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Hollander, W. den

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OSTEOARTHRITIS SUSCEPTIBILITY POLYMORPHISM RS225014 MODULATES EPIGENETIC REGULATION OF *DIO2* IN ARTICULAR CARTILAGE

W. den Hollander^{1,2,4}, N. Bomer^{1,3}, Y.F.M. Ramos^{1,3}, S.D. Bos^{1,4}, R. van der Breggen¹, N. Lakenberg¹, B.A. Peppers⁵, A.E. van Eeden¹, E.W. Tobin^{1,3}, B.J. Duijnsveld⁶, B.T. Heijmans^{1,3}, W.M.C. van Roon-Mom⁵, P.E. Slagboom^{1,3,4}, R.G.H.H. Nelissen⁶, I. Meulenbelt^{1,2,3,4}.

¹Dept. of Molecular Epidemiology, LUMC, Leiden, The Netherlands; ²Reumafonds, Dutch Arthritis Foundation; ³IDEAL; ⁴The Netherlands Genomics Initiative, sponsored by NCHA, Leiden-Rotterdam, The Netherlands; ⁵Dept. of Human Genetics, LUMC, Leiden, The Netherlands; ⁶Dept. of Orthopedics, LUMC, Leiden, The Netherlands.

ABSTRACT

OBJECTIVES: An earlier observed allelic imbalance and up-regulation of the osteoarthritis (OA) susceptibility gene Type II Deiodinase (*DIO2*) in OA affected articular cartilage indicate that *DIO2* associated OA susceptibility is mediated by aberrant expression of *DIO2*. We set out to characterize possible regulatory properties of putative CCCTC-binding factor (CTCF) sites, proximal and distal CpG dinucleotides in the *DIO2* locus.

METHODS: Electrophoretic Mobility Shift Assays were performed to assess binding of CTCF to the rs225014 locus. OA affected and macroscopically preserved articular cartilage were sampled from end stage OA patients undergoing total joint arthroplasty (RAAK study) of the knee (N = 29) or hip (N = 23) to assess CpG dinucleotide methylation and *DIO2* expression. Chromatin Immunoprecipitation assays were performed to investigate the relation between methylation dependent CTCF binding and *DIO2* expression in three primary chondrocyte cell lines.

RESULTS: OA susceptibility polymorphism rs225014 alleles did not influence local CTCF binding, however a functional CTCF binding site was present 90 base pairs upstream of rs225014. We observed that *DIO2* expression is under epigenetic control of a CpG dinucleotide located in a CTCF binding site 2031 base pairs upstream of the *DIO2* transcription start site. Furthermore, *DIO2* expression in carriers of the rs225014 risk allele showed an enhanced up-regulation upon methylation changes at this CpG dinucleotide, compared to homozygous wildtype carriers.

CONCLUSIONS: *DIO2* associated OA susceptibility is likely to be brought about by aberrant up-regulation of *DIO2* in articular cartilage, due to methylation at a CpG dinucleotide sensitive to the OA process.

INTRODUCTION

Osteoarthritis (OA) is a common disease in the elderly causing pain and disability in articular joints. (1, 2) Although OA is characterized by diseased joint tissues due to wear and tear, it has a significant genetic component. As with many complex genetic diseases, multiple minor genetic defects are considered to contribute to the onset and development of OA. (3) Research aiming to identify determinants conferring osteoarthritis susceptibility, by applying molecular epidemiological approaches, has resulted in a number of successes. Early genetic studies on OA have provided several compelling genes harboring OA susceptibility alleles including the growth differentiation factor 5 gene *GDF5* (4, 5), *SMAD3* (6) and the deiodinase iodothyronine type II and III genes (*DIO2* and *DIO3*), of which the latter two are identified by our own group. (7, 8) In addition, large European consortia (such as arcOGEN and TREAT~OA) have compiled genome wide association (GWA) data and performed meta-analyses on OA and OA-related quantitative traits such as joint space width, which resulted genome wide significant signals. (3, 9-12)

