Original Reports

Genome-Wide Epigenomic Analyses in Patients With Nociceptive and Neuropathic Chronic Pain Subtypes Reveals Alterations in Methylation of Genes Involved in the Neuro-Musculoskeletal System

Ludwig Stenz,* Joane Le Carré,†,‡ François Luthi,†,§,¶ Philippe Vuistiner,†,‡ Cyrille Burrus,†,§ Ariane Paoloni-Giacobino,*,* and Bertrand Léger†,†,*,a

†Department of Genetic Medicine and Development, Geneva University, Medicine Faculty, Geneva, Switzerland, †Institute for Research in Rehabilitation, Clinique romande de réadaptation, Sion, Switzerland, ‡Department of Medical Research, Clinique romande de réadaptation, Sion, Switzerland, §Department of Musculoskeletal Rehabilitation, Clinique romande de réadaptation, Sion, Switzerland, ¶Department of Physical Medicine and Rehabilitation, Orthopaedic Hospital, Lausanne University Hospital, Lausanne, Switzerland

Abstract: Nociceptive pain involves the activation of nociceptors without damage to the nervous system, whereas neuropathic pain is related to an alteration in the central or peripheral nervous system. Chronic pain itself and the transition from acute to chronic pain may be epigenetically controlled. In this cross-sectional study, a genome-wide DNA methylation analysis was performed using the blood DNA reduced representation bisulfite sequencing (RRBS) technique. Three prospective cohorts including 20 healthy controls (CTL), 18 patients with chronic nociceptive pain (NOCI), and 19 patients with chronic neuropathic pain (NEURO) were compared at both the single CpG and differentially methylated region (DMR) levels. Genes with DMRs were seen in the NOCI and NEURO groups belonged to the neuro-musculoskeletal system and differed between NOCI and NEURO patients. Our results demonstrate that the epigenetic disturbances accompanying nociceptive pain are very different from those accompanying neuropathic pain. In the former, among others, the epigenetic disturbance observed would affect the function of the opioid analgesic system, whereas in the latter it would affect that of the GABAergic reward system. This study presents biological findings that help to characterize NOCI- and NEURO-affected pathways and opens the possibility of developing epigenetic diagnostic assays.

Perspectives: Our results help to explain the various biological pathways modifications underlying the different clinical manifestations of nociceptive and neuropathic pains. Furthermore, the new targets identified in our study might help to discover more specific treatments for nociceptive or neuropathic pains.

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*Contributed equally.

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Address reprint requests to Bertrand Léger, PhD, Department of Medical Research, Clinique romande de réadaptation, Avenue Grand Champsec 90 1951, Sion, Switzerland. E-mail: Bertrand.Leger@crr-suva.ch

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Chronic pain conditions demonstrate complex interactions between biological, psychological, environmental, and social factors, and their etiology and physiopathology remain obscure. In 2019, the International Association for the Study of Pain (IASP) defined 2 main types of pain, nociceptive and neuropathic, and established various criteria for their description. Concerning chronic nociceptive pain (NOCI), the trigger is an activation of the nociceptors, either at the surface of the body (ie, skin) or at a deeper level (ie, muscle, bones, joints, or tendons), without damage to the somatosensory nervous system. Patients experiencing NOCI report pain localized in the area of the injury or of musculoskeletal origin. In chronic neuropathic pain (NEURO), the trigger is an alteration in the central or peripheral nervous system, such as trauma or infection. Patients experiencing NEURO report sensations of burning, numbness, tingling, mechanical hypersensitivity, or allodynia or hyperalgesia.

These 2 types of pain, in particular that which occurs due to an inflammatory response, induce transcriptional and translational changes in neurons at the central level that induce pain hypersensitization by altering the pain threshold.

The epigenome is the interface between signals from the environment and genetic modifications that affect gene expression. Epigenetic factors modify the DNA structure through the (de)methylation of subsets of CpG islands, named differentially methylated regions (DMRs). Methylation in the promoter region generally induces a decrease in the expression of the corresponding gene. Although epigenetic modifications are needed for normal development, they may also be responsible for some disease states. Various environmental factors, such as diet, pollutants, and psychological stressors, have been associated with epigenetic changes.

