Abnormal recruitment of extracellular matrix proteins by excess Notch3ECD in CADASIL

Iman Haddad[1], Marie Monet-Leprêtre[2,3], Céline Baron-Menguy[2,3], Maï Fouillot-Panchal[2,3], Meriem Riani[2,3], Valérie Domenga-Denier[2,3], Claire Dussaule[2,3], Emmanuel Cognat[2,3], Anne Joutel[2,3]and Joelle Vinh[1]

1 ESPCI-ParisTech, Spectrométrie de Masse Biologique et Protéomique, CNRS USR 3149, 10 rue Vauquelin 75231 Paris, France
2 INSERM, U740, Paris, F-75010, France
3 Univ Paris Diderot, Sorbonne Cité, UMR S740, Paris, F-75010, France

Introduction

Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy, or CADASIL, one of the most common inherited small vessel diseases of the brain, is characterized by a progressive loss of vascular smooth muscle cells and extracellular matrix accumulation. The disease is caused by highly stereotyped mutations within the extracellular domain of the NOTCH3 receptor (Notch3(ECD)) that result in an odd number of cysteine residues. While CADASIL-associated NOTCH3 mutations differentially affect NOTCH3 receptor function and activity, they all are associated with early accumulation of NOTCH3(ECD)-containing aggregates in small vessels.

Material & Method

Material

We used TghNotch3(WT), and TghNotch3(R90C) mice, the transgene is expressed at the homozygous state. Cerebral arteries, including arteries of the circle of Willis and the medium-sized branches, were dissected under microscope, immediately snap frozen in liquid nitrogen and stored at 80°C until use. The experimental procedures conformed to the national guidelines for the use of animals in research and were approved by the Ethics committee on animal experiment (Local committee of University Paris Diderot, Lariboisiere-Villemin).

Murine brain artery samples were fractionated using RIPA and then SDS-containing buffer. Each murine sample was prepared with vessels pooled from four transgenic mice. The final pellet was extracted in 40 ml of Laemmli buffer containing 20mM dithiothreitol between 20–48 h and clarified by centrifugation. 30 µL of protein extracts were denatured by incubation at 95°C for 5 min, electrophoresed on a 4–12% NUPAGE Bis-tricine gel run for 12 min at 200 V. The gel was recovered, washed in water, stained with Coomassie blue (Bio-Rad) and extensively washed in water for 2 to 3 h at room temperature (three changes). Each lane was divided in three parts based on size, cut into 1mm cubes and put into 1% acetic acid. Hence, each sample further comprised three subsamples.

Digestion

In-gel tryptic digestion method was used on the purified samples. Briefly, after reduction–alkylation (5mM dithiothreitol in 50mM NH_4HCO_3, 30 min at 56°C; 25mM iodoacetamide in 50mM NH_4HCO_3, 20 min in dark at room temperature), gel pieces were digested by incubation with 12.5 ng/ml Trypsin (modified sequencing grade, Roche) in NH_4HCO_3, overnight at 37°C with gentle shaking. The reaction was stopped with a volume of 5% formic acid.

Mass spectrometry analysis

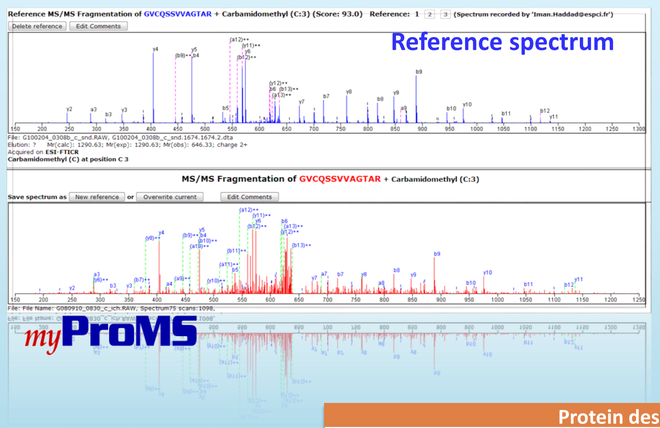
For each analysis, 8 µL of the peptide solution was loaded on a precolumn (C18 Acclaim PepMap 100 Å, 300µm i.d. x 5mm length, Dionex) and eluted on a capillary reversed phase column (nano C18 Acclaim PepMap100 Å, 75 µm i.d. x 15 cm length, Dionex), at a constant flow rate of 220 nL/min, with a gradient from 2% to 40% B in 45min (buffer A: water/ACN/FA 98:2:0.1 (v/v/v); buffer B water/ACN/FA 10:90:0.1 (v/v/v)). The MS analysis was performed on a FT ICR mass spectrometer (LTQ-FT Ultra, ThermoFisher Scientific, San Jose, CA) with the top 7 acquisition method: MS resolution 60,000, mass range m/z 500-2000, followed by 7 MS/MS (LTQ) on the 7 most intense peaks, with a dynamic exclusion for 90 s.