A considerable number of OA susceptibility genes have been found to be part of a common pathways involved in the developmental process of endochondral ossification. (13) Furthermore, it has previously been shown that activation of such genes in articular cartilage occurs during the OA disease process. (14, 15) To ensure cartilage integrity, articular chondrocytes should remain in a differentiated and maturational arrested state; however a hypothesized loss of epigenetic control in articular cartilage could result in reactivation of genes involved in endochondral ossification, leading to loss and mineralization of articular cartilage, a process known to contribute to OA. (7, 16-18)

The linkage signal that identified *DIO2* as OA susceptibility gene was significantly explained by the minor allele (C) of the T>C single nucleotide polymorphism (SNP) rs225014. Rs225014 is a non-synonymous SNP located in exon 2 of *DIO2* causing a Thr92Ala transition. (7) Although this transition does not influence enzyme velocity (19), we have recently reported a highly consistent allelic imbalance (AI) in articular cartilage of the Research and Articular Osteoarthritis Cartilage (RAAK) study, where the OA-associated rs225014 C allele was being 1.3 times more abundantly expressed relative to the T allele. (20) No SNP was found in high linkage disequilibrium (LD) with rs225014 (7) that could explain the AI, therefore it was hypothesized that the observed expression of *DIO2* is directly influenced by rs225014 alleles. A putative CCCTC-binding factor (CTCF) site is overlapping with rs225014, indicative of potential regulatory properties of the locus. (21) CTCF is considered to facilitate long-range chromatin interactions in order to regulate gene expression; distal transcriptional elements on the genome are brought in close proximity to transcriptional start sites (TSSs) of genes to alter expression. (22) We hypothesize that *DIO2* associated OA susceptibility is brought about by aber-

rant up-regulation of *DIO2* expression in articular cartilage. Here we set out to identify genetic and epigenetic elements that regulate *DIO2* expression in articular cartilage and how these are related to rs225014 alleles and the ongoing OA disease process.

MATERIALS AND METHODS

SUBJECTS

Macroscopically, we identified and sampled weight bearing preserved (away from the affected area) and OA affected cartilage (within the affected area) from 52 Caucasian end stage OA patients who underwent a total knee replacement (N = 29) or a total hip replacement (N = 23) in the Leiden University Medical Centre. Ethical approval was obtained from the medical ethics committee of the LUMC (P08.239) and informed consent was obtained from all study participants.

NUCLEIC ACID ISOLATION AND GENOTYPING

Snap frozen cartilage was powderized using a Retsch Mixer Mill 200 with continuous liquid nitrogen cooling. DNA was isolated using the Promega Wizard Genomic DNA Purification kit according to the manufacturer's protocol. RNA was isolated using the Qiagen RNeasy Mini kits, followed by cDNA synthesis using 1 µg of RNA and random hexamer primers (First Strand cDNA Synthesis Kit, Thermo Scientific). Samples were genotyped for rs225014 using restriction fragment length polymorphism analysis with *RsaI* (Forward primer (F): 5'-AGTGGCAATGTGTTTAAATGTGA-3', Reverse primer (R): 5'-CACACACGTTCAAAGCTACC-3'). DNA fragment length of wildtype alleles were called after gel electrophoresis and were 121, 30 and 389 base pairs. The risk allele C affects the first cut site resulting in two fragments consisting of 151 and 389 base pairs. All primers were ordered at Invitrogen.

MEASUREMENTS

For Electrophoretic mobility shift assays (EMSAs) synthetic oligonucleotides containing the putative CTCF binding site were 5'-end labeled by γ - (32)P-ATP and subsequently purified by gel filtration on Sephadex G-25 Medium columns. The CTCF-11 zinc finger (11ZF) DNA binding domain, full-length CTCF using pIVEX1.4 WG CTCF-11ZF and CTCF-FL constructs were synthesized with the RTS 100 Wheat Germ CEF kit (5 PRIME). For binding reactions, we used buffer containing standard PBS with 5 mM MgCl₂, 0.1 mM ZnSO₄, 1 mM DTT, 0.05% NP40, 50 ng/µl poly(dI-dC) and 10% glycerol. The reaction mixtures were incubated for 30 min at RT and analyzed by 5% native PAGE in 0.5x Tris-borate-EDTA buffer. **Supplementary Table 1** contains the sequences of the used probes.

