

Short communication

Differential cerebro spinal fluid proteome investigation of Leber hereditary optic neuropathy (LHON) and multiple sclerosis

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Abstract

Leber's hereditary optic neuropathy (LHON) is a genetic disease leading to the loss of central vision and optic nerve atrophy. The existence of occasional cases of LHON patients developing a Multiple Sclerosis (MS)-like illness and the hypothesis that mtDNA variants may be involved in MS suggest the possibility of some common molecular mechanisms linking the two diseases. We have pursued a comparative proteomics approach on cerebrospinal fluid (CSF) samples from LHON and MS patients, as well as healthy donors by employing 2-DE gel separations coupled to MALDI-TOF-MS and nLC-MS/MS investigations. 7 protein spots showed significant differential distribution among the three groups. Both CSF of LHON or MS patients are characterized by lower level of transthyretin dimer adduct while a specific up regulation of Apo A-IV was detected in LHON CSF.

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1. Introduction

Leber's hereditary optic neuropathy (LHON), the first disease to be linked with a maternally inherited mtDNA point mutation (Wallace et al., 1988), is a genetic form of retinal

ganglion cell degeneration leading to loss of central vision and optic nerve atrophy occurring predominantly in young males (Riordan-Eva and Harding, 1995). A subset of LHON patients present extraocular symptoms involving the central nervous system (CNS) and these cases are referred to as LHON "plus" (Riordan-Eva and Harding, 1995). Amongst the LHON "plus" cases a specific phenotype is the association of LHON with a disorder indistinguishable from Multiple Sclerosis (MS) (Harding et al., 1992).

Three pathogenic point mutations at nucleotides 11,778, 3460 and 14,484 of mtDNA, affecting, respectively, ND4, ND1 and ND6 subunit genes of complex I of the respiratory chain,

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are most often associated with the disease (over 95% of LHON cases) (Riordan-Eva and Harding, 1995) even though other rare pathogenic mutations have been reported associated to both complex I and III (Carelli et al., 2004).

The occurrence of cases with the LHON “plus” MS-like variant has been reported with all three LHON pathogenic mutations (Vanopdenbosch et al., 2000) fostering various studies on the possible involvement of mtDNA in MS (Vanopdenbosch et al., 2000).

Additional genetic (nuclear or mitochondrial) and/or environmental factors are thought to be involved in incomplete penetrance and phenotypic expression in LHON (Riordan-Eva and Harding, 1995; Baracca et al., 2005). The complex I dysfunction in LHON leads to both a bioenergetic defect and a chronic increase in reactive oxygen species (ROS) production (Beretta et al., 2004). A neuropathological study by Kovacs and colleagues (Kovacs et al., 2005) on a case of LHON, complicated by a devastating widespread CNS demyelination including vacuolation of white matter, concludes that mtDNA mutations affect the nervous system on a metabolic basis, but occasionally may aggravate or initiate autoimmune processes such as in the Multiple Sclerosis-like disorder. Recently, a

micro-arrays investigation on post-mortem motor cortex from MS patients identified twenty-six nuclear-encoded mitochondrial genes and the functional activities of mitochondrial respiratory chain complexes I and III to be decreased (Dutta et al., 2006), suggesting a possible mitochondrial dysfunction in association to axonal degeneration in MS patients. However, the link between LHON and MS remains enigmatic due to our poor understanding of the molecular mechanisms leading to neurodegeneration in both diseases.

New data available from proteome studies may offer not only an improved understanding of the pathophysiology of MS and LHON, but also identify valuable biomarkers. We here report a comparative cerebrospinal fluid (CSF) proteome investigation of two LHON cases carrying the 11778/ND4 mutation, five MS patients and five healthy individuals.

2. Materials and methods

The two LHON cases were both during the acute phase within 1 month from the disease onset. The five MS patients were classified as relapsing-remitting MS and the lumbar puncture was performed during the acute phase. More details

