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Genome instability in Maple Syrup Urine Disease correlates with impaired mitochondrial biogenesis

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ABSTRACT

Objective. The mitochondrial branched-chain ketoacid dehydrogenase (BCKD) catalyzes the degradation of branched-chain amino acids (BCAA), which have been shown to induce oxidative stress. Maple Syrup Urine Disease (MSUD) is caused by impaired activity of BCKD, suggesting that oxidative stress and resulting DNA damage could contribute to pathology. We evaluated the potential effect of BCKD deficiency on genome integrity and mitochondrial function as a downstream target.

Methods. Primary fibroblasts from MSUD patients and controls were either cultivated under normal conditions or exposed to metabolic or oxidative stress. DNA was analyzed for damage and mitochondrial function was evaluated by gene expression analyses, functional assays and immunofluorescent methods.

Results. Patient fibroblasts accumulated damage in mitochondrial DNA (mtDNA) and nuclear DNA, with a corresponding reduction in mitochondrial transcription, mtDNA copy number and pyruvate dehydrogenase. We found no evidence of increased level of reactive oxygen species (ROS) in patient fibroblasts under normal conditions, suggesting that the genotoxic effect is ascribed to accumulating metabolites.

Conclusions. Impaired BCKD activity as in MSUD, results in accumulation of DNA damage and corresponding mitochondrial dysfunction.

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1. Introduction

MSUD is an autosomal recessive disease, caused by mutations in one of the genes encoding subunits of BCKD. MSUD has been classified into five distinct forms depending on the severity of the disease, affected gene and responsiveness to thiamine treatment [1]. Mutations in the *DBT* gene coding for the transacylase (E2) subunit are often associated with the intermittent form of the disease, where patients have some residual enzyme activity left [2]. Our laboratory previously identified new mutations in the E2 subunit in patients with the intermittent variant of MSUD and showed that the

Abbreviations: MSUD, Maple Syrup Urine Disease; BCKD, Branched chain ketoacid dehydrogenase; BCKA, branched chain ketoacids; BCAA, branched chain amino acids; ROS, reactive oxygen species; mtDNA, mitochondrial DNA; GDH, glutamate dehydrogenase; RT, room temperature; nt, non-treated; RT-qPCR, real-time qPCR; PDH, pyruvate dehydrogenase; Sirtuins, Silent information regulator of gene transcription-family.

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mutations lead to instability of the E2 protein with subsequent lower cellular level [3].

The BCKD complex catalyzes the rate-limiting step in the degradation of the three branched-chain amino acids (BCAAs), leucine, isoleucine and valine. During MSUD, BCAAs and their corresponding keto acids (BCKAs; α-ketoisocaproate, α-keto-β-methylvalerate and α-ketoisovalerate) accumulate, which can lead to encephalopathic crisis, mental retardation or death if left untreated. Neuropathology in MSUD, which includes dysmyelination [4,5] is reported to correlate with energy deficiency [6,7], impaired pyruvate uptake [8], disturbed neurotransmitter balance [9], increased apoptosis [10], inhibited glutamate uptake [11] and oxidative stress [12–14].

One of the many cellular targets of oxidative stress is DNA. Both the nuclear DNA and the mitochondrial DNA are sensitive to ROS, and if the inflicted damage is not removed, it can have deleterious consequences for the cell. Chronic administration of BCAAs has been shown to induce DNA damage in rats [15]. Prolonged DNA damage can affect signaling pathways, gene expression, transcription level and cause replication errors and genomic instability [16].

Besides being essential for BCKD activity, the E2 subunit of BCKD is ascribed as a core factor in the mitochondrial nucleoid [17–19]. The functional impact of the association to the mtDNA is unknown but alludes to a role in regulating mtDNA-associated processes or even protecting the mitochondrial genome. Thus, altered BCKD activity as in MSUD may have genotoxic consequences, either via oxidative stress, mitochondrial alterations or both.

To investigate this, we used fibroblasts from MSUD patients with reduced levels of E2 protein to evaluate the effect on DNA integrity and mitochondrial function.