Epigenetic factors might be involved in the transition from acute to chronic pain and chronic pain maintenance. A few studies have confirmed the epigenetic control of the 2 main types of chronic pain, NOCI and NEURO, in humans. Concerning NOCI, 2 genome-wide methylation analyses have been performed in patients with chronic musculoskeletal pain showing DMRs at sites enriched in neurological pathways and in the immune and GABAergic signaling systems. In NEURO patients, epigenetic mechanisms have been shown to control the altered expression of neuronal ion channels and receptors, as well as glial cell and macrophage proinflammatory cytokine and chemokine production. Methylation patterns associated with chronic neuropathic pain occurring in chemotherapy or diabetes have also been described. Peripheral neuropathy induced in some breast cancer patients by chemotherapy treatment was associated with differential methylation in the hypoxia-inducible factor I (HIF-I) signaling pathway. A genome-wide methylation study of diabetic mice with neuropathic pain allowed identifying gene pathways contributing to diabetes or pain. However, no genome-wide methylation analysis has been performed to date in patients with NEURO.

A systematic characterization of the DNA methylation profiles of the 2 main types of chronic pain, NOCI and NEURO, is needed in humans. Furthermore, it would be interesting to compare the epigenetic signatures of patients with NOCI and NEURO to better understand their molecular identities. Therefore, by recruiting relatively large cohorts of patients, we aimed to compare blood DNA methylation levels in NOCI and NEURO patients with healthy controls (CTL). The main hypothesis guiding our investigations was that DNA methylation levels of patient NOCI and NEURO differs both from CTL and also from each other. To test this hypothesis we performed a genome wide blood DNA methylation analysis of patients with different origin of pain and healthy controls.

Methods

Study Population

Patients were recruited from the Musculoskeletal Rehabilitation Department of the Clinique romande de réadaptation between August 2015 and January 2018. Patients came to our service to complete their reeducation program after a trauma because of a need for intensification of therapies that was not possible on an ambulatory basis, or when the evolution needed the evaluation of multidisciplinary specialists. Patients were addressed to our center by their physician or by the insurance in charge after their injury. In our department, patients mainly suffer from musculoskeletal deficiency after fractures, ligamentous injuries or soft tissue contusions. Patients over 18-year-old, suffering from chronic post-traumatic pain with neuropathic or nociceptive characteristics were included. Exclusion criteria were: history of diabetes, blood or infectious disorders (hepatitis, thalassemia, HIV or systemic infections), widespread pain syndrome and severe psychiatric comorbidity. Patients were also excluded if they suffered from major health conditions such as malignancy or chronic inflammatory disease (eg, rheumatoid arthritis, systemic lupus erythematosus). Upon admission, a senior clinician assessed whether the patients met the inclusion criteria. Based on a thorough medical examination and the results of the DN4 questionnaire patients were classified into one of the 2 groups of chronic pain (NEURO or NOCI) according to the recommendations of the IASP. Categorization as NEURO patients was done after a specialized neurological evaluation including ENMG and/or imaging to confirm the plausibility of the peripheral lesion causing the neuropathic pain. Twenty healthy volunteers matched for sex, age, and BMI who reported no pain and taking no medication were recruited as the control group. An approximate number of patients were estimated from a previous study on DNA methylation profile in pain patients.

In order to minimize potential sources of bias, patients were matched on the basis of their age, sex, BMI, time since injury, location of the lesion, pain medication, pain levels, anxiety and depression levels. Psychological status and pain level data were obtained
using validated questionnaires, while clinical information and medication status were collected from medical records. After obtaining informed consent, all volunteers were scheduled for blood sampling, as previously described.\textsuperscript{15} The study protocol was approved by the local ethics committee (CCVEM 034/12) and was conducted according to the recommendations of the Declaration of Helsinki.

Questionnaires

The DN4 is a validated questionnaire that has been developed to diagnose polyneuropathy.\textsuperscript{6} DN4 is a clinician-administered questionnaire composed of 7 items related to pain symptoms, and 3 items linked with neuropathic pain examination. Healthcare professionals assess whether there is a reduced sensation to touch or a pinprick (hypoesthesia) and whether light brushing increases or causes pain (allodynia).