Expression of *DIO2* was assessed using TaqMan probe Hs00988260_m1 (Applied Biosystems), normalized for *GAPDH* expression (real-time PCR, F: 5'-TGCCATGTAGACCCCTGAAG-3', R: 5'-ATGG-TACATGACAAGGTGCGG-3') and subsequently log transformed for downstream analysis.

Using the ZymoResearch EZ DNA Methylation kit isolated genomic DNA was treated with sodium bisulphite (BS), thereby reducing unmethylated cytosine residues to uracil, while methylated cytosines remain unchanged. The methylated fraction of CpG dinucleotides was assessed with MALDI-TOF mass spectrometry (Epityper, Sequenom), a commonly applied to quantify CpG methylation. (23-25) Samples were randomly distributed on PCR plates prior to BS treatment and PCR amplification. PCR amplification and MALDI-TOF measurements were performed in triplicate as technical replicates.

Using MethPrimer 9 amplicons (**Supplementary Table 1**) were designed, covering a total of 23 measurable CpG dinucleotides upstream, downstream and intragenic of *DIO2*. Amplicons were designed to cover conserved transcription factor binding sites (TFBS) according to Human Genome Assembly, build 19. Methylation of several CpG dinucleotides was measured redundantly by separate amplicons, measurements of CpG dinucleotides with the most successful observations per amplicon were used for downstream analyses. Finally, the 9 amplicons constituted 4 independent regions.

CELL CULTURE AND CHROMATIN IMMUNOPRECIPITATION

Primary cartilage chondrocytes were isolated from cartilage derived from three OA patients who underwent total joint arthroplasty of the hip (RAAK study). Cartilage tissue was incubated overnight in DMEM (high glucose; Gibco, Bleiswijk, The Netherlands) supplemented with 10% fetal bovine serum (FBS; Gibco), antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin; Gibco) and 2 mg/ml collagenase Type I at 37 °C in a humidified 5% CO₂/95% atmosphere. Subsequently, primary chondrocytes were resuspended and filtered through a 100 µm mesh to remove undigested cartilage fragments and extracellular matrix debris. Cells were expanded at 37 °C in a humidified 5% CO₂/95% atmosphere in DMEM supplemented with 10% FBS, penicillin (100 units/mL), streptomycin (100 units/mL) and 0.5 ng/ml FGF-2 (PeproTech, Heerhugowaard, The Netherlands) for 2 passages. 24 Hours into the second passage 1.5 µM of the demethylating agent 5-aza-2-deoxycytidine (AZA) (Sigma Aldrich; Zwijndrecht, The Netherlands) was added. Cells were harvested for DNA and RNA isolations after being grown to confluence, obtained after three more days.

For chromatin immunoprecipitation (ChIP) chromatin was prepared and immunoprecipitation was performed as previously described. (26) Sonification was optimized to result in ~500 base pair long fragments. For immunoprecipitation rabbit anti-CTCF antibody was used (Anti-CTCF; 07-729 Millipore). Protein A and G sepharose beads were ordered from Sigma (P3391) and GE Healthcare

(17-0618-01) respectively. Finally the immunoprecipitated chromatin was used as input for real-time-PCR to assess the relative binding of CTCF, used primers are listed in **Supplementary Table 1**.

DATA ANALYSIS

Methylation of CpG dinucleotides with fewer than two out of three triplicate measurements or with a SD > 0.1 were discarded prior to analysis. CpG site-containing fragments that had equal or overlapping mass, making them irresolvable by mass spectrometry, and CpG sites containing fragments whose measurement was confounded by SNPs were removed prior to analysis. (27) Samples with bisulphite conversion rates < 98% were discarded.

Principal Component Analysis (PCA) was performed to reduce redundant variation of methylation at CpG dinucleotides which had a high in-between correlation. (27) For each of the 4 regions, Principal Components (PCs) were constructed if the KMO sampling adequacy was < 0.5 and Bartlett's test of sphericity was < 0.01. PCs with Eigen values > 1 were considered relevant. The individual loadings of each of the PCA were checked to assess whether all CpG dinucleotides were covered by the constructed PCs, CpG dinucleotides for which all component loadings were < 0.4 were considered not significant and these CpG dinucleotides were discarded from the PCA and analyzed separately in subsequent analyses.

