.d. 9%). CYP24A1 showed only a MR of 7% (s.d. 5%) without any outliers.

As expected vitamin D metabolites were positively correlated (R2 = 0.18, $p = 0.0004$) with only 25-OH-D3 showing a strong seasonal variation.

Furthermore, the MR autocorrelated within a given CpG island, while the MR of both genes correlated neither with season of examination nor with 25-OH-D3 and 1,25-(OH)2-D3 serum levels except CYP27B1 site #15–17 that showed a weak association with 25-OH-D3 (R2 ∼ −0.11, all $p < 0.05$). There seemed to be also tendency of higher MRs of CYP27B1 with lower 25-OH-D3 serum values (Fig. 2A) which is observed also in late German winter and early spring months (Fig. 2B). Results did not change when using multivariate models that adjusted for confounding factors like age, sex and city of origin.

4. Discussion

This pilot study represents an early approach by applying recent in vitro concepts of gene methylation [1] in a clinical study.

DNA obtained from peripheral blood lymphocytes (PBLs) in families show either no (CYP24A1) or only weak methylation (CYP27B1) except of the most upstream site #5. This point towards an extension of the actively methylated area beyond the sequenced fragment. Shi [13] conducted bisulfite sequencing of CYP27B1 and also assumed that it just touched

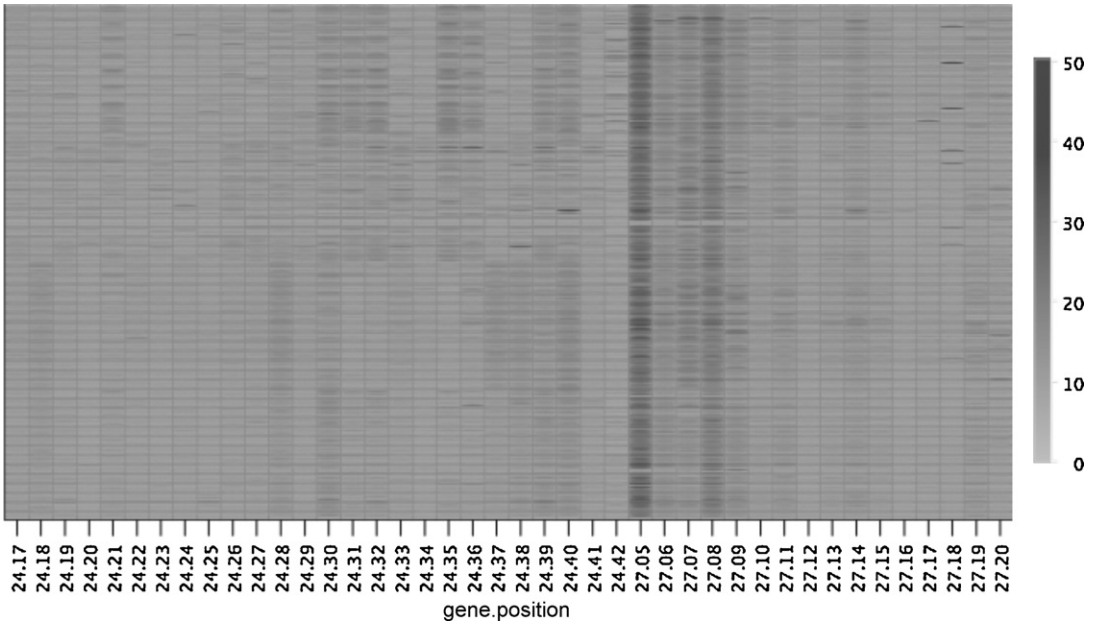

Fig. 1. CpG methylation ratios of CYP24A1 and CYP27B1 genes in PBLs of 383 individuals (German Asthma Family Study). CpG sites are sorted at increasing positions from left to right (CYP24A1 CpGs are labelled as 24.xx and CYP27B1 CpGs are labelled as 27.xx) and individual samples are ordered from top to bottom. Only CYP27B1 #5 shows a major methylation signal in nearly all samples.

the boundary of the CpG island in the 5′ upstream region of CYP27B1.