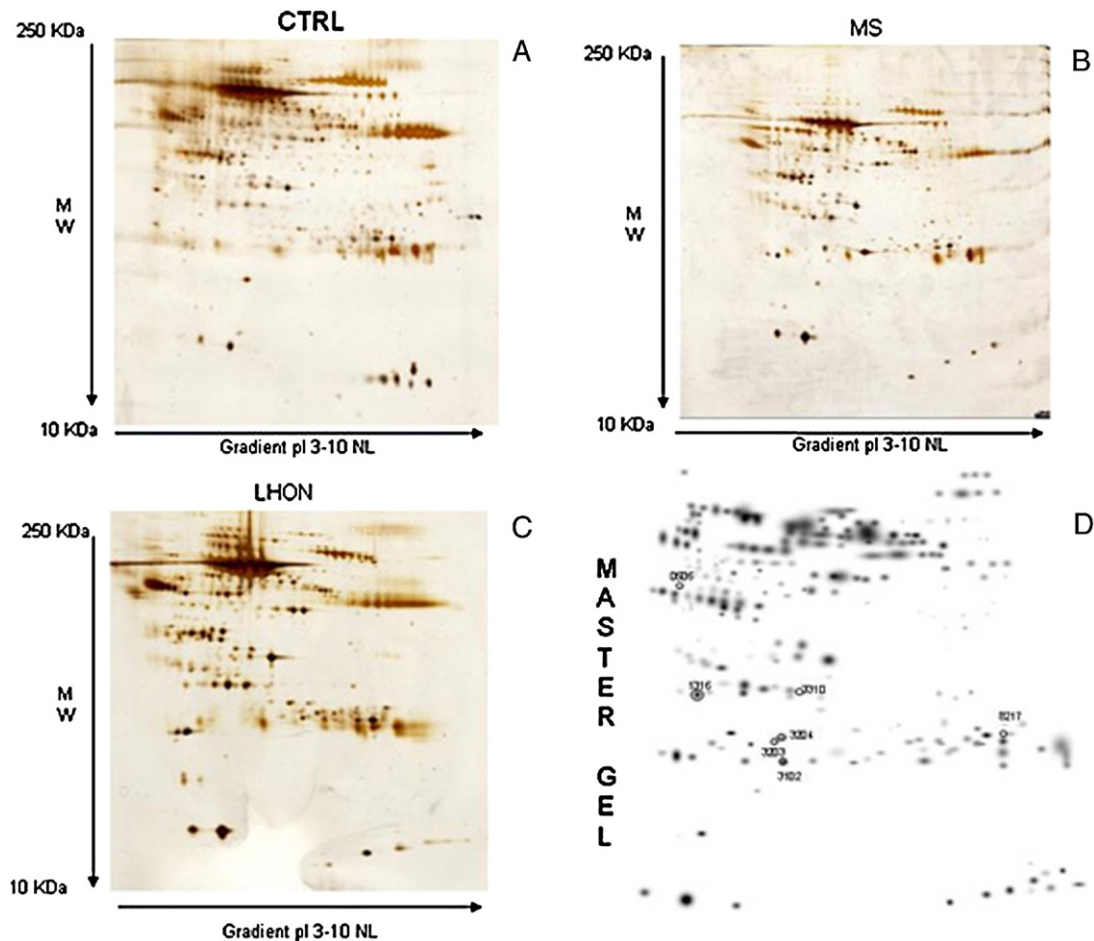


Fig. 1. 2-DE slab gel of CSF protein from a Healthy Control individual (A), a MS affected individual (B) and a LHON affected individual (C). 30 μ g of sample were loaded on non linear pH 3–10 IPG strips. The second dimension was performed on a 9–16% acrylamide gradient. After silver staining an average number of 544, 659 and 597 protein spots were detected in the three different group respectively and included in the synthetic master gel (D).

about samples collection, preparation and mass spectrometry analysis are reported in Supplemental data file. Samples from individual subjects have been analysed independently in replicate experiments after separation of the CSF proteins on 2D electrophoresis (2DE) on non-linear 3–10 pH range 1st dimension and 2nd dimension on gradient SDS-PAGE 9–16% (Fig. 1, see Supplemental data file for details). An average digital CSF proteome map have been created from every single subject. Protein profile differences have been therefore analysed imposing the presence of such a protein spot within all the subjects within the same group.

3. Results and discussion

Seven significant differentially expressed spots were identified after the statistical analysis of protein spots common to all the individuals of a clinical group. Because of the limited number of cases analysed we considered as significant only changes in protein level above three folds.

These spots were isolated by excision from the 2-DE gels and identified by MALDI-TOF and LC-MS/MS analysis (see Supplemental data file for details) — (Table 1) from multiple gels plugs.

Spots 3102, 3204, and 3203 are found only in MS samples. These spots were identified by Peptide Mass Fingerprinting (PMF) as albumin fragment, Ig K V1–5, and Ig κ C protein respectively. Spots 3310 and 8217 are more expressed in MS patients. The former is an albumin fragment whereas the latter is a Ig λ light chain VLJ region.

