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The DNA methylation landscape of CD4$^+$ T cells in oligoarticular juvenile idiopathic arthritis

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ABSTRACT

Juvenile idiopathic arthritis (JIA) is presumed to be driven by an adverse combination of genes and environment. Epigenetic processes, including DNA methylation, act as a conduit through which the environment can regulate gene activity. Altered DNA methylation has been associated with adult autoimmune rheumatic diseases such as rheumatoid arthritis, but studies are lacking for paediatric autoimmune rheumatic diseases including JIA. Here, we performed a genome-scale case-control analysis of CD4$^+$ T cell DNA methylation from 56 oligoarticular JIA (oJIA) cases and 57 age and sex matched controls using Illumina HumanMethylation450 arrays. DNA methylation at each array probe was tested for association with oJIA using RUV (Remove Unwanted Variation) together with a moderated *t*-test. Further to this 'all-inclusive' analysis, we stratified by age at diagnosis (≤6yrs, >6yrs) and by sex as potential sources of heterogeneity. Following False Discovery Rate (FDR) adjustment, no probes were associated with oJIA in the all-inclusive, >6yrs-diagnosed, or sex-stratified analyses, and only one probe was associated with oJIA in the ≤6yrs-diagnosed analysis. We attempted technical validation and replication of 14 probes ($p_{unadj}<0.01$) at genes of known/potential relevance to disease. At *VPS53*, we demonstrated a regional shift towards higher methylation in oJIA (all-inclusive) compared to controls. At *REEP3*, where polymorphism has been previously associated with JIA, we demonstrated higher DNA methylation in male oJIA compared to male controls. This is the most comprehensive JIA case-control analysis of DNA methylation to date. While we have generated some evidence of altered methylation in oJIA, substantial differences are not apparent in CD4$^+$ T cells. This may indicate a lesser relevance of DNA methylation levels in childhood, compared to adult, rheumatic disease.

1. Introduction

Juvenile idiopathic arthritis (JIA) is a complex childhood autoimmune rheumatic disease that encompasses seven subtypes, as defined by the International League of Associations for Rheumatology (ILAR) [1,2]. These are based on clinical presentation, phenotypic features, and serologic markers, and are characteristically different in their age of onset and ratios of affected females to males [1]. It is generally accepted that the current subtype classification system requires refinement, because there remains significant within-subtype heterogeneity [3]. Disease heterogeneity represents a major challenge in uncovering the pathogenesis of JIA.

Numerous lines of evidence have established JIA as a complex disease [4] [5], arising from the interaction of genetic and environmental risk factors. Comprehensive twin studies to determine the relative impact of genes and environment on JIA disease susceptibility are lacking. However, there is some evidence that monozygotic twin discordance may be in the range of 25–40% [6]

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indicating environmental contribution to disease risk. Recent work to quantify the contribution of common genetic variants to paediatric autoimmune diseases, including JIA, demonstrated that around three quarters of JIA susceptibility appears determined by such genetic variation, suggesting a moderate environmental component [7,8]. The presence of an environmental component is supported by recent data indicating sibling exposure, antibiotic exposure and sex as relevant environmental factors for JIA [9–12]. A key mediator of environmental influence on gene expression is epigenetic modification, defined as mitotically-stable alterations in gene expression in the absence of changes to DNA sequence [13]. The epigenome encompasses many layers including DNA methylation, histone modifications and non-coding RNAs, which interact to influence gene transcription, cell function, and disease risk [14].

There is a growing body of data associating epigenetic alterations with adult autoimmune rheumatic diseases, including rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) [15,16]. RA is an adult rheumatic disease with clinical and pathological similarities to JIA. At a genetic susceptibility level, RA and JIA overlap considerably [17]. However, evidence suggests that the environmental contribution to susceptibility may be greater for RA than for JIA, with RA monozygotic twin concordance rates around 15% [18], and several well-established environmental factors, such as smoking, that are known to impact risk [19]. DNA methylation variation has been associated with RA in disease-relevant cell types including whole blood [20], peripheral blood mononuclear cells (PBMCs) [21], synovial fibroblasts [22], T cells [23], and B cells [23]. In SLE, in which onset can occur at any age but is far more common in adulthood [24], there is also substantial evidence for the presence of DNA methylation alterations in various immune cell populations [25].

Determining the role of epigenetics in autoimmune rheumatic (and other) diseases provides opportunity to unveil previously-unrecognised disease pathways and new therapeutic molecular targets. Additionally, therapeutic approaches that correct epigenetic aberration by targeting epigenetic machinery are developing rapidly, and are of growing interest in rheumatic disease [26]. Given the evidence for a role for DNA methylation in adult autoimmune rheumatic diseases, but mindful of the fact that the environment may be relatively less relevant to JIA disease risk, we sought to determine whether substantial differences in DNA methylation are also associated with JIA.

