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Research Paper

A Molecular Signature of Myalgia in Myotonic Dystrophy 2

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Background: Chronic muscle pain affects close to 20% of the population and is a major health burden. The underlying mechanisms of muscle pain are difficult to investigate as pain presents in patients with very diverse histories. Treatment options are therefore limited and not tailored to underlying mechanisms. To gain insight into the pathophysiology of myalgia we investigated a homogeneous group of patients suffering from myotonic dystrophy type 2 (DM2), a monogenic disorder presenting with myalgia in at least 50% of affected patients.
Methods: After IRB approval we performed an observational cross-sectional cohort study and recruited 42 patients with genetically confirmed DM2 plus 20 healthy age and gender matched control subjects. All participants were subjected to an extensive sensory-testing protocol. In addition, RNA sequencing was performed from 12 muscle biopsy specimens obtained from DM2 patients.
Findings: Clinical sensory testing as well as RNA sequencing clearly separated DM2 myalgic from non-myalgia patients and also from healthy controls. In particular pressure pain thresholds were significantly lowered for all muscles tested in myalgic DM2 patients but were not significantly different between non-myalgic patients and healthy controls. The expression of fourteen muscle expressed genes in myalgic patients was significantly up or down-regulated in myalgic compared to non-myalgic DM2 patients.
Interpretation: Our data support the idea that molecular changes in the muscles of DM2 patients are associated with muscle pain. Further studies should address whether muscle-specific molecular pathways play a significant role in myalgia in order to facilitate the development of mechanism-based therapeutic strategies to treat musculoskeletal pain.
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1. Introduction

Chronic pain affects 11–24% of the world's population and as such represents a major burden on health services (Breivik et al., 2006). A substantial proportion of pain patients suffer from musculoskeletal pain (McBeth and Jones, 2007). Treatments for muscle pain and myalgia are often ineffective and have not been tailored to treat pain which is associated with a diverse range of pathologies. In order to gain insight into the molecular pathology of pain in a genetically uniform group we have studied patients with myotonic dystrophy type 2 (DM2) (Udd and Krahe, 2012). DM2 is an autosomal dominant multisystem disorder caused by a CCTG repeat expansion of the cellular nucleic acid-binding protein, *CNBP* (Liquori et al., 2001). Mutant transcripts lead to a toxic RNA gain of function and miss-splicing of several effector genes (Charlet-B et al., 2002; Tang et al., 2012). Many, but not all DM2 patients, complain of chronic muscle pain (George et al., 2004; Suokas et al., 2012). The clinical features are highly variable and include late-onset progressive muscle weakness, myotonia, cardiac conduction defects, early-onset cataracts and insulin resistance (Rhodes et al., 2012; Savkur et al., 2004; Udd and Krahe, 2012; Wahbi et al., 2009). How the *CNBP* mutation confers risk for muscle pain in some patients but not others is unknown and is the key question addressed in this study.

Quantitative sensory testing (QST) is a standardized technique to assess human somatosensory function and document altered nociceptive signal processing (Backonja et al., 2013). By determining pain and detection threshold to external mechanical and thermal stimuli, sensory

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profiles are generated that can potentially trace underlying pathophysiological mechanisms. QST profiles have been made of patients with muscle-related disorders such as fibromyalgia, chronic back pain and myogenic temporomandibular disease revealing similarities as well as differences that may mirror distinct neurobiological mechanisms (Blumenstiel et al., 2011; Pfau et al., 2009). We used a comprehensive QST assessment to characterize the sensory phenotype of our cohort, a pre-requisite for identifying molecular signatures of muscle pain. Our analysis of the clinical and molecular profile of muscle pain in DM2 has enabled us to identify molecular signals in the affected muscle that segregate with muscle pain.

2. Methods

2.1. Study design and participants

In this cross-sectional study we investigated a cohort of 42 DM2 patients and 20 age and gender-matched healthy controls between March 2013 and January 2015. All DM2 patients were recruited from Muscle Disorders Outpatient Clinic at Charité Campus Buch, Berlin, Germany. The local ethics committee (EA1-127-14) approved the study. All patients and healthy subjects signed the written informed consent forms. Inclusion criteria were age > 18 years and molecularly confirmed diagnosis of DM2. Exclusion criteria were additional neurological disorders that could affect sensory function (e.g. stroke) or treatment with opioid analgesics (Supplementary Fig. 1). Healthy volunteers were excluded if they had diabetes, hypertension, neurological disorders affecting sensory function, took analgesics or had muscle pain in the last 3 months. We also obtained written informed consent from 12 DM2 patients, who underwent muscle biopsies for diagnostic purposes between 2004 and 2014, to subject their stored muscle biopsy specimens to RNA Seq analysis.