Given the content of albumin in CSF the presence of various HSA fragments have been often observed in 2DE CSF maps. Finehout et al. (2004) found 102 different protein spots belonging to albumin or albumin fragment. Our data highlight in 5 different cases a well defined fragmentation of HSA in MS affected individuals. These fragments might be generated by proteases present in lymphocytes (Kam et al., 2000), which infiltrate in the CNS (Antel and Owens, 1999).

Moreover, in MS cases we found higher level of lambda and kappa immunoglobulin chains. Intrathecally generated Igs, specifically IgG, are found in 95% of MS patients (D'Aguanno et al., 2007) although oligoclonal bands may be produced in other condition such as CNS infections and paraneoplastic syndromes (Sindic and Laterre, 1991). The presence of two or more oligoclonal bands is considered a positive test for MS diagnosis (D'Aguanno et al., 2007). Goffette et al. (2004) by using an immunoaffinity mediated capillary blotting technique reported the frequent occurrence of CSF specific oligoclonal free kappa bands (92%), and the less frequent appearance of free lambda bands (69%) in MS. Interestingly, they found that few samples negative for oligoclonal IgG in the CSF contained visible amounts of free kappa bands. This observation suggested that an intrathecal immune reaction could be more sensitively detected by the presence of such free kappa chain. In fact, free kappa bands are poorly represented in the CSF of patients without neurological disorders and rarely detected in non-MS neurological diseases (Sindic and Laterre, 1991; Goffette et al., 2004). Even if this finding is not a novelty in

Table 1
Differentially expressed proteins in MS, Control and LHON CSF identified by Peptide Mass Fingerprinting (PMF) and nLC-MS/MS

SSP ^a	Control ^b	Standard deviation	LHON ^b	Standard deviation	MS ^b	Standard deviation	Ratio (MS/CRTL)	Ratio (LHON/CRTL)	NCBI accession number	Identified protein by PMF	Mascot Score	Sequence coverage	Theoretical M _r /pI	Identified peptide by nLC-MS/MS (Mascot score)
3102	<20	—	<20	—	3375	2804	—	—	gi 55669910	Human Serum Albumin	115	20%	68/5.6	—
3204	<20	—	<20	—	3538	2697	—	—	<i>gi 21410817</i>	IGKV1–5 protein	73	29.6%	26.3 /5.9	—
8217	<1000	—	<1000	—	4299	3704	—	—	<i>gi 21669539</i>	immunoglobulin lambda light chain VLJ region	66	25%	28.7/8.8	—
3203	<20	—	<20	—	2812	2630	—	—	gi 66267321	IGKC protein	72	26.9%	26.4/7.6	—
3310	1384	729	<1000	—	2386	834	1.7	—	gi 55669910	Human Serum Albumin	125	16.8%	68/5.6	—
1316	6806	3287	<1000	—	<1000	—	—	—	<i>gi 55669576</i>	Dimer Of Transferrin	138	86%	12.8/5.2	AADDTWPEFASGK (100)
506	1025	594	3248	2835	1167	800	1.1	3	gi 178761	<i>Apolipoprotein A-IV</i>	163	28%	45.3/5.1	ISASAEELR (68) IDQNVVELK (79)

All reported spots are statistically significant at 95% both with T-Student and Mann Whitney analysis in all replicate group.

^a Index in the reference gel.

^b Average spot intensity in each replicate group expressed in ppm; ppm intensity values <20 i.e. spot values under our experimental detection limit; ppm intensity values <1000 i.e. detectable spot with intensity values under our experimental threshold for quantitation.

the study of MS it could validate the other evidences presented below.