Previously, we performed a small genome-scale analysis of $CD4^+$ T cell DNA methylation for JIA using medium density Illumina Infinium HumanMethylation27 (HM27) BeadChip arrays [27]. We chose $CD4^+$ T cells specifically since they play an established role in disease pathogenesis [28], and JIA-associated genetic variants are linked to $CD4^+$ T cell function [29]. We generated some evidence in this prior study to suggest that changes to DNA methylation may be associated with JIA. Therefore, in order to build on this past work, we have now carried out a larger and more comprehensive $CD4^+$ T cell JIA case-control analysis using the higher density HM450 array platform, with measurement of DNA methylation at >485,000 genomic sites [30,31]. Case heterogeneity was minimised by focusing on a single ILAR disease subtype – oligoarticular JIA (oJIA) [32], characterised by four or fewer joints affected at six months post-diagnosis, younger average age at onset, and a 2–3 times higher frequency in girls. We accounted for other sources of confounding by matching cases and controls by age and sex, by selecting only cases with active disease and no exposure to disease modifying anti-rheumatic drugs (DMARDs), and through the use of the RUV (Remove Unwanted Variation) algorithm [33] in our analyses. Further, we tested the hypothesis that accounting for age at onset and sex as sources of case heterogeneity [12,34] may more clearly reveal associations by re-performing our analyses following stratification by these factors.

2. Materials and methods

2.1. Participants

Subjects were drawn from the ChiLdhood Arthritis Risk factor Identification sTudY (CLARITY) [35]. Briefly, cases were diagnosed according to ILAR criteria by a paediatric rheumatologist at the Royal Children's Hospital (RCH) in Melbourne Australia. Controls were otherwise healthy children ≤16 years undergoing a minor surgical procedure in the RCH Day Surgery Unit. A peripheral blood sample was collected from each participant. The project was approved by the RCH Human Research Ethics Committee #27127.

For this study, 57 oJIA cases and 57 healthy controls were selected as the 'discovery' sample. Of these, 51 pairs were matched for both age (within 1 year) and sex. All cases had active disease and no prior exposure to methotrexate or biologic DMARDs. One case was subsequently excluded due to inactive disease. The average time from diagnosis to blood collection was 8.5 months. The female to male ratio was approximately 4:1. Using the same criteria, 19 oJIA age and sex matched case-control pairs were selected as a 'replication' sample. One additional unmatched male case and two additional unmatched female controls were also available for analysis and included to augment study power. Subsets of the replication sample were used for replication of different stratified analyses. The characteristics of the discovery and replication samples are shown in Supplementary Table 1.

2.2. $CD4^+$ T cell isolation and DNA extraction

The procedure for the isolation of total $CD4^+$ T cells from selected participants has been described previously [27]. Briefly, peripheral blood mononuclear cells (PBMCs) were isolated from blood samples using standard Ficoll gradient procedures within 24 h of blood collection and stored in vapour phase nitrogen until use. PBMCs were thawed at 37 °C. To isolate $CD3^+$ $CD4^+$ T cells, PBMCs were positively sorted using flow cytometry. Cells were gated according to viability, lymphocytes, then double positivity for CD3 and CD4. T-cell purity was typically ~98% and viability averaged 85%. $CD4^+$ T cell DNA was extracted using the Qiagen Flexigene kit (Qiagen, Hilden Germany) according to manufacturer's protocol.

2.3. Genome-scale DNA methylation raw data generation and preprocessing

Genomic DNA was submitted as a single batch to ServiceXS (Leiden, The Netherlands) and bisulphite converted using the EZ DNA Gold kit (Zymo, Irvine, USA). DNA methylation was measured using Illumina Infinium HumanMethylation450 BeadChip arrays (HM450) (San Diego, USA). To avoid potential confounding due to inter-array variability, cases were distributed randomly across arrays, with case-control pairs applied to the same array. DNA samples from two case-control pairs were used as technical replicates.

Raw data (idat files) were analysed in R using Bioconductor software packages [36]. The Minfi package [37] was used to preprocess raw signal intensities to methylation measurements, and also to detect any low-quality samples by inspecting the median of the methylated and unmethylated signal intensities. The SWAN (Subset-quantile Within Array Normalization) package [38] was used for probe type normalization by correcting for technical differences between type I and type II probes (which measure methylated and unmethylated signals using two paired probes, or use a single probe, respectively). X and Y chromosome probes were

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