Clinica Chimica Acta 454 (2016) 33–38

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

Development of new quantitative mass spectrometry and semi-automatic isofocusing methods for the determination of Apolipoprotein E typing

Christophe Hirtz [a,1], Jerome Vialaret [a,1], Georges Nouadje [b], Susanna Schraen [c], Pascale Benlian [d,e], Sandrine Mary [d], Pascal Philibert [f], Laurent Tiers [a], Pauline Bros [a], Constance Delaby [a], Audrey Gabelle [a,g], Sylvain Lehmann [a,*]

[a] CHRU de Montpellier, Hôpital St Eloi Université de Montpellier, INSERM U1183, IRMB, Laboratoire de Biochimie Protéomique Clinique, Montpellier, France
[b] SEBIA, 91008 Evry, France
[c] Inserm U837, Lille, FRANCE Université Paris 7-Denis Diderot, France
[d] Université Lille 2 and UMR CNRS 8199 Integrative Genomics and Metabolic Disease Modeling, LILLE France
[e] CHRU de Lille, Unité de Médecine Moléculaire des Maladies Métaboliques (U4M), Lille, FRANCE
[f] CHRU de Montpellier, Service de Biochimie et d'Hormonologie, Montpellier, France
[g] Centre Mémoire de Ressources et de Recherche Languedoc-Roussillon, Département de Neurologie, CHRU de Montpellier, Université de Montpellier, Montpellier, France

ARTICLE INFO

Article history:
Received 20 November 2015
Received in revised form 11 December 2015
Accepted 17 December 2015
Available online 19 December 2015

Keywords:
Apolipoprotein E
Mass spectrometry
Isoelectric focusing
Immunoprecipitation
Immunofixation
Phenotype
Genotype
Alzheimer's disease

ABSTRACT

Background: Apolipoprotein E (Apo E) is a 36 Kda glycoprotein involved in lipid transport. It exists in 3 major isoforms: E2, E3 and E4. ApoE status is known to be a major risk factor for late-onset Alzheimer's and cardiovascular diseases. Genotyping is commonly used to obtain ApoE status but can show technical issues with ambiguous determinations. Phenotyping can be an alternative, not requiring genetic material. We evaluated the ability to accurately type ApoE isoforms by 2 phenotyping tests in comparison with genotyping.
Methods: Two phenotyping techniques were used: (1) LC–MS/MS detection of 4 ApoE specific peptides (6490 Agilent triple quadripole): After its denaturation, serum was either reduced and alkylated, or only diluted, and then trypsin digested. Before analysis, desalting, evaporation and resuspension were performed. (2) Isoelectric focusing and immunoprecipitation: serum samples were neuraminidase digested, delipidated and electrophoresed on Hydragel ApoE (Sebia agarose gel) using Hydrasys 2 Scan instrument (Sebia, Lisses, France). ApoE isoforms bands were directly immunofixed in the gel using a polyclonal anti human ApoE antibody. Then, incubation of the gel with HRP secondary antibody followed by TTF1/TTF2 substrate allowed the visualization of ApoE bands. The results of the two techniques were compared to genotyping.
Results: Sera from 35 patients previously genotyped were analyzed with the 2 phenotyping techniques. 100% concordance between both phenotyping assays was obtained for the tested phenotypes (E2/E2, E2/E3, E2/E4, E3/E3, E3/E4, E4/E4). When compared to genotyping, 3 samples were discordant. After reanalyzing them by both phenotyping tests and DNA sequencing, 2/3 discrepancies were confirmed. Those can be explained by variants or rare ApoE alleles or by unidentified technical issues. 102 additional samples were then tested on LC–MS/MS only and compared to genotyping. The data showed 100% concordance.
Conclusion: Our 2 phenotyping methods represent a valuable alternative to genotyping. LC–MS/MS has the advantage of being fully specific, with identification of the different isoforms and can be considered as a reference method. Sebia isofocusing technique was concordant with LC–MS/MS. Plus, it is a rapid, semi-automated assay that can be easily implemented in clinical laboratories.