All statistical analyses were performed by fitting Generalized Linear Mixed Models (GLMMs). To account for inter-individual differences a random effect for sample donor was added to each model. Homozygous carriers (N = 3) of the rs225014 risk allele were pooled with heterozygous carriers (N = 27). Analyses were carried out using the R programming language with the *lme4* (GLMMs) (28), *psych* (29) and *GPArotation* (PCA) (30) packages. To assess the relation between the cartilage phenotype and methylation of separate features, we fitted the following model: $Methylation_i \sim (1|Donor) + Phenotype$. To identify functional CpG dinucleotides, we fitted the following model: $DIO2\ Expression \sim (1|) Donor + Methylation_i$. Where, in both models, $Methylation_i$ represents the methylated fraction of the i -th CpG feature. To explore other possible significant covariates, we fitted the following model: $DIO2\ Expression \sim (1|Donor) + Joint\ site + rs225014\ alleles + Methylation_{CpG-2031}$. Finally, p-values were adjusted for multiple testing using Bonfferoni correction.

RESULTS

THE PUTATIVE CTCF BINDING SITE OVERLAPPING RS225014 IS NOT FUNCTIONAL IN ARTICULAR CHONDROCYTES

To assess the regulatory properties of the rs225014 overlapping CTCF binding site, we investigated binding of CTCF at this locus and tested whether rs225014 alleles directly influence local binding of CTCF. We performed EMSAs using full-length CTCF (CTCF-FL) and a truncated protein, containing just the 11 Zinc Finger binding domain (CTCF-11ZF). We have used three different probes containing the rs225014 common allele (T, **Figure 1**, lane 1-2), the minor allele (C, **Figure 1**, lane 3-4) and a non-existent allele (G, **Figure 1**, lane 5-6). The latter is in highest agreement with the consensus CTCF-binding sequence. (31) No band shift was observed for either protein, irrespective of the three rs225014 alleles, indicating that CTCF does not bind to the putative CTCF sequence at SNP rs225014. Nevertheless, the EMSA confirmed a CTCF site 90 base pairs upstream of rs225014 (DIO2-CTCF2, **Figure 1**, lane 7-8), as a clear band shift was observed for this predicted CTCF binding site.

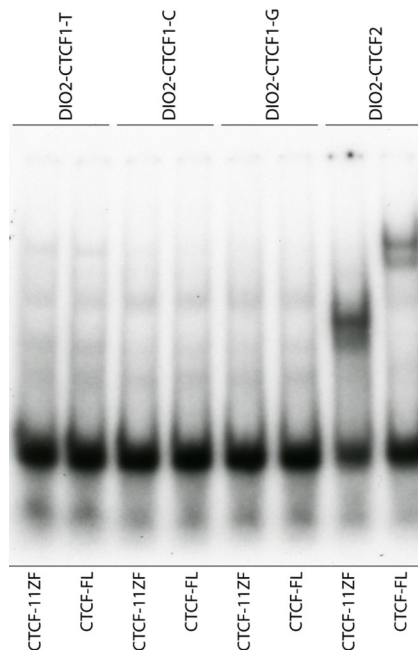


Figure 1. EMSAs for two CTCF binding sites located on (DIO2-CTCF1) and next to (DIO2-CTCF2) rs225014. Full length CTCF (CTCF-FL) and truncated CTCF (CTCF-11ZF) containing just the 11 Zinc Finger binding domain were used. No band shift was observed for DIO2-CTCF1, irrespective of rs225014 alleles (lane 1-6). A band shift for DIO2-CTCF2 was observed for both protein constructs (lane 7-8).

