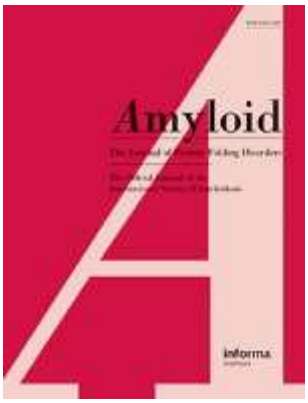

α-SYNUCLEIN AGGREGATION IN THE SALIVA OF FAMILIAL AMYLOIDOTIC POLYNEUROPATHY PATIENTS: A POTENTIAL BIOMARKER

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α-SYNUCLEIN AGGREGATION IN THE SALIVA OF FAMILIAL AMYLOIDOTIC POLYNEUROPATHY PATIENTS: A POTENTIAL BIOMARKER

Running title: α-SYNUCLEIN AGGREGATION AS A BIOMARKER FOR FAP

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Abbreviations

Familial amyloidotic polyneuropathy – FAP, Transthyretin – TTR, Parkinson's disease – PD, domino liver transplantation – DLT, orthotropic liver transplantation – OLT.

Keywords

Familial amyloidotic polyneuropathy / Saliva / Systemic amyloidosis / Transthyretin/ α-synuclein

Abstract

Familial amyloidotic polyneuropathy (FAP) is an autosomal dominant disease characterized by the formation of transthyretin (TTR) amyloid deposits. This crippling and fatal disease is associated with point mutations in TTR, a protein mainly produced in the liver. Hence, liver transplantation is the only treatment capable of halting disease progression. Ideally, liver transplantation should be performed as early as possible in the disease course before significant neurologic disability has been incurred. Early detection of disease before serious pathological lesions occur is crucial for the clinical management of patients and for morbidity delay. Unfortunately, the presence of TTR mutations by itself is not a predictor of disease onset or progression. In the present work, we observed an increased oligomerization of α-synuclein in the saliva of FAP symptomatic individuals comparatively to asymptomatic carriers of the same TTR mutation and healthy control subjects. Based on this observation, we propose monitoring α-synuclein oligomers in saliva as a biomarker of FAP progression. Since α-synuclein plays a major role in several neurodegenerative disorders, assessing its oligomerization state in this fluid provides a non-invasive approach to survey these pathologies.

Introduction

Familial amyloidotic polyneuropathy (FAP) is an autosomal dominant neurodegenerative disease where transthyretin (TTR), a homotetrameric plasma protein, is the main component of amyloid fibril deposits. This is a systemic and fatal disease, hindering organ functions and ultimately causing death.

More than eighty TTR point mutations are known to be associated with systemic amyloid diseases [1, 2]. The most common amyloidogenic mutation is the replacement of valine at position 30 by methionine, the V30M variant [3]. TTR V30M carriers are heterozygous in the large majority of cases hence both wild type TTR and TTR V30M are expressed [4]. Currently, the main hypothesis for FAP development is based on TTR tetramer instability caused by point mutations, favoring its dissociation to non-native monomeric species with the ability to self-associate and forming amyloid deposits [5].

As plasma TTR is produced in the liver [6,7] the only definitive therapy for FAP is orthotropic liver transplantation (OLT) from cadaveric donors [8]. In OLT, the mutated TTR form in circulation is readily substituted with the one produced by the transplanted liver, i.e. non-mutated TTR. Domino liver transplantation (DLT) was introduced to recover the liver of a FAP patient donor to a patient suffering from fatal liver failure. Thus the FAP patient receives a cadaveric liver (OLT) and donates his liver to another subject for increased life expectancy (DLT). For this procedure to be most effective, FAP patients must receive a new liver as early as possible in the disease course [9], something that is difficult to evaluate. In addition, a considerable number of TTR mutation bearers are asymptomatic and do not qualify for liver transplantation. Thus, earlier detection of disease onset, through the use of suitable biomarkers, is a strategy to overcome these problems.

In this work, we focused on saliva protein profile changes by comparing healthy individuals, asymptomatic TTR V30M mutation carriers and FAP patients (TTR V30M mutation bearers). We also analysed patients subjected to OLT and DLT. We confirmed the presence of α-synuclein in human saliva, as previously reported [10], and found an increased α-synuclein oligomerization in FAP patients in comparison with control subjects or asymptomatic TTR V30M carriers. Moreover, DLT subjects also present an increased oligomerization of α-synuclein in saliva. Notably, in the case of OLT, α-synuclein aggregation is decreased. These observations suggest the use of α-synuclein and its oligomerization state in human saliva as a FAP biomarker that can be monitored non-invasively.

Methods

Saliva collection

Human saliva samples were collected from control subjects (two females and one male, age range 35-45), FAP patients (three females, age range 26–33 years), TTR V30M asymptomatic carriers (two females and one male, age range from 18-28 years) and patients subjected to liver transplantation, both domino liver transplantation (DLT) and cadaveric donor liver transplantation (OLT) (three males each, age range 35–42 years). Saliva samples were centrifuged at 16000 g for 5 min at 4°C and the soluble fraction was used for further analysis. Human subjects gave informed written consent and the protocol was approved according to EEC ethic rules.