The Brief Pain Inventory (BPI) is a self-administered questionnaire that allows patients to rate the severity of their pain and the degree to which their pain interferes with the common components of feeling and function. It has been broadly used in studies on pain.\textsuperscript{13}

The Hospital Anxiety and Depression Scale (HADS) was used to detect states of anxiety and depression. It is a self-administered questionnaire that has been specifically developed in a hospital medical outpatient clinical setting.\textsuperscript{54}

DNA Extraction and Quality Control

DNA was extracted from blood samples in 3 cohorts composed of 20 CTL, 20 patients with NOCI, and 20 patients with NEURO using the DNeasy Blood and Tissue kit (Qiagen, Hildon, Germany) with minor modifications. Briefly, 40 μL of proteinase K and 200 μL of PBS were added to 200 μL of whole blood. RNA was then removed following the addition of 800 μg of RNase A. After incubation for 2 minutes, 400 μL of Buffer AL was added to the RNA-free mix and incubated for 15 minutes at 56°C. Before loading the solution onto the spin column, 400 μL of EtOH was added to the mixture. The next steps of the protocol were followed according to the manufacturer’s instructions. The elution step was performed twice with 200 μL in 2 separate microtubes to increase DNA yield. DNA concentrations were measured using the Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific, Waltham, MA). DNA quality (relative to fragmentation levels) was assessed using the Fragment Analyzer and the DNF-488 High Sensitivity Genomic DNA Analysis Kit (Agilent, Santa Clara, CA). Three samples did not pass QC (pens at 12.3, 12.4 and 11.1 kb). Fifty-seven samples passed QC (size of DNA presenting a pen at 17 ± 2 kb); consequently 20 CTL, 18 patients with NOCI, and 19 patients with NEURO were included in the analysis.

Preparation of RRBS Libraries and Sequencing

DNA was analyzed for cytosine methylation on a genome-wide scale using reduced representation bisulfite sequencing (RRBS). RRBS experiments were conducted using DNA methylation profiling (RRBS Service, Diagenode, Cat. G02020000). Libraries were generated and sequenced in paired-end mode of 50 bp using a HiSeq4000 instrument (Illumina Inc., San Diego, CA) using one full lane for 5 multiplexed samples.

Analyses of RRBS data were performed using a bioinformatics pipeline written in Bash, Python, and R developed by Matthias Beyens (BISC Global, Gent, Oost-Vlaanderen, Belgium). We implemented the pipeline directly in a high-performance computing cluster (HPC) named “Baobab” that belongs to the University of Geneva. We performed 3 comparisons: CTL versus NOCI, CTL versus NEURO, and NOCI versus NEURO. The pipeline performed the bseq-based detection of DMRs and the Fisher test of methylation levels at CpG sites on each chromosome separately. For each comparison, the different tables containing the results obtained on each chromosome in R were merged before the execution of the downstream steps (overlapping, consistency, and Wilcoxon testing, explained afterward).

Read Trimming and Mapping

We trimmed the reads using TrimGalore (version 0.6.0) with the following options: --non-directional, --paired, --rrbs, --phred33, and with cutadapt (version 2.3) using the following options: --quality 30, --length 35. Trimmed reads were mapped against the human bisulfite genome (assembly GRCh38) using Bismark (version 0.22.1) running with Bowtie 2 (version 2.3.5.1) and with the specified options: --q, --phred33, --score-min L0,0,2, --ignore-quals, --no-mixed, --no-discordant, --devtail, --maxins 1000, option-nondirectional. Alignment was performed on all strands (OT, OB, CTOT, and CTOB). We used the 3-letter aligner Bismark to avoid overestimation of methylation levels appropriate for the wildcard aligner.\textsuperscript{5}

Methylation Extraction

Methylation calls for every analyzed cytosine were performed using the Bismark methylation extractor algorithm with options --paired-end, --gzip, --bed Graph, --multicore 10, --cytosine_report, --ignore 3, --ignore_r2 3, --ignore_sPrime_r2.\textsuperscript{28} The “ignore” options corrected for the bias in methylation values found at the extremities of the reads.