CPG DINUCLEOTIDES LOCATED ACROSS *DIO2* ARE METHYLATED IN ARTICULAR CARTILAGE

Being unable to confirm binding of CTCF at the putative CTCF binding site overlapping the rs225014 locus, we set out to quantify CpG dinucleotide methylation across the *DIO2* locus. We therefore selected macroscopically preserved and OA affected cartilage from patients undergoing total joint arthroplasty of the knee (N = 29) or hip (N = 23) of the RAAK study (**Supplementary Table 2**). Amplicons containing CpG dinucleotides were designed across TFBSs located intragenic, upstream and downstream of the *DIO2* open reading frame. **Figure 2** shows the 4 various regions (A-D) across the *DIO2* locus, methylation measurements of respective CpG dinucleotides per region and the overall mean across pooled samples. Two, four and two CpG dinucleotides were discarded from region A, B and C respectively prior to analysis, due to the inability to discriminate them by mass spectrometry. Region A, B and D showed relatively high levels of methylation, whereas region C, overlapping the *DIO2* promotor, showed overall hypomethylation. Where applicable, we summarized CpG dinucleotides per region in Principal Components (PCs), which resulted in 15 independent variables (5 PCs and 10 individual CpGs), constructed from the original 23 (**Supplementary Table 3** and **Supplementary Table 4**).

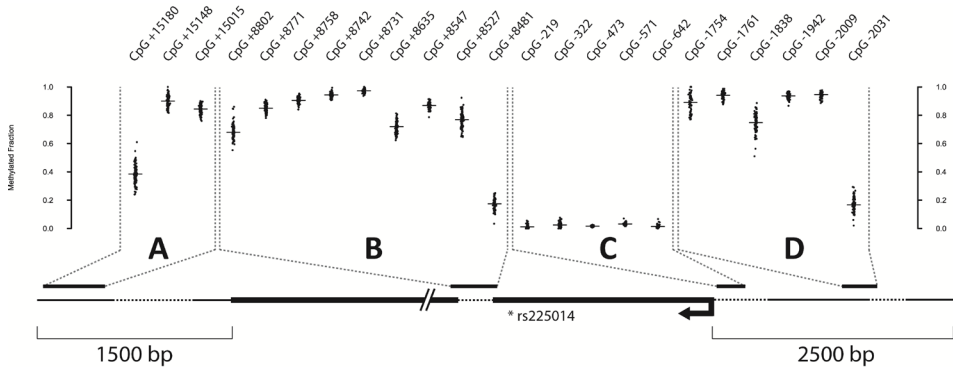


Figure 2. Schematic overview of the *DIO2* open reading frame (ORF) and flanking regions. Lower panel shows the coding strand of the genome (chr14:80,661,868-80,680,970, hg19) with the *DIO2* ORF located on the complementary strand, depicted as a solid bold line. Conserved TFBSs are depicted as dashed lines. CpG dinucleotides are indicated by their relative position to the *DIO2* TSS. (A-D) Observed methylation values of CpG dinucleotides in 4 interrogated regions of pooled preserved and OA cartilage samples.

A CPG DINUCLEOTIDE 2031 BASE PAIRS UPSTREAM OF THE *DIO2* TSS (CPG -2031) MODULATES EXPRESSION SIGNIFICANTLY

Upon investigating differential methylation and expression between OA and preserved cartilage, we observed that methylation at multiple CpG features across *DIO2* were responsive to the OA disease process as reflected by significant differential methylation between matched preserved and OA

cartilage samples (**Table 1**). For the single CpG-2031 in region D, we observed the most significant difference in methylation between OA and preserved cartilage (GLMM, $N = 103$, $\text{Beta} = 0.028$, $P = 0.0007$, Bonferroni adjusted, **Figure 3A**). Suggestive evidence was observed for a difference in *DIO2* expression between preserved and OA affected cartilage, albeit not significant (**Figure 3B**, GLMM, $N = 87$, $\text{Beta} = 0.22$, $P = 0.063$). To prioritize on functional CpG dinucleotides, we subsequently focused on those features that additionally associated with *DIO2* expression (**Supplementary Table 5**) in cartilage. As seen in **Figure 3C**, we observed a significant positive association between methylation and expression of *DIO2* only for CpG-2031 (GLMM, $N = 87$, $\text{Beta} = 4.959$, $P = 0.0016$, Bonferroni adjusted).