Higher levels of spot 1316, corresponding to the transthyretin (TTR) dimer, were present in control samples compared to MS and LHON cases. This transport protein, synthesized primarily in liver, choroid plexus and the retina, is one of the 23 human proteins known to be associated with local or systemic amyloidosis (Reixach et al., 2004). TTR is a homotetramer that carries retinol-binding protein loaded with retinol and thyroxine (T₄) in the plasma and CSF involved in amyloidosis. Senile systemic amyloidosis is a sporadic disorder resulting from the deposition of wild-type TTR fibrils in cardiac and other tissues. Familial amyloidotic polyneuropathy and cardiomyopathy are hereditary autosomal-dominant diseases, in which the deposited TTR fibrils are derived from one of 80 known amyloidogenic mutations, and primarily affect the peripheral and autonomic nervous systems, and heart, respectively. Brain TTR is secreted into the cerebrospinal fluid, where becomes the major thyroid hormone-binding protein. During ontogeny, the maximum TTR synthesis in the choroid plexus precedes that of the growth rate of the brain and occurs during the period of maximum neuroblast replication. TTR is a component of the network determining thyroid hormone (TH) distribution (Fernandez et al., 2004). It is well established that TH is required for the normal timing of differentiation and maturation of oligodendrocyte precursor cells (OPCs), the most important source of remyelinating oligodendrocytes in the adult CNS (Fernandez et al., 2004). OPCs are present in early (fresh) demyelinating lesions in MS where they fail to differentiate (Fernandez et al., 2004). It is reported that the administration of TH during the acute phase of experimental autoimmune encephalomyelitis (EAE) in Lewis rats and in Dark Agouti (DA) female rats, the most commonly used experimental models for MS, is able to channel OPCs and/or progenitors into oligodendroglial lineage, thus leading to a faster morphological reorganization of myelin sheaths in the white matter (Fernandez et al., 2004). Our findings of higher levels of TTR dimer (SSP 1316) in control individuals compared to MS patients seem to support the idea of a neuroprotective effect played by thyroid hormone.

The specific Apolipoprotein A-IV spot (SSP 506) has a three-fold relative concentration increase in CSF of LHON patients. ApoA-IV is a plasma protein that circulates freely in solution or associates with chylomicrons and high-density lipoprotein (HDL). Although ApoA-IV is a major circulating apolipoprotein, its physiological function is not completely clear. Several functions have been ascribed to this apolipoprotein. For example, it has been shown that ApoA-IV promotes cholesterol efflux from extra-hepatic tissues, is a ligand for HDL binding to hepatocytes, activates lecithin:cholesterol acyltransferase, and modulates the activation of lipoprotein lipase by ApoCII (Qin et al., 1998). These functions can also be fulfilled by other apolipoproteins, particularly ApoAI. Overexpression of either human or mouse ApoA-IV in transgenic mice confers significant protection against diet-induced atherosclerosis in cholesterol-fed animals and apoE-deficient mice (Qin et al., 1998). It has been suggested that ApoA-IV may play a protective role against atherosclerosis acting as endogenous

inhibitor of lipid oxidation (Qin et al., 1998). Moreover ApoA-IV aggregates in the regenerating sciatic nerve for myelin biosynthesis (Olmarker et al., 1996) and is up regulated in CSF patients with lumbar disc herniation (Liu et al., 2006). The finding of higher levels of ApoA-IV in CSF from LHON patients is in agreement with their pathologic conditions characterized by degeneration of the optic nerve and possible ROS oxidative stress leading to lipid oxidation.

4. Conclusion

This pilot proteomics investigation of CSF protein repertoire from LHON and MS have highlighted protein patterns, which allow to differentiate the two diseases, supporting the hypothesis of different molecular mechanisms at the basis of the two disorders. Moreover, our results underline two specific potential candidate proteins for further clinical validation studies in order to foster their application as univariate molecular biomarkers, which may complement the molecular genetic diagnosis.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jneuroim.2007.10.004.