C/T SNP Filtration

Using a BASH script, we removed the methylation levels determined using the RRBS analysis that were located within known single nucleotide polymorphisms (SNPs) involving cytosine to thymine changes (C/T-SNP, 1000 genome project phase 3, ftp://ftp.ensembl.org/).

Identification of Differentially Methylated CpG

We used the fisherTests in the Bseq package to test for differences in methylation between 2 groups for each of the 3 comparisons for all CpGs obtained after the SNP filtering process.\textsuperscript{22} We only kept CpGs with a P-
value resisting Bonferroni correction for multiple testing (P < 1.7e-9) and with a finite value for the logarithm in base 2 of the odds ratio, “log_{2}(OR).” Infinite values of log_{2}(OR) are due to division by zero and were not considered for further analysis.

Identification of Differentially Methylated Regions

The BSmooth open source package was used to identify DMRs in R, taking biological variation into account. The quantile-based cutoff of the t-statistic was set at 0.025 and 0.975, and methylation changes had to be present in at least 3 samples of the group with an absolute change in methylation of ≥ 0.25. The bseq::dmrFinder function identified the DMRs reported based on the meanDiff and areaStat.

Identification of Overlap Between DMRs and CpG

This step validated DMRs and CpGs using 2 different approaches, resulting in the concordance of evidence. The GenomicRanges package was used for overlapping purposes. Files containing the results of the Fisher exact test performed on individual CpGs and files containing the annotated DMRs were converted into GRanges objects using the “makeGRangesFromDataFrame” function in R. The “subsetByOverlaps” function in R identified DMRs overlapping with CpGs and CpGs overlapping with DMRs. Finally, we selected CpGs and DMRs that demonstrated consistent changes.

Annotation of DMRs and CpGs

DMRs were annotated depending on their overlap notably with the official gene symbols, as well as with the following genes structures: “1 to 5kb” (5-kb region upstream of promoters), “promoters”, “5UTRs”, “3UTRs”, “exons”, “introns” and “intronexonboundaries” (200 bp up/downstream of any boundary between an exon and intron). CpGs were annotated depending on their distance from a CpG island (CGI) as “islands” (overlapping), “shores” (2 kb of distance from one end), “shelf” (2–4 kb distance), and “inter” (>4 kb) using the Bioconductor package, “annotatr.” We manually annotated the CpGs that overlapped with DMRs.

Consistency

We defined consistency as methylation changes observed between significant CpGs and overlapping DMRs varying in the same direction according to the 2 groups compared. This means that if a CpG is hypermethylated in one group, its associated overlapping DMR should be hypermethylated in the same group and vice versa. Consistency was assessed using logical tests in Excel.

Functional Analysis

Functional networks were detected using STRING (https://string-db.org/) following online submission of the lists of genes that were either hyper- or hypomethylated in each of the 3 comparisons performed. The reported gene functions were derived from the available literature.

Statistical Analyses

The differences in socio-demographic and psychological data between the groups were evaluated by the Wilcoxon-Mann-Whitney rank-sum test. No corrections for potential batch effects were performed as all the samples were treated as a single batch for all experimental steps, including bisulfite treatment, RRBS libraries preparation and sequencing. We also performed nonparametric Wilcoxon tests between the