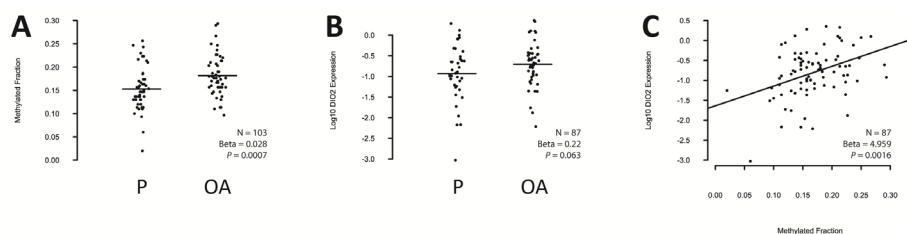


Figure 3. Summary statistics and functional evaluation of methylation at CpG-2031 in articular cartilage. **(A)** A significant difference in methylation was found at CpG-2031 between preserved and OA affected cartilage. **(B)** Suggestive evidence for a difference in *DIO2* expression between preserved and OA affected cartilage. **(C)** Methylation at CpG-2031 correlated significantly with *DIO2* expression.

Table 1. Independent features tested for differential methylation between preserved and OA affected cartilage. Asterisks indicate significant *P*-values after Bonferroni adjustment.

Region	Feature	N	Beta	p-value	Adjusted p-value
A	PC 1	102	0.079	0.56018	1
B	PC 1	95	0.223	0.03132	0.4699
	PC 2	95	-0.152	0.26965	1
	CpG +8802	76	0.001	0.92605	1
	CpG +8742	101	-0.003	0.36266	1
	CpG +8635	103	0.001	0.58860	1
C	CpG-219	94	0.000	0.92106	1
	CpG-322	98	0.002	0.60637	1
	CpG-473	102	0.001	0.15828	1
	CpG-571	97	0.002	0.28463	1
	CpG-642	98	0.003	0.08636	1
D	PC 1	96	-0.520	0.00015	0.0023 *
	PC 2	96	-0.301	0.04685	0.7028
	CpG-1754	63	0.032	0.00449	0.0673
	CpG-2031	103	0.028	0.00005	0.0007 *

To confirm the regulatory properties of CpG-2031 on *DIO2* expression, we performed ChIP assays for a predicted CTCF binding site overlapping the location of CpG-2031. To test whether CpG methylation influences local CTCF binding we treated three primary chondrocyte cell lines, derived from OA patients undergoing total joint arthroplasty of the hip, with 1.5 μ M AZA, a widely used demethylating agent. As shown in **Figure 4A**, all cell lines show a decrease in methylation at CpG-2031 that corresponded to an increase in bound CTCF at the respective position (**Figure 4B**) and subsequent down regulation of *DIO2* expression (**Figure 4C**), confirming that methylation at CpG-2031 affects expression of *DIO2*, which may be attributable to local methylation dependent binding of CTCF.

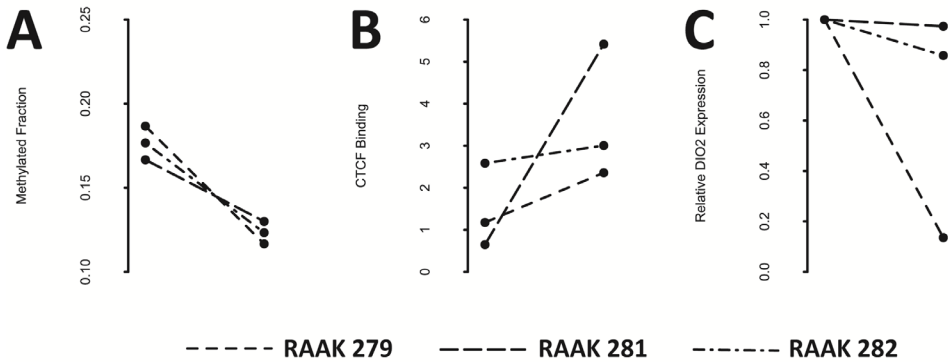


Figure 4. Response of primary chondrocyte cell lines upon treatment with 1.5 μ M AZA. **(A)** Decrease of CpG -2031 methylation upon AZA treatment. **(B)** Increase in CTCF binding at CpG-2031 upon AZA treatment. **(C)** Decrease in *DIO2* expression upon AZA treatment.