1. Introduction

Human Apolipoprotein E (ApoE) is a 34 kDa glycoprotein that has two main polymorphic codons at position 112 and 158. Depending on the amino acid substitutions, three isoforms ApoE2 (cys112, cys158), ApoE3 (cys112, arg158), and ApoE4 (arg112, arg158) are thus generated [1]. These substitutions modified both the structure (molecular weight, isoelectric point, conformation) and the functional properties of the protein. ApoE, which is expressed mainly in the liver and the brain [2] exists mainly as a component of lipoprotein complexes such as Intermediate-density lipoprotein (IDL) [3] and is involved in the transport/clearance of lipids like cholesterol among various tissues.

* Corresponding author.
E-mail address: s-lehmann@chu-montpellier.fr (S. Lehmann).
[1] These authors have equally contributed to this work.

http://dx.doi.org/10.1016/j.cca.2015.12.020
0009-8981/

ApoE is the ligand for binding to specific cell-surface receptors, including the LDL receptor family members and heparan sulfate proteoglycans (HSPGs). It has also been involved in many cellular processes including cytoskeletal assembly, mitochondrial function, and dendritic formation [4].

Importantly, the ApoE gene encoding the three above isoforms represents the major genetic risk factor of Alzheimer's disease (AD). While the Apo ε2 allele is protective against AD, the presence of one ApoE4 allele is associated with 3 to 4 times risk increase for the disease, and the presence of two copies with a 10–14 times risk increase [5–8]. Its relationship with AD pathophysiology is primarily related with amyloid beta (Aβ) peptide metabolism. In fact, many in vitro and in vivo data suggest that ApoE interacts with Aβ peptides, promotes its aggregation or transport (clearance) and is linked with an increased deposition in the brain of patients. Aβ independent mechanism has been also proposed through for example the generation of toxic fragments, impacts on synaptic plasticity, neurite outgrowth or neuronal cytoskeleton and tau proteins [9]. The exact mechanism of ApoE involvement in AD pathogenesis is still the matter of debate. The determination of ApoE status is anyway valuable to estimate AD risk and to predict the safety and efficacy of new AD therapeutic approaches [4]. In addition, ApoE status is also associated with cardiovascular disease risk [10] and longevity in humans [11].

Historically, the determination of the ApoE status was performed using isoelectrofocusing (IEF) and immunoblotting of plasma or serum [12]. ApoE phenotyping is based on the charge differences and separation of common isoforms. ApoE4 has one more positive charge than apoE3, and apoE2 has one less, resulting in differences in their apparent pI values. However, charge alterations could result from post-translational modification such as physiological sialylation/desialylation and diabetes-induced glycation or oxidative modification such as imine adduct formation. Besides, thanks to high sensitive mass spectrometers, adapted measurement methods like Multiple Reaction Monitoring (MRM) and pre-fractionation approach, the quantitative detection of protein/peptide biomarkers in low concentrations is now feasible from complex biological fluid [13]. LC-MRM is considered as a method of choice for protein multiplexing [14]. The general principle is to follow selected specific ions (i.e. proteotypic peptides) and fragments arising from these ions known as “transition fragments”. This method relies mainly on the use of triple quadrupole mass spectrometer analysers and is highly specific and reproducible [15]. We described here a novel LC-MRM ApoE blood typing method using stable isotope-labeled peptides. This method is based on the detection of four different tryptic peptides including the polymorphic sites of ApoE. The goal of our study was to develop and validate the ApoE phenotype obtained with these two novel (LC-MRM and semi-automatic isofocusing) methods in a cohort of human sera.