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Neuropathic (N = 19)</th>
<th>Nociceptive (N = 18)</th>
<th>Controls (N = 20)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>44 (14)</td>
<td>43 (10)</td>
<td>40 (10)</td>
<td>.54</td>
</tr>
<tr>
<td>Sex F</td>
<td>4 (21%)</td>
<td>3 (17%)</td>
<td>7 (35%)</td>
<td>.40</td>
</tr>
<tr>
<td>BMI</td>
<td>27 (3)</td>
<td>27 (5)</td>
<td>25 (3)</td>
<td>.08</td>
</tr>
<tr>
<td>Time since injury (days)</td>
<td>488 (90–1349)</td>
<td>452 (213–1061)</td>
<td>-</td>
<td>.62</td>
</tr>
<tr>
<td>DN4 ≥ 4</td>
<td>19 (100%)</td>
<td>0 (0%)</td>
<td>-</td>
<td>&lt;10^{-6}***</td>
</tr>
<tr>
<td>Pain location</td>
<td></td>
<td></td>
<td></td>
<td>1.00</td>
</tr>
<tr>
<td>- Upper limb</td>
<td>7 (37%)</td>
<td>6 (33%)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>- Lower limb</td>
<td>7 (37%)</td>
<td>7 (39%)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>- Rachis</td>
<td>4 (21%)</td>
<td>5 (28%)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>- Multiple pain locations</td>
<td>1 (5%)</td>
<td>0 (0%)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Medication (patients taking any medication)</td>
<td>15 (79%)</td>
<td>14 (78%)</td>
<td>-</td>
<td>1.00</td>
</tr>
<tr>
<td>BPI pain severity (0-10)</td>
<td>4.5 (2.1)</td>
<td>4.5 (0.1)</td>
<td>-</td>
<td>.96</td>
</tr>
<tr>
<td>HADS Anxiety (0-21)</td>
<td>9.9 (4.4)</td>
<td>10.2 (3.6)</td>
<td>-</td>
<td>.84</td>
</tr>
<tr>
<td>HADS Depression (0-21)</td>
<td>7.4 (3.9)</td>
<td>8.1 (4.1)</td>
<td>-</td>
<td>.63</td>
</tr>
</tbody>
</table>

Abbreviations: BMI, body mass index; DN4, Douleur Neuropathique en 4 Questions; BPI, Brief Pain Inventory; HADS, Hospital Anxiety and Depression Scale; TSK, Tampa Scale of Kinesiophobia; PCS, Pain Catastrophizing Scale; CIRS, Cumulative Illness Rating Scale.

The values are expressed as mean (standard deviation), median (range) or number (%).

***P < .001.
compared groups in R using methylation percentages recorded at the single cytosine level taking into account coverage. We extracted the methylated (Cmethyl) and unmethylated (Cunmethyl), as well as the methylation levels from all samples, for all selected sites from the Bis-mark SNP-filtered coverage files. The percentage of methylation was equal to Cmethyl/(Cmethyl + Cunmethyl) x 100, and coverage was equal to (Cmethyl + Cunmethyl)/number of samples.

Results

Patient Characteristics

There was no significant difference in clinical or psychological features (pain severity, anxiety and depression) between the 2 groups of patients, except for the results of the DN4 questionnaire, which was designed to discriminate neuropathic pain from other types (Table 1). As opioids were previously demonstrated to potentially influence methylation levels, we carefully monitored medication intake for the entire cohort. Interestingly, there was no significant difference in medication use between the 2 groups of patients for the different classes of medications investigated (Supplementary Table 1).

Global DNA Methylation Changes at DMR and CpG Sites

Through the RRBS analysis, we tested for differences in the distribution of methylation and unmethylation signals using the Fisher exact test for approximately 6 million CpGs in each comparison (CTL vs NOCI, CTL vs NEURO, NOCI vs NEURO). We found 56,572 CpGs for the CTL versus NOCI, 35,774 for the CTL versus NEURO, and 70,730 for the NOCI versus NEURO comparison were below the genome-wide significant P-value threshold of 1.7e-9 and produced finite values for the log2(OR) parameter. The analysis of DMRs showed 2,066 significant DMRs in the CTL versus NOCI, 1,098 in the CTL versus NEURO, and 765 in the NOCI versus NEURO comparison (Fig 1A).

Based on the overlap and consistency criteria, as well as the Wilcoxon test results (see methods), 26 DMRs were finally identified in the CTL versus NOCI, 8 DMRs in CTL versus NEURO and 20 DMR in NOCI versus NEURO.

The genomic distribution of DMRs in the 3 groups was analyzed. Approximately 78 to 84% of DMRs were located in intergenic or intragenic regions, 13 to 17% in the promoter region, and 3 to 5% in the exon region (Supplementary Fig 1). The distribution of DMRs between chromosomes is shown in Supplementary Fig 2.