RS225014 RISK ALLELE CARRIERS SHOW AN ENHANCED CORRELATION BETWEEN CPG -2031 METHYLATION AND *DIO2* EXPRESSION

Having observed the association between *DIO2* expression and OA reflective methylation at CpG -2031, we explored in a multivariate analysis the individual effects of CpG-2031 methylation, joint site and rs225014 alleles as independent variables on *DIO2* expression in articular cartilage as dependent variable. We could ratify the significant association between CpG-2031 methylation and *DIO2* expression (GLMM, N = 87, $\text{Beta}_{\text{CpG-2031}} = 4.526$, $P_{\text{CpG-2031}} = 0.0006$) and observed a slight effect for joint site (GLMM, N = 87, $\text{Beta}_{\text{joint}} = -0.264$, $P_{\text{joint}} = 0.048$). However, the most compelling observation was the significant effect of rs225014 alleles on *DIO2* expression (GLMM, N = 87, $\text{Beta}_{\text{rs225014}} = 0.448$, $P_{\text{rs225014}} = 0.0003$), independently of methylation at CpG-2031.

Finally, to further elucidate these effects, we explored whether the earlier observed results were affected by stratification of the rs225014 risk allele. We observed an increase in methylation (**Figure 5A**, GLMM, N = 59, $\text{Beta} = 0.034$, $P = 0.00002$) and expression (**Figure 5B**, GLMM, N = 51, $\text{Beta} = 0.35$, $P = 0.012$) differences between preserved and OA affected cartilage in carriers of the rs225014

risk allele. Most strikingly, expression of *DIO2* in cartilage derived from rs225014 risk allele carriers was more responsive to methylation changes at CpG-2031 (**Figure 5C**, GLMM, $N = 51$, $\text{Beta} = 5.58$, $P = 0.0006$). Together, these data indicate that *DIO2* expression among carriers of the rs225014 risk allele, compared to homozygous common allele carriers, is more sensitive to subtle methylation changes at CpG-2031 in articular cartilage.

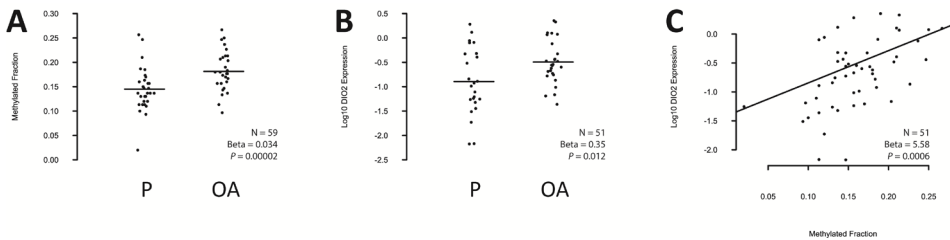


Figure 5. (A) A more pronounced difference between preserved and OA affected cartilage was observed in carriers of the rs225014 risk allele. (B) *DIO2* expression in carriers of the rs225014 risk allele is significantly higher in OA affected cartilage compared to preserved cartilage. (C) The functional relation between CpG-2031 and *DIO2* expression is more pronounced in carriers of the rs225014 risk allele.

DISCUSSION

In the current study, we have identified a CpG dinucleotide at 2031 base pairs upstream of the *DIO2* TSS that modulated *DIO2* expression significantly (**Figure 3C**) and appeared highly sensitive to the ongoing OA process (**Table 1** and **Figure 3A**). Furthermore, by means of ChIP analyses it was shown that the regulatory properties of methylation at CpG-2031 are most likely to be exerted via methylation dependent binding of CTCF at the respective position (**Figure 4**). Moreover, regarding epigenetically regulated *DIO2* expression, we showed that subtle differential methylation at this particular CpG dinucleotide associated with up regulation of *DIO2* expression, most vividly among rs225014 risk allele carriers (**Figure 5C**). Given the role of *DIO2* in growth plate chondrocytes, i.e. the initiation of endochondral ossification (32, 33), such an up-regulation should be considered detrimental to articular cartilage integrity and increasing the propensity to develop OA. Together these data provide compelling insights into how the *DIO2* OA susceptibility allele of rs225014 confers risk to OA; upon repeated challenges throughout life, chondrocytes residing in articular cartilage of rs225014 risk allele carriers are less able to maintain cartilage homeostasis due to the fact that subtle changes in methylation at CpG-2031 result in detrimental up-regulation of *DIO2*.

