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Research Paper

A Minimally-invasive Blood-derived Biomarker of Oligodendrocyte Cell-loss in Multiple Sclerosis

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ABSTRACT

Multiple sclerosis (MS) is a neurodegenerative disease of the central nervous system (CNS). Minimally invasive biomarkers of MS are required for disease diagnosis and treatment. Differentially methylated circulating-free DNA (cfDNA) is a useful biomarker for disease diagnosis and prognosis, and may offer to be a viable approach for understanding MS. Here, methylation-specific primers and quantitative real-time PCR were used to study methylation patterns of the myelin oligodendrocyte glycoprotein (MOG) gene, which is expressed primarily in myelin-producing oligodendrocytes (ODCs). MOG-DNA was demethylated in $O4^+$ ODCs in mice and in DNA from human oligodendrocyte precursor cells (OPCs) when compared with other cell types. In the cuprizone-fed mouse model of demyelination, ODC derived demethylated MOG cfDNA was increased in serum and was associated with tissue-wide demyelination, demonstrating the utility of demethylated MOG cfDNA as a biomarker of ODC death. Collected sera from patients with active (symptomatic) relapsing-remitting MS (RRMS) demonstrated a higher signature of demethylated MOG cfDNA when compared with patients with inactive disease and healthy controls. Taken together, these results offer a minimally invasive approach to measuring ODC death in the blood of MS patients that may be used to monitor disease progression.

1. Introduction

Multiple sclerosis (MS) is a neurodegenerative disease characterized by demyelination of axons in the central nervous system (CNS). The cells that produce myelin in the CNS, oligodendrocytes (ODCs) (Bunge et al., 1962; Bunge, 1968), die from an autoimmune response that results in demyelinated lesions in the brain and spinal cord (Noseworthy et al., 2000; Olsen and Akirav, 2015). Axonal demyelination reduces the signal strength of nerve impulses (Waxman, 1977; Felts et al., 1997) and may leave the axon exposed to degeneration (Ferguson et al., 1997). Demyelinated lesions can be visualized by magnetic resonance imaging (MRI); however, this procedure is costly and cannot detect diffused or mild myelin degeneration or measure the active loss of ODCs. Although new innovations in MS therapy are currently being explored (Olsen and Akirav, 2015), there are currently no clinically approved molecular biomarkers of ODC death in MS. This unmet need impairs MS diagnosis, prognosis, and assessment of clinical intervention.

DNA methylation is a mechanism involved in the control of tissue-specific gene expression (Jones, 2012). DNA hypermethylation of CpG dinucleotides acts as an inhibitor of transcription while hypomethylation is associated with increased gene expression. Differences in DNA methylation patterns between different cell types can be used to determine the origin of the DNA. Accordingly, methylated circulating-free DNA (cfDNA) is used as a biomarker of tumor progression (Nawroz et al., 1996; Chen et al., 1996; Crowley et al., 2013). Our group has successfully adapted this approach for the detection of β-cell loss in patients with autoimmune type 1 diabetes (Akirav et al., 2011), where differentially methylated β-cell derived insulin cfDNA was used to detect β-cell loss. A recent report by Lehmann-Werman et al. showed the utility of using cfDNA to detect cell loss in MS, by measuring demethylated cfDNA of MBP and WM1 genes, showing an increase in the levels of demethylated DNA in patients with MS (Lehmann-Werman et al., 2016).

In contrast to MBP, which is found in both ODCs and Schwann cells, and WM1, whose tissue expression patterns are largely unknown, myelin oligodendrocyte glycoprotein (MOG) is a CNS-specific protein expressed solely by ODCs as an integral part of the myelin sheath (Linnington et al., 1984; Lebar et al., 1986; Gardinier et al., 1992; Johns and Bernard, 1999). The specificity of MOG in ODCs suggests that the

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Table 1
Primer sequences and PCR protocols for mouse MOG analysis.

PCR type	Primer designation	Primer sequence 5′ → 3′	Product length	PCR protocol
First-step PCR	Forward	GAGTGATAGGATTAGGGTATTTTATT	169 bp	50 cycles, annealing temperature 57 °C
	Reverse	TCTACATCTTAATCCTTACCATTTC		
Methylation-specific nested qPCR	Common forward	GAGTGATAGGATTAGGGTATTTTATT	87 bp	40 cycles, annealing temperature 64 °C
	Hypermeth-specific reverse	TAACGTTCTTCCCAAAAAATATACG		
	Hypometh-specific reverse	TAACATTCTTCCCAAAAAATATACA		
Native sequence first-step PCR	Forward	GAGTGATAGGACCAGGGTATCCCATC	169 bp	Cycles no. variable, annealing temperature 57 °C
	Reverse	CTGCATCTTGGTCCTTGCCATTTC		

MOG gene may present with unique methylation patterns in ODCs, which can be used to detect ODC-derived DNA in the blood and assess disease activity in patients with MS. Here we identify demethylated CpG dinucleotides in ODCs from mouse and human origin that are absent in other cell types in the brain and periphery. Methylation-specific primers for demethylated MOG-DNA were able to detect ODC-specific DNA in purified primary mouse ODCs and primary human oligodendrocyte precursor cells (OPCs). When used to detect ODC MOG-DNA in the sera, these primers showed an increase in ODC-derived cfDNA in mice treated with cuprizone. Testing of human primers successfully detected ODC MOG cfDNA in sera of patients with relapsing-remitting MS (RRMS) and showed a correlation with disease activity.