2.2. Clinical assessment of DM2 patients

Patients were asked about current unpleasant or painful sensations in their muscles lasting for more than 3 months. They were asked to rate the (1) unpleasant muscle sensation/pain, (2) muscle weakness and (3) muscle stiffness on a visual analogue scale (score of 0 for "no symptom" score of 10 for "worst imaginable intensity of symptom"). Patients with DM2 were allocated to either the myalgia or no myalgia group based on positive history of muscle pain and pain rating on the testing day. Patients indicated on a drawing where the pain was located. Frequency, duration, and modulating factors of pain such as temperature and movement were also recorded. Patients completed the German version of the McGill Pain Questionnaire (MPQ). Pain rating index (MPQ-PRI), number of words chosen (MPQ-NWC) and present pain index (MPQ-PPI) were calculated. Past medical history was obtained including presence of comorbidities, recent laboratory values, current pain medication, smoking status, education and work status. Genetic diagnosis of DM2 was performed at the Institute for Medical Genetics, University of Würzburg, Germany.

2.3. Sensory testing protocols

We used a comprehensive, multimodal, QST protocol to generate somatosensory profiles for each DM2 patient and healthy controls (Rolke et al., 2006). We assessed pain thresholds for skin and muscle tissue. Skin thermal and mechanical testing was performed in a unilateral fashion (dominant hand side) over the hand dorsum, shoulder and thigh. Thresholds for pressure pain were obtained over eight muscles on the left and right side of the body: extensor digitorum communis, deltoid, quadriceps and anterior tibialis. The repertoire of pain tests included pressure pain threshold (PPT), mechanical pain threshold (MPT), mechanical pain sensitivity (MPS), dynamic mechanical allodynia and wind-up ratio (WUR). Thermal detection tasks included warm detection threshold (WDT), cold detection threshold (CDT), heat pain threshold (HPT) (Frenzel et al., 2012) and the paradoxical heat detection (PHS). Pain testing procedures, instruments and methods were used strictly according to those prescribed by the German research network on neuropathic pain (Rolke et al., 2006).

2.4. Transcriptomic analysis of muscle tissue

Human muscle biopsy specimens were obtained from *M. quadriceps femoris* under sterile conditions and frozen in liquid nitrogen. Total RNA from 12 DM2 biopsy specimens (6 patients with and 6 patients without muscle pain) was isolated with RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. RNA quantity and purity were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, USA) and an Agilent 2100 bioanalyser (Agilent Technologies, USA). The cDNA libraries were prepared using TruSeq Stranded mRNA library preparation kit (Illumina, USA). RNA paired-end sequencing was performed using the Illumina HiSeq platform (Illumina, USA). Differentially expressed genes were validated by qPCR using Sybr green assay at Stratagene Mx3000P cycler (Agilent Technologies, USA) according to manufacturer's instructions. Sequences of the oligonucleotides are provided in Supplementary Table 1.

2.5. Statistical analysis

All statistical calculations were performed using R software. The QST parameters – CDT, WDT, PPT, WUR, MPS, MDT, and MPT – are usually normally distributed in log-space and thus were log-transformed. The QST-profiles of DM2 patients were compared to controls using repeated measure two factorial (for group and tested site) ANOVA. All QST measures from each patient were then standardized by z-transformation with respect to the age- and sex-matched healthy subject group.

$$\mathrm{Z-score} = \left(\mathrm{Mean}_{\mathrm{individual\ DM2}} - \mathrm{Mean}_{\mathrm{healthy\ subjects}}\right) / \mathrm{SD}_{\mathrm{healthy\ subjects}}$$

where mean $_{\mathrm{individual\ DM2}}$ is the value of the QST parameter in a DM2 patient, and $\mathrm{mean}_{\mathrm{healthy\ subjects}}$ and $\mathrm{SD}_{\mathrm{healthy\ subjects}}$ are mean and standard deviation of the corresponding QST parameter in the healthy control group. Z-scores signs were adjusted so that a z-score > 0 indicated means gain of sensory function (lower threshold), and z-score < 0 means loss of function (increase in threshold). The advantage of graphical representation of QST profiles as z-transformed data was to directly compare between sensory modalities of different units and ranges between groups and tested sites.

Gene expression data was analysed with CLC Genomic Workbench v7.0 (Qiagen, Germany) and Qlucore Omics Explorer v3.1 (Qlucore, Denmark). Samples obtained from patients without muscle pain were considered as a reference group. Preprocessed raw sequences were imported and trimmed in CLC Genomics Workbench and all trimmed reads were aligned to the human reference genome (GRCh37) and mapped back to the human transcriptome (v.19). Mapped read counts were normalized using Trimmed mean of M-values (TMM) method implemented in Edge-R package. Normalized read counts were used for analysis of differential gene expression at Qlucore Omics Explorer. P values were calculated by two group comparison T-test. Genes with P-value <0.05 and fold change >±1.8 were considered to be differentially expressed and were presented as a heatmap with hierarchical clustering of the samples. Differentially expressed genes from RNAseq data were additionally confirmed with qPCR using ΔΔCt method with the average of Ct values for GAPDH and cyclophilin A used as a reference. Ct values were calculated by MxPro qPCR software v4.1 (Agilent Technologies, USA). Gene

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