Results

Database search and Validation (2008):

The raw data were processed using Proteome Discover v.1.3 (ThermoFisher Scientific) with Mascot v.2.3 (Matrix Science) as algorithm search. Each sub-sample was first analyzed in triplicate. The database search was done on merged data using Mascot search engine on all taxa using SwissProt database (version 2012_06, 536489 sequences). The following parameters were used: up to 2 miss cleavages; MS tolerance 10 ppm; MS/MS tolerance 1 Da; full tryptic peptides; carbamidomethylation (C) and oxidation (M) as partial modifications. Validation on DAT files was performed on proteins using MyproMS software (1) using four steps:

1. proteins identified with at least 2 peptides with MASCOT score above 25 were selected,
2. the list was filtered out on Mouse taxonomy or Human Notch3,
3. a database containing the resulting reference MS/MS spectra was built using identifications on all runs,
4. MyproMS was further used to look for every similar fragmentation patterns on every run to extend the identifications to the reference MS/MS database
5. Protein abundance between the 2 conditions was evaluated by peptide count.



Results (2008)

2 functionally important extracellular matrix proteins were identified by peptide count : Tissue inhibitor of metalloproteinases 3 (TIMP-3) and Vitronectin (VTNC) that are sequestered into Notch3ECD-containing aggregates (Table1).

Protein description	WT		R90C	
	mean	STD	mean	STD
NOTC3_HUMAN:Neurogenic locus notch homolog protein 3 precursor	0	0.3	8	0.6
TIMP3_MOUSE:Metalloproteinase inhibitor 3 precursor	1	0.3	6	0.3
VTNC_MOUSE:Vitronectin precursor	1	0.3	4	1.0

Table 1: Proteins identified by peptide count

5 years later

Label-free protein quantification (2013):

The database search was processed by Xtandempipeline with the same parameters used on MASCOT , the peaks were aligned by the “ms2 alignement” method and areas calculated using MassChroQ software (2). Then, areas from all the peptides of each protein were summed for each sample, allowing the comparison of this protein quantity between the 2 conditions (Table 2).

Results (2013)

The quantification of proteins identified by peptide count is according with a significant ratio of the 2 conditions.

Protein description	Ratio	p-Student	p-signe
VTNC_MOUSE:Vitronectin precursor	2.342238	0.1083	0.25
NOTC3_HUMAN:Neurogenic locus notch homolog protein 3 precursor	7.469592	0.034273	0.25
TIMP3_MOUSE:Metalloproteinase inhibitor 3 precursor	1.468485	0.294107	0.25

Table 2: Results of area ratio 90C/WT

Conclusions

Different partners of NOTCH3, the disease marker in CADASIL, were identified following mass spectrometry analysis, using the peptide count method and subsequently confirmed by immunohistochemical analysis (3). Today, it is not possible to present the results of differential analysis by mass spectrometry without using any quantification. From the data collected in 2008, it has been possible to reproduce the results obtained by peptide count by using label-free quantification. However, it would be interesting to study other proteins whose initial identification by peptide count remains ambiguous but whose ratio changes significantly according to the label free method.

References

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Contact: iman.haddad@espci.fr