Protein quantification and polyacrylamide gel electrophoresis

Saliva proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (12% SDS-PAGE) in mini-gel format (7x7 cm Tetra system from Bio-Rad). Fifteen micrograms of protein were used per lane. Protein concentration was determined by the Bradford protein assay (Bio-Rad) using bovine serum albumin as standard. Samples were diluted 10 fold in MilliQ water and mixed with reduction buffer (62.5 mM Tris-HCl, pH 6.8, 20% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol). Prior to electrophoresis, samples were heated at 100 °C for 5 min. Proteins were stained with Coomassie Brilliant Blue R-250 (Bio-Rad). Phoretix 1D Advanced Software (NonLinear Dynamics) was used to convert individual gel lanes to densitometric profiles. These profiles were then subjected to background subtraction, band detection and Rf calibration.

Western blotting

For Western blot analysis, proteins were transferred from the polyacrylamide gel to PVDF membranes (Millipore) and stained with Ponceau S to monitor protein transfer. Membranes were blocked overnight at 4 °C with TBS-T (10 mM Tris–HCl, 150 mM NaCl, pH 7.5 with 0.1% (v/v) Tween 20) containing 5% (w/v) skimmed milk. Membranes were then incubated overnight at 4 °C with the primary antibody diluted in TBS-T containing 1% (w/v) skimmed milk. The antibodies used were: anti-human TTR polyclonal antibody (Santa Cruz Biotechnology) at a dilution of 1:5000 and anti-human α-synuclein (Santa Cruz Biotechnology) at a dilution of 1:5000. Membranes were washed three times for 10 min with TBS and incubated for 1 h at room temperature with anti-rabbit IgG (Roche) (at 1:10000 dilution) and anti-mouse IgG (Roche) (at 1:10000 dilution). Immunoreactivity was detected with diaminobenzidine (DAB) as a chromomeric substrate, following the manufacturer's instructions (Pierce).

In gel protein hydrolysis

Individual gel lanes in SDS-PAGE gels were sliced according to its densitometric profile and digested using trypsin as previously described [11]. Briefly protein bands were washed in milliQ water and destained in 50% (v/v) acetonitrile (ACN) and subsequently with 100% ACN. Cysteine residues were reduced with 10 mM DTT and alkylated with 50 mM iodoacetamide. Gel pieces were dried by centrifugation under vacuum and rehydrated in digestion buffer containing 50 mM NH_4HCO_3 and 6.7 ng/µL of trypsin (modified porcine trypsin, proteomics grade, Promega) at 4 °C. After 30 min, the supernatant was removed and discarded and 20 µL of 50 mM NH_4HCO_3 were added.

Digestions were allowed to proceed at 37 °C overnight (16-18 hours). After digestion, the remaining supernatant was removed and stored at -20 °C.

Mass spectrometry

For protein identification, a MALDI-TOF-TOF 4800 Plus mass spectrometer (Applied Biosystems) was used. Desalting and concentration of tryptic peptides was carried out with home-made chromatographic microcolumns using GELoader tips packed with POROS R2 (Applied Biosystems). Peptides were directly eluted from the microcolumns onto the MALDI plate using 5 μg/μL of α-ciano-4-hydroxycinnamic acid (CHCA, Fluka) in 50% (v/v) ACN with 0.1% (v/v) formic acid. MS experiments were performed in positive reflectron mode for monoisotopic peptide mass determination. The mass spectrometer was externally calibrated using des-Arg-Bradykinin (904.468 Da), angiotensin 1 (1296.685 Da), Glu-Fibrinopeptide B (1570.677 Da), ACTH (1-17) (2093.087 Da), and ACTH (18-39) (2465.199) (4700 Calibration Mix, Applied Biosystems). MS spectra were collected in a result-independent acquisition mode, typically using 1000 laser shots per spectra and a fixed laser intensity of 3000V. For tandem experiments, fifteen of the strongest precursors were selected for MSMS, the weakest precursors being fragmented first. MSMS analyses were performed using CID (Collision Induced Dissociation) with 1 kV collision energy and 1 x 10^6 torr air pressure. 2000 Laser shots were collected for each MSMS spectrum using a fixed laser intensity of 4000V. Raw data were generated by the 4000 Series Explorer Software v3.0 RC1 (Applied Biosystems) and tryptic peptide contaminant *m/z* peaks resulting from trypsin auto digestion (842.508 Da; 1045.564 Da; 2211.108 Da; 2225.119 Da) were excluded when generating the peptide mass list used for comparison with the theoretical tryptic

digest. Proteins were identified with the GPS Explorer (Applied Biosystem) and further confirmed using the ProteinPilot software (Applied Biosystem).

Results and discussion

Saliva protein profile and protein identification

Despite slight inter-individual variations in whole saliva composition all samples are very similar among themselves. Protein bands (assigned in Figure 1) were excised and proteins identified by MSMS (Table 1). Among 22 identified protein band in saliva, we found α-synuclein in different protein bands (Figure 1), suggesting the presence of aggregates and fragments. Average sequence coverage for all α-synuclein identified proteins was around 50% and several peptides were fully sequenced, making α-synuclein identification in saliva unequivocal (Figure 2).