2. Material and methods

2.1. Chemicals used

Tris(hydroxymethyl)aminomethane (Tris) ref. ACRO140505000 (VWR, Fontenay-sous-Bois, France); 2,2,2 Trifluoroethanol (TFE) ref. 05841-50ML; DL-Dithiothreitol (DTT) ref. 43819-25G; Iodoacetamide (IAA) ref. I1149-25G (Sigma-Aldrich); Water ULC-MS, ref. 23214102, Formic Acid ULC-MS (FA) ref. 069141A8, Acetonitrile ULC-MS (ACN) ref.01204101, Methanol ULC-MS (MeOH) ref. 13684101 all from Biosolve (Dieuze, France); Protein LoBind tube 1.5 mL, ref. 022431081 Eppendorf (Le Pecq, France); Trypsin Gold, Mass Spectrometry Grade, ref. 5280 (Promega, Charbonnières-les-Bains, France); Polypropylene Vials (Agilent Technologies, ref. 9301–0978); Sep.-Pak tC18 1 cm^3 Vac Cartridge, ref. WAT054960 (Waters, Guyancourt, France).

The following peptides were purchased from Eurogentec (Angers, France) with incorporated stable-isotope-labeled arginine (R*, 13C615N4): LAVYQAGAR*, LGADMEDVR*. LGADMEDVCGR* and CLAVYQAGAR*.

2.2. Collection and sampling patients

Blood samples originated from a sample collection of patients from Montpellier neurological and Clinical Research Memory Centers (CMRR) for cognitive or behavioral disorders (Biobank officially registered # DC-2008-417). All patients gave informed consent including for genetic determination. Blood samples were initially collected by venous puncture in PET BD Vacutainer® Tubes. After collection of the whole blood, the blood was allowed to clot by leaving it undisturbed at room temperature. Samples were then centrifuged at 2500 rpm at room temperature for 10 min. The resulting supernatant, designated as serum sample was aliquoted and then frozen at −80 °C until assays were undertaken.

2.3. Sample preparation for IEF analysis

For isofocusing 20 μL of sera were digested with 5 μL of neuraminidase (5 μg/mL), for 1 h at 45 °C. Samples treated with neuraminidase were delipidated using 25 μL of delipidation solution for 1 h at 45 °C. Samples were vortexed and centrifuged at 5000 rpm for 5 min. The supernatant was kept and frozen at −80 °C until IEF analysis.

2.4. IEF analysis

Pretreated samples (18 samples simultaneously per gel) were analyzed on Hydrasys 2 Scan instrument using a ready to use agarose gel (Hydragel 18 ApoE Isofocusing) containing carrier ampholytes chosen to have pIs which match the pIs of ApoE isoforms. Migration was carried out under 500 V until 300Vh has been accumulated and under 1000 V until 100 Vh has been accumulated (final 400 Vh), at 20 °C controlled by Peltier effect (for about 45 min).

2.4.1. Immunofixation

ApoE Isoform bands were directly immunofixed in the gel using a polyclonal anti human ApoE (Sebia PN 2115) for 10 min at 20 °C controlled by Peltier effect. There is no need to transfer ApoE isoform bands from the gel to a membrane. After a cycle of wash, the gel was incubated for 10 min with a secondary antibody conjugated with horseradish peroxidase (SIGMA, PN A9452-1VL).

2.4.2. Visualization of protein bands

In order to visualize ApoE isoform bands, the gel was incubated for 10 min with specific Sebia substrate TTF1/TTF2 (Sebia, PN 2151) that is converted into a blue color with HRP. The total time for ApoE isoform analysis of 18 samples using Hydragel ApoE method was 2 h 15 min.

2.5. Sample preparation for LC-MRM analysis

After denaturation with 50% trifluoroethanol/20 mM Tris (1 h, 65 °C), half of the samples were reduced using 5 mM DTT (1 h, 37 °C) then alkylated using 20 mM Iodoacetamide, the other half were diluted with 10% trifluoroethanol. The samples were then digested using 2 μg of trypsin 3 h at 37 °C. Before analysis, samples were desalted using SepPak, evaporated and resuspended in 20 μL of A phase (3% acetonitrile, 0.1% formic acid) containing heavy internal standard peptides of ApoE2, E3 and E4 isoforms. Peptides were detected using LC–MS/MS in MRM mode.

2.6. LC-MRM

2.6.1. Liquid chromatography (LC) separation

LC separation was carried out on a 1290 LC system (Agilent technologies) with a RRHD Eclipse Plus C18, 2.1 × 150 mm, 1.8 μm (959759-