References

- Antel, J.P., Owens, T., 1999. Immune regulation and CNS autoimmune disease. *J. Neuroimmunol.* 100, 181–189.
- Baracca, A., Solaini, G., Sgarbi, G., Lenaz, G., Baruzzi, A., Schapira, A.H., Martinuzzi, A., Carelli, V., 2005. Severe impairment of complex I-driven ATP synthesis in Leber's hereditary optic neuropathy cybrids. *Arch. Neurol.* 62, 730–736.
- Beretta, S., Mattavelli, L., Sala, G., Tremolizzo, L., Schapira, A.H., Martinuzzi, A., Carelli, V., Ferrarese, C., 2004. Leber hereditary optic neuropathy mtDNA mutations disrupt glutamate transport in cybrid cell lines. *Brain* 127, 2183–2192.
- Carelli, V., Ross-Cisneros, F.N., Sadun, A.A., 2004. Mitochondrial dysfunction as a cause of optic neuropathies. *Prog. Retin. Eye Res.* 23, 53–89.
- D'Aguzzo, S., Del Boccio, P., Bernardini, S., Ballone, E., Di Ilio, C., Federici, G., Urbani, A., 2007. Electrophoretic separations of cerebrospinal fluid proteins in clinical investigations. *Clin. Chem. Lab. Med.* 45 (4), 437–449.
- Dutta, R., McDonough, J., Yin, X., Peterson, J., Chang, A., Torres, T., Gudz, T., Macklin, W.B., Lewis, D.A., Fox, R., Rudick, R., Mirmics, K., Trapp, B.D., 2006. Mitochondrial dysfunction as a cause of axonal degeneration in multiple sclerosis patients. *Ann. Neurol.* 59, 478–489.
- Fernandez, M., Giuliani, A., Pirondi, S., D'Intino, G., Giardino, L., Aloe, L., Levi-Montalcini, R., Calzà, L., 2004. Thyroid hormone administration enhances remyelination in chronic demyelinating inflammatory disease. *Proc. Natl. Acad. Sci.* 101, 16363–16368.
- Finehout, E.J., Frank, Z., Lee, K.H., 2004. Towards two-dimensional electrophoresis mapping of the cerebrospinal fluid proteome from a single individual. *Electrophoresis* 25, 2564–2575.
- Goffette, S., Schlupe, M., Henry, H., Duprez, T., Sindic, C.J.M., 2004. Detection of oligoclonal free kappa chains in the absence of oligoclonal IgG in the CSF of patients with suspected multiple sclerosis. *J. Neurol. Neurosurg. Psychiatry* 75, 308–310.
- Harding, A.E., Sweeney, M.G., Miller, D.H., Mumford, C.J., Kellar-Wood, H., Menard, D., McDonald, W.I., 1992. Occurrence of a multiple sclerosis-like illness in women who have a Leber's hereditary optic neuropathy mitochondrial DNA mutation. *Brain* 115, 979–989.

- Kam, C.M., Hudig, D., Powers, J.C., 2000. Granzymes (lymphocyte serine proteases): characterization with natural and synthetic substrates and inhibitors. *Biochim. Biophys. Acta* 1477, 307–323.
- Kovacs, G.G., Hoftberger, R., Majtenyi, K., Horvath, R., Barsi, P., Komoly, S., Lassmann, H., Budka, H., Jakab, G., 2005. Neurophatology of white matter disease in Leber's hereditary optic neuropathy. *Brain* 128, 35–41.
- Liu, X.D., Zeng, B.F., Xu, J.G., Zhu, H.B., Xia, O.C., 2006. Proteomic analysis of the cerebrospinal fluid of patients with lumbar disk herniation. *Proteomics* 6, 1019–1028.
- Olmarker, K., Nordborg, C., Larsson, K., Rydevik, B., 1996. Ultrastructural changes in spinal nerve roots induced by autologous nucleus pulposus. *Spine* 21, 411–414.
- Qin, X., Swertfeger, D.K., Zheng, S., Hui, D.Y., Tso, P., 1998. Apolipoprotein AIV: a potent endogenous inhibitor of lipid oxidation. *Am. J. Physiol.* 274, H1836–H1840.
- Reixach, N., Deechongkit, S., Jiang, X., Kelly, J.W., Buxbaum, J.N., 2004. Tissue damage in the amyloidoses: Transthyretin monomers and non native oligomers are the major cytotoxic species in tissue culture. *Proc. Natl. Acad. Sci.* 101, 2817–2822.
- Riordan-Eva, P., Harding, A.E., 1995. Leber's hereditary optic neuropathy: the clinical relevance of different mitochondrial DNA mutations. *J. Med. Genet.* 32, 81–87.
- Sindic, C.J., Laterre, E.C., 1991. Oligoclonal free kappa and lambda bands in the cerebrospinal fluid of patients with multiple sclerosis and other neurological diseases. An immunoaffinity-mediated capillary blot study. *J. Neuroimmunol.* 33, 63–72.
- Vanopdenbosch, L., Dubois, B., D'Hooghe, M.B., Meire, F., Carton, H., 2000. Mitochondrial mutations of Leber's hereditary optic neuropathy: a risk factor for multiple sclerosis. *J. Neurol.* 247, 535–543.
- Wallace, D.C., Singh, G., Lott, M.T., Hodge, J.A., Schurr, T.G., Lezza, A.M., Elsas, L.J.D., Nikoskelainen, E.K., 1988. Mitochondrial DNA mutation associated with Leber's hereditary optic neuropathy. *Science* 242, 1427–1430.