2. Methods

2.1. Mice

C57BL/6 female mice purchased from Jackson Laboratory (Bar Harbor, ME) were started on a diet of rodent chow with 0.2% cuprizone (Research Diets Inc., New Brunswick, NJ) at 8 weeks of age (Day 0). Mice were divided into 2 groups of 6 (total $n = 12$) with blood sampling by cheek pouch bleed on Days 0, 7, 21, 35, and 49 for group 1 and Days 0, 14, 28, and 42 for group 2. On Day 56 blood was obtained from all mice via heart puncture. Brain tissues were collected for histological analysis of myelination. All animal care was approved by the Winthrop-University Hospital Institutional Animal Care and Use Committee.

2.2. Cell and Cell Lines

SW10 immortalized neuronal murine Schwann cells were purchased from American Type Culture Collection (Manassas, VA) and cultured using the provided protocols. Cells were pelleted for DNA extraction. Human OPC genomic DNA extracts (ScienCell Research Laboratories, Carlsbad, CA) were used as a positive control sample for demethylated MOG-DNA. This genomic DNA sample was obtained from ScienCell Research Laboratories from early passage human OPCs using the AllPrep DNA/RNA Mini Kit (Qiagen N.V., Valencia, CA).

2.3. Mouse Tissues

Mouse liver, kidney, and brain tissue were obtained from a C57BL/6 mouse (Jackson Laboratory, Bar Harbor, ME) and homogenized for DNA extraction.

2.4. Enrichment of $O4^+$ Cells

$O4^+$ cells were purified from the brains of five C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) and treated with the Neural Dissociation Kit (P) and Anti-O4 MicroBeads, followed by magnetic purification on the autoMACS Pro Separator (Miltenyi Biotec Inc., Auburn, CA).

2.5. FACS Staining

Evaluation of $O4^+$ fraction purity was performed by fluorescence-activated cell sorting (FACS) analysis. Magnetically purified ODCs were washed twice and suspended in FACS buffer containing anti-O4 labeled antibody or isotype control and analyzed using an Accuri FACS analyzer (BD Biosciences, San Diego, CA). FACS data was analyzed using FlowJo software (FlowJo, Ashland, OR).

2.6. Plasmid Injections

Four C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) were each administered an intravenous injection of TOPO plasmid containing the demethylated mouse MOG sequence. Five minutes following injection, blood was obtained by heart puncture.

2.7. Histology

Brain tissues from cuprizone or sham treated mice were collected immediately following euthanasia, embedded in O.C.T. compound (Fisher, Waltham, MA) and snap frozen in liquid nitrogen. Tissues were sectioned at 7 μm thickness and mounted on poly-D-lysine coated slides, followed by formalin fixation. Myelin staining was done using a NovaUltra Luxol Fast Blue Staining Kit according to the manufacturer's instructions (IHCworld, Woodstock, MD). Stained slides were imaged using a bright field on a Nikon Eclipse Ti confocal microscope (Nikon, Melville, NY).

2.8. Human Subject Samples

Serum samples were obtained from BioServe Biotechnologies, Ltd. (Beltsville, MD). Samples were designated as "Active" if having a relapse at the time of sampling, "Inactive" if in remission at the time of sampling, or "Healthy Control" based on donor information. Disease activity was determined by specialized neurologists at the time of sampling and included a combination of disease activity by patient admission, EDSS scoring and MRI imagining where applicable. In some cases, MS activity

Table 2
Primer sequences and PCR protocols for human MOG analysis.

PCR type	Primer designation	Primer sequence 5′ → 3′	Product length	PCR protocol
First-step PCR	Forward	GGGTAGTTTAGAGTGATAGGATTAAGATAT	152 bp	50 cycles, annealing temperature 57 °C
	Reverse	TAAAAATAAACCACCCTAAAAAAAA		
Methylation-specific nested qPCR	Common forward	GGGTAGTTTAGAGTGATAGGATTAAGATAT	97 bp	40 cycles, annealing temperature 64 °C
	Hypermeth-specific reverse	TAACGTTTTTCTCAAAAAATATACG		
	Hypometh-specific reverse	TAACATTCTTCCCAAAAAATATACA		