2. Materials and methods

2.1. Cell material, culture and treatment

This study was approved by the Regional Committee for Medical and Health Research Ethics of South East Norway, and the skin fibroblasts for culture were collected and stored in approved biobanks according to Norwegian framework. Primary fibroblasts from four intermittent MSUD patients and four healthy controls were used. The patients carrying the E2 amino acid alterations R301C, G62X, W84C and R376C, respectively, have previously been described [3]. The fibroblasts were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mmol/L glutamine and 1% Pencillin/Streptomycin supplement. During treatment, the fibroblasts were exposed to 10 mmol/L BCAA or 10 mmol/L BCKA (Sigma Aldrich) for 24 h before the cells were harvested and analyzed.

2.2. Nucleic acid isolation and quantification

Total DNA was isolated from cells with Blood and Tissue kit (Qiagen) according to manufacturer's protocol. Total RNA was isolated with RNeasy mini kit (Qiagen) according to manufacturer's protocol, including an optional DNase treatment step. Nucleic acid concentrations were estimated by Nanodrop Spectrophotometer (Thermo Scientific), or alternatively by Epoch Microplate Spectrophotometer (Bio-Tek).

2.3. DNA damage and copy number estimation

DNA damage was estimated by a method based on the ability of DNA damage to inhibit restriction enzyme cleavage. DNA damage introduced into a TaqI sensitive restriction site will result in altered cutting frequency of the DNA, which ultimately will affect PCR amplification of the target sequence spanning the restriction site. The resulting Δct gives an estimate of the DNA damage level. The method has been developed by us previously [20]. mtDNA copy number was estimated by real-time qPCR quantification of the mtDNA-encoded *mt-RNR1* relative to the nuclear *NDUFA9* using the ΔΔct method and presented relative to average of controls.

2.4. Gene expression

Gene expression analysis was performed by qPCR on StepOne Plus Real-time PCR system (Applied Biosystems/Life Technologies) using Power SYBR green master mix (Applied Biosystems/Life Technologies) and primers listed in Table 1. cDNA was synthesized from total RNA with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Expression levels were determined with the relative standard curve method, and the housekeeping gene 18S rRNA was used as internal control.

2.5. ROS Analysis, ATP measurement and citrate synthase activity

Mitochondrial ROS generation *in vivo* was quantified after staining plated fibroblasts with 100 nmol/L rhosamine (MitoTracker® Red CMXRos, 1 mmol/L stock solution in DMSO, Invitrogen/Molecular Probes, Eugene, OR) and monitoring for 30 min. The cells were assessed by confocal imaging at regular intervals up to 30 min, with a 40× oil objective on a Leica TCS SP8 as described below. Intensity histograms were collected for each time point for two cells in eight different locations from four patients and four controls, and mitochondrial ROS

Table 1 – Oligonucleotides used in this study.

Target	Forward primer (5'–3')	Reverse primer (5'–3')
MTRNR1	cctccccaataaagctaaaa	gctattgtgtgttcagatat
MTND2	gccctagaaataaacatgcta	gggctattcctagttttatt
MTND6	caaccagtaactactactaa	actttaatagtgtaggaagc
NDUFA9	attccccttgccgctttttg	atgtgcatccgctccacttt
ATP5	tgggtcgtgtagttgatgcc	cagccttaatgccagtctgc
SIRT1	aagttgactgtgaagctgtacg	ggacatcgaggaactacctga
SIRT3	acccagtggcattccagac	gcttggggttgtgaaagaagaa
SIRT4	aagatgagctttgcgttgact	gctggggtttgcgatccaa
SIRT5	cggccaagttcaagtatggca	tttctgcactaacaccagctc
PDHE2	ctcccacaggtcctggaatg	gtgcaataacccgacgaatgt
18S	cttccacaggaggcctacac	ggcaaaggctattttccgcc
MTRNR1-DNA DAMAGE	aaactgctcgccagaacact	catgggctacaccttgacct
NDUFA9-DNA DAMAGE	gcaagggtccctatgagagaa	caagaacgaggggaaaagtg

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