Genome-Wide Blood DNA Methylation Changes in NOCI and NEURO Patients

When NOCI and NEURO patients were compared to CTL, more DMRs were identified in the NOCI group than in the NEURO group. Specifically, 39 CpGs overlapping 26 DMRs in the NOCI group compared with 8 CpGs overlapping 8 DMRs in the NEURO group were identified. This represented 4.9 and 3.3 more CpGs and DMRs, respectively in the NOCI group than in the NEURO group.
**Functional Analyses of DMR Genes in NOCI and NEURO Patients Shows Enrichment in Neuro-Musculoskeletal System-Related Genes**

The direction of the methylation changes and the biological role of the genes with DMRs identified in the CTL versus NOCI, CTL versus NEURO, and NOCI versus NEURO comparisons are shown in Fig 2. For the first functional analysis, DMR genes were segregated according to the tissue or system to which they belong, and 5 main families were identified: the nervous system (central and peripheral); skeletal muscle, bone, and connective tissue; and the inflammatory and immune systems. A few genes had 2 different tissues or system affiliations, which are also taken into account in Fig 2. Finally, networks were identified in each group comparison, as shown in Fig 2.

**CTL versus NOCI**

In the CTL versus NOCI comparison, robust enrichment was observed in the genes with DMRs associated with the neuro-musculoskeletal system (12/26 genes with DMRs, 48%). Ten genes with DMRs were found to be related to the nervous system, 2 to the skeletal muscle, and 3 to the bone. Eight genes with DMRs were related to the immune system or inflammation.

Functional analyses of genes with DMRs resulted in the identification of significant enrichment for the protein domain family PF12414 (false discovery rate [FDR] = .001) in RBFOX-1 and RBFOX-3. RBFOX-1 mediates the neuron-specific splicing of the calcitonin gene, allowing for the neuronal production of calcitonin gene-related peptide (CGRP). Interestingly, RBFOX-3 was also associated with CNP in the STRING cluster (CL:619, FDR = .02), which is involved in neuronal adhesion (Fig 2, upper panel).

**CTL versus NEURO**

In the comparison between the CTL and NEURO groups, a large prevalence of genes with DMRs associated with the neuro-musculoskeletal system was observed (6/8 DMR genes, 75%). Five genes with DMRs were related to the nervous system, one was related to the skeletal muscle, and one was related to the bone. One gene with DMRs was associated with the immune system.

Functional analyses of genes with DMRs resulted in the identification of 2 independent significant enrichments. The first enrichment (FDR = .02) was observed in the histone acetylation pathway (HAS-3214847) in the ELP2 and KANSL1 genes. A second enrichment (FDR = .01) was associated with the calcium homeostasis STRING cluster (CL:9220) in the STOM and PVALB genes (Fig 2, middle panel).

**NOCI versus NEURO**

In the comparison between the NOCI and NEURO groups, a robust enrichment in genes with DMRs associated with the neuro-musculoskeletal system was observed (9/20 genes with DMRs, 45%). Six genes with DMRs were related to the nervous system, 2 were related to the skeletal muscle, and 4 were related to the bone. No genes with DMRs were associated with inflammation.

Functional analyses identified 3 independent enrichments. The first enrichment (FDR = .003) is the “vesicle-mediated transport” (GO:0006892) involving the Golgi-related protein TGN2 in the RAB10 genes. The second enrichment (FDR = .03) is the GO term “negative regulation of steroid” (GO:0010894) in the GFI1 and DKK3 genes. The third enrichment is “hematopoietic stem cell differentiation” (CL:17858) in the DIDO1 and C5orf56 genes (Fig 2, lower panel).

Supplementary Table 2 presents a list of the genes described in Fig 2, organized by selected tissues, cell types, and systems.

**Discussion**

The results of this study, keeping in mind the relatively limited size of the cohorts, suggest the existence of NOCI and NEURO-related epigenetic signatures. The 2 patient cohorts in this study were relatively small compared with those in previous chronic pain epigenome-wide studies; however, the different types of pain, ie, NOCI and NEURO, make the interpretation of the results particularly interesting.

Our epigenome-wide study was performed on whole blood because of the impossibility of obtaining human peripheral or central nervous system samples in living individuals. Previous studies have shown a correlation between brain tissue and blood methylation patterns. Davies et al reported that some interindividual variations were reflected between brain and blood, although between-tissue variations could exceed it. Strong interindividual correlation between brain and blood was observed in humans and rats. It is generally admitted that blood DNA methylation can be used as a proxy for brain and/or other tissue-specific alterations. This justifies the extrapolation of data obtained from the whole blood to different human tissues, and blood was used as a proxy for the nervous system.
NOCI and NEURO differ in their etiology and consequences but have a common main symptom, pain, although it has different characteristics. At the statistical stringency used in this study, the epigenetic signatures in NOCI and NEURO groups differ from each other with no overlap. Specific epigenetic effects can be induced either by the cause or secondary effects of chronic pain.