MR at site #5, however, did not correlate with month of examination nor 25-OH-D3 levels making a direct interference of vitamin D metabolites and immune function via CYP methylation unlikely. It should be noted, however, that CYP27B1 gene is expressed primarily in kidney [7] where the CYP27B1 promotor may indeed undergo changes of the MR. Furthermore, as PBLs represent a heterogeneous pool a further analysis of methylation pattern in B or T cell subsets is warranted [14]. Chung [3] who described epigenetic silencing of CYP24A1 by 1,25-OH2-D3 only in tumor-derived endothelial cells but not in normal human cells. Similarly, Khorchide [4] concluded that human prostate cells expressing vitamin D hydroxylases may be under epigenetic control, pointing also towards the strong influence of the tissue context of DNA methylation.

Unfortunately, a cross-sectional study is not optimally suited to resolve any season dependent effects. The observed tendency of higher MRs of CYP27B1 with lower 25-OH-D3 serum values point towards a rather long lagging phase of gene regulation as an immediate silencing of an anabolic enzyme seems to be counterintuitive when substrate is being needed. There might, however, also be some artifacts involved as some of our children are being treated with steroids [15]. In summary, these preliminary results highlight the feasibility of gene methylation studies also in larger clinical studies. It is expected that the rapidly evolving field of epigenetics

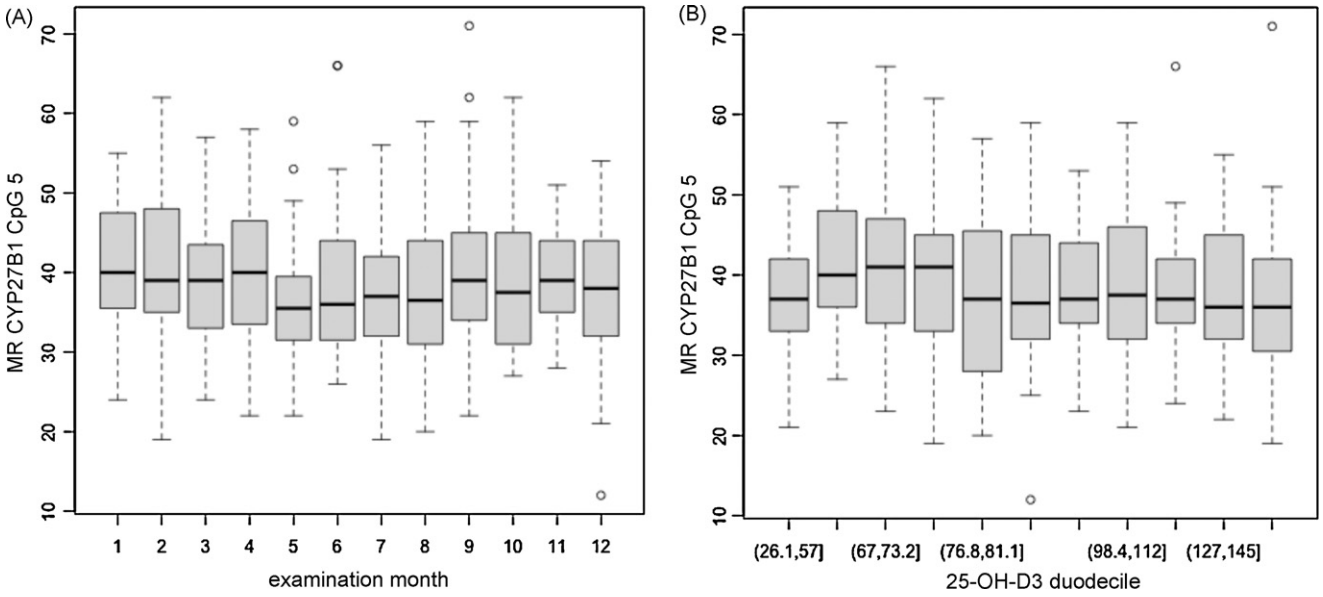

Fig. 2. Cpg methylation rate at CYP27B1 CpG site #5 by month of examination (A) and duodecile groups of 25-OH-D3 serum levels (B) in PBLs of 383 individuals (German Asthma Family Study). There is a tendency of higher MRs with lower 25-OH-D3 serum values (except for the lowest 25-OH-D3 category) which is observed also in late German winter and early spring month where 25-OH-D3 serum values are usually low in the population.

is going to provide new insights [16] not only in enzyme kinetics but also in the origin of complex human diseases [1,17,18].

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