In previous studies we have demonstrated an early mode of action of *DIO2* susceptibility alleles on hip morphology (34), whereas in articular cartilage we have reported on up regulation of *DIO2*

protein in OA as compared to preserved cartilage and a highly consistent AI of *DIO2* in heterozygous carriers of the rs225014 risk allele. (20) In the current study, we have prioritized on CpG dinucleotides that significantly modulated expression of *DIO2* and have provided insight into the mechanisms how the rs225014 risk allele confers risk to OA at later ages. Nevertheless, we were unable to unravel the molecular mechanism of the AI marked by the rs225014 alleles. This implies that *DIO2* expression in articular chondrocytes is subject to multiple regulatory mechanisms, possibly intertwined, as is suggested by the vivid *DIO2* up-regulation upon CpG-2031 methylation changes in rs225014 risk allele carriers.

Demethylation of CpG dinucleotides commonly up-regulates gene expression, however, here a positive correlation between methylation at CpG-2031 and *DIO2* expression was observed. This positive correlation is likely to be mediated via the confirmed methylation dependent binding of CTCF, a general insulator protein known to facilitate regulation of gene expression, at CpG-2031. We hypothesize that a putative three dimensional structure, brought on about by bound CTCF in hypomethylated samples, exerts inhibitory effects on *DIO2* expression, possibly via recruitment of inhibitory transcription factors.

A significant up-regulation of *DIO2* mRNA and protein in OA affected cartilage compared to healthy controls is reported in literature (14, 15, 20), however, when comparing preserved with OA affected cartilage we were unable to detect a significant difference in *DIO2* expression. Hence, it is likely that preserved cartilage from OA affected joints has already to some extent been seized by the ongoing OA disease process, but has not yet manifested in macroscopically observable differences. However, we did observe a significant difference in expression between preserved and OA affected cartilage in carriers of the rs225014 risk allele. Even so, our approach does minimize environmental influences, to which CpG methylation is highly susceptible (35-37), as the preserved and matched OA affected cartilage originate from a single joint, thereby allowing robust pair wise comparisons. To study the epigenetic mechanisms underlying the reported up-regulation of *DIO2* mRNA in OA affected cartilage compared to unaffected cartilage, CpG dinucleotide methylation in true healthy controls should be quantified. However, appropriate sample sizes are required, as large variation in methylation of CpG dinucleotides is observed among subjects.

It has been shown that regulatory elements on the genome, such as CpG dinucleotides, quite commonly do not influence gene expression of the nearest gene (38); i.e. differential methylation of CpG dinucleotides does not imply an effect on gene expression of the nearest gene whatsoever (**Table 1** and **Supplementary Table 5**). Therefore, in the view of the authors, functional follow up by means of epigenetic regulation of OA susceptibility genes, should have a focus on CpG dinucleotides correlating significantly with expression of the gene of interest.

In conclusion, we have shown that a CpG dinucleotide 2031 base pairs upstream of the *DIO2* promoter is differentially methylated between preserved and OA affected cartilage. The functionality of this CpG dinucleotide is illustrated by local methylation dependent CTCF binding and, moreover, by a significant correlation with *DIO2* expression. Furthermore, compelling evidence was observed for an enhanced response of *DIO2* expression upon methylation changes at CpG-2031 in rs225014 risk allele carriers in articular cartilage.

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Supplementary tables can be found at doi: 10.1136/annrheumdis-2013-204739

Supplementary Table 1.

Used EMSA probes and Epityper primers.

Supplementary Table 2.

Characteristics of samples used in methylation and expression assessment.

Supplementary Table 3.

PCA eigenvalues per region. PCs with eigenvalues < 1 were discarded.

Supplementary Table 4.

PCA loadings for each region. A dash indicates inappropriate KMO sampling adequacy or failed Bartlett's test for sphericity, these CpG dinucleotides were analyzed separately.

Supplementary Table 5.

Independent features tested for their association with *DIO2* expression in articular cartilage. Asterisks indicate significant *P*-values after Bonferroni adjustment.