**Figure 2.** Functional analysis of genes with DMRs in NOCI and NEURO patients. Genes with DMRs were identified in the comparison groups as shown: CTL versus NOCI in the upper panel, CTL versus NEURO in the middle panel, and NOCI versus NEURO in the lower panel. For each comparison, genes with DMRs were segregated according to the direction of the methylation changes observed: hypomethylation in the left and hypermethylation in the right column. Genes that belong to a functional network as detected by STRING appear surrounded and linked together by a line, with the corresponding description and code as well as the false discovery rate (FDR). If there is no line between 2 genes, the interaction between these genes is not detectable by the STRING program. This may either reflect a true absence of connection or insufficient knowledge. Functional networks may imply Homo sapiens (HSA) pathways either based on cellular processes (Kyoto Encyclopedia of Genes and Genomes, KEGG), or based on molecular reactions (Reactome), a gene ontology term (GO), functionally related protein family (INTERPRO), conserved protein domain (PF), and STRING cluster (CL). Genes with a functional link to the nervous system are shown in purple, those linked to the muscle in red, those linked to the bone and connective tissue (referred to as bone) in white, and those linked to the inflammatory system or immunity in green. For genes linked to 2 different systems, 2 different colors were used, one for the gene name and the other for the surrounding frame. Genes with no links to the above systems are shown as dashed squares.
When interpreting the results, it should be kept in mind that false positives may still be present among the statistically significant genes with DMRs, but maximum care was taken to minimize their possibility. The criteria for selection could be classified as follows: a strong statistical significance of the comparisons, the biological relevance of the findings, and the presence of significantly enriched pathways. The statistical significance refers to the distribution of the C methylation and T unmethylation signals at the tested CpGs using the Fisher test; second, the methylation levels at the CpGs (nonparametric Wilcoxon tests) and third, converging identifications with 2 independent approaches (a smoothing algorithm for DMRs and CpGs tested individually).

**Top Statistically Significant Candidates**

The 5 DMRs present not only in the NOCI versus CTL comparison, but also in the NOCI versus NEURO comparison may be considered top candidates. The relevance of the 5 top candidates was validated based on the observation that the direction of the methylation changes was the same in the 2 comparisons. RAB10, BMP1, and LRRC59 were hypomethylated in the NOCI group in both comparisons (NOCI vs CTL and NOCI vs NEURO), whereas PNPLA6 and P3H3 were hypermethylated.

**Biological Relevance**

In the NOCI and NEURO groups, the function of the 4 genes might be associated with the causes or effects of pain: RAB10, RBFOX1/CGRP, MAGI2, and PVALB. RAB10, a member of the small GTPase family, was reported to increase the cell surface expression of the δ-opioid receptor (DOPr) in the nervous system and was therefore proposed to increase DOPr-agonist-mediated analgesia. RAB10 hypomethylation in patients with NOCI would most likely increase its expression. Upregulation of the opioid system was observed under other conditions. Inflammation was associated with hippocampal and spinal cord hyperalgesia and reduced expression of D2 dopamine receptor (D2R) in the spinal cord. The mechanism by which RAB10 hypermethylation was associated with decreases in D2R expression is unknown. RAB10 hypomethylation might therefore be associated with decreases in D2R expression. Additionally, RAB10 hypermethylation might contribute to increases in the expression of the opioid system by increasing the expression of these genes in neurons. The observation that MAGI2 and PVALB are differentially methylated in the NEURO group is in line with the involvement of GABAergic signaling in neuropathic pain.

**Significantly Enriched Pathways**

The involvement of a gene in an enrichment pathway should validate the relevance of its association with either the NOCI or NEURO.

The first enriched pathway detected in the NOCI group (Fig 2) involved RBFOX1 and RBFOX3, both of which play an important role in the nervous system. It is known that RBFOX family proteins interact, forming homo- and hetero-oligomeric complexes. RBFOX1 knockout has been found to result in brain defects in cytoskeletal membranes and synaptic proteins. RBFOX1 is connected with brain-derived neurotrophic factor (BDNF), a potent modulator of brain synaptic plasticity and stimulates the neuronal synthesis of CGRP involved in the transmission of nociceptive pain. The second enrichment pathway detected in the NOCI group involved RBFOX3 and CNP. The latter gene is expressed in myelin-forming oligodendrocytes, and a missense variant of CNP is the cause of hypomyelinating leukodystrophy in humans.1

The 4 genes implicated in the 2 enrichments detected in the NEURO group were all associated with the neuromuscularoskeletal system. ELP2 is an inhibitor of osteoblast differentiation and KANSL1, found to be genetically associated with intellectual disability, should play a role in neurons. PVALB is associated with both muscles and neurons involved in muscle relaxation and GABAergic interneurons. Finally, STOM encodes stomatin, which has been shown to be involved in sensory neuron mechanotransduction.

Three enrichment pathways were identified in the NOCI versus NEURO comparison. The first involves TGOLN2 and RAB10, the latter of which was found to sustain DOP receptor action, as well as CCDC91, which is associated with hip bone geometry and mechanical stress-induced ossification. A second enrichment involves GF11 and DKK3, with the latter implicated in muscle atrophy and impaired osteogenesis.

It is noteworthy that PVALB and RAB10, 2 of the 3 genes with DMRs highlighted above for their particular functional relevance to pain are found in the enriched pathways, supporting the fact that they might be strong candidates for chronic pain regulation.

**Differences Between NOCI and NEURO Signatures**

Comparison of the NOCI and NEURO DMRs shows that the 2 types of pain have different epigenetic targets.

NOCI epigenetically affects the opioid receptor by possibly modulating RAB10 and CGRP via RBFOX1 and 3, whereas NEURO epigenetically affects the GABAergic system by possibly modulating MAG12 and PVALB. This suggests that NOCI and NEURO epigenetically affect...
pain genes involved in different pathophysiological processes. These results are consistent with a recent study by our group that showed completely different miRNA signatures in NOCI and NEURO patients. Genes with DMRs connected to the inflammatory system were detected only in the NOCI group, suggesting the more specific involvement of the inflammatory system in NOCI. Genes connected to the musculoskeletal system, although predominant in the NOCI, were also present in the NEURO group. Even if NEURO patients were selected in a population of patients with peripheral nervous system injury they all had musculoskeletal trauma that may explain this observation.

Limitations

Nociplastic combination may be part of the process and was not addressed. We cannot rule out some nociplastic contribution to the chronic pain after a long postinjury evolution. The totality of our NOCI population suffered of musculoskeletal injury and in some cases had undergone surgery following trauma. Patients with widespread pain conditions or other nociplastic pain features (mechanical or thermal allodynia and/or any other clinically evoked pain hypersensitivity phenomena) were not included. Nevertheless, some nociplastic adaptations may have developed in combination with nociceptive or neuropathic pain processes.

Epigenetic analyses and data obtained from blood cells were used here as a proxy for other tissues.

Hydroxymethylation of DNA cannot be discriminated from methylation using the RRBS technique.

The size of the cohorts is limited, which may reduce the statistical power and do not allow, for example, discrimination of the results according to gender. Moreover, this cross-sectional study does not allow us to exclude the possibility that DNA methylation levels change over time. A longitudinal study, would be necessary to evaluate possible variations in DNA methylation linked to the process of pain chronicization.

Conclusions

In conclusion, this epigenome-wide study identified CpGs methylation signatures in the blood, discriminating nociceptive from neuropathic types of chronic pain. Despite a relatively low number of patients, our model is quite robust and allows to reach a good level of generalization as our cohorts were matched for several variables known to influence methylation process (age, sex, medication, psychological factors, . . .). Genes associated with these signatures appear to have essential functions in the neuro-musculoskeletal system. Additional studies are required to confirm these results.
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methylation landscape of prefrontal cortex and peripheral T cells. Sci Rep 6:19615, 2016