Increased levels of α-synuclein oligomers in FAP saliva

Considering the migration of α-synuclein, consistent with high molecular weight species, most likely aggregates as previously reported [12, 13], a Western blot analysis was performed with a specific α-synuclein antibody (Figure 3). We analysed samples from asymptomatic and symptomatic FAP individuals to evaluate the differences in α-synuclein electrophoretic profile associated with the disease progression. In all individuals the protein band containing monomeric α-synuclein is observed by western-blot analysis (Figure 3A). Western blot analysis shows that in FAP individuals, despite of some inter-individual variations, α-synuclein presents a much higher number of bands at higher molecular mass than asymptomatic or control individuals. This could be interpreted as a sign of α-synuclein aggregation, as already described [12, 13]. Of interest, asymptomatic FAP individuals present an α-synuclein electrophoretic profile very similar to control individuals, indicating that the observed

aggregation is associated with the progression of FAP. To validate the use of α-synuclein as a FAP biomarker we analyzed transplanted individuals, both after an orthotropic liver transplantation (OLT) and domino liver transplantations (DLT). In OLT, FAP individuals receive a cadaveric liver while in DLT the transplanted FAP patient donated his liver to a patient suffering from a potentially fatal liver pathology. Western blot analysis of α-synuclein shows that individuals subjected to OLT present α-synuclein forms as a control individual, suggesting that the high molecular mass form of α-synuclein are mainly produced in individuals presenting FAP symptoms (Figure 3B). It is apparent that patients subjected to DLT have some high-molecular mass aggregates of α-synuclein (Figure 3B). In this analysis, TTR was used as loading control.

α-synuclein aggregation as a biomarker of FAP progression

Since the liver synthesizes more than 90% of blood circulating TTR, hepatic transplant stops the production of the amyloidogenic TTR variant and halts disease progression. The first liver transplantation procedures for FAP was performed in Sweden in 1990 and the favorable clinical outcome for the first 4 transplanted patients prompted a widespread use of this procedure [14]. Liver transplantation for FAP V30M is now widely performed to halt the progression of the clinical manifestations of the disease. Liver transplantation sometimes leads to gradual fibril reabsorption and disease stabilization, especially of neurologic complications. Ideally, the transplant should be performed as early as possible in the disease course [15, 16]. Unfortunately, the presence of a given TTR mutation is not a predictor of disease onset or progression given the considerable number of asymptomatic mutation carriers. Finding early warning biomarkers unequivocally associated to FAP onset and progression would

allow the preventive use of liver transplantation. At present, many groups recommend this procedure early in the course of the disease [17 - 20]. In this work, we focused on saliva as a potential source of protein biomarkers, having previously shown that the wild-type/V30M TTR ratio in saliva is identical to the one found in plasma [21]. α-Synuclein was found in different bands of high molecular mass in FAP patients, confirmed by western blot analysis with a specific antibody. It likely corresponds to oligomeric forms of α-synuclein, as previously observed in the serum and used as biomarker for Parkinson's disease [22]. Since these oligomers are not evident on healthy subjects and asymptomatic TTR V30M mutation carriers, it is of discriminating value for these conditions. It is also noteworthy that OLT individuals presented much lesser oligomeric forms of α-synuclein than DLT individuals. Hence, we propose the detection of α-synuclein oligomers as a FAP biomarker. α-Synuclein is associated with Parkinson's disease and the formation of Lewy bodies [23]. A previous study showed elevated levels of both α-synuclein oligomers and the ratio between oligomer and total α-synuclein in the cerebrospinal fluid of Parkinson's disease patients [23, 24]. In the present work we found similar results, with α-synuclein oligomers increased in the saliva of TTR V30M carriers presenting FAP symptoms. The physiological and molecular mechanisms underlying this observation are yet unknown although it is likely that α-synuclein plays a major role in several conformational pathologies.

Conclusions

Through a simple and non-invasive method of sample collection, we were able to find evidences of α-synuclein oligomerization in whole saliva that can be used as biomarker for FAP progression. The presence of α-synuclein in saliva opens the way for a simple and non-invasive analysis of this protein in the clinical context of other conformational diseases such as Parkinson.

Declaration of Interest statement

There are no conflicts of interest.

Acknowledgments

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Tables

Table 1- Proteins identified in saliva of control individuals

Band	Protein Identification	Accession code	Protein Score	Total Ion Score	Matched MSMS peptides
1	Mucin-5B	MUC5B_HUMAN	123	123	4
2	α -synuclein	SYUA_HUMAN	172	141	3
3	α -synuclein	SYUA_HUMAN	288	239	5
4	Polymeric immunoglobulin receptor	PIGR_HUMAN	217	157	5
	α-synuclein	SYUA_HUMAN	159	136	3
5	α -synuclein	SYUA_HUMAN	167	128	3
6	Serum albumin	ALBU_HUMAN	334	215	6
	α -synuclein	SYUA_HUMAN	217	186	3
7	Serum albumin	ALBU_HUMAN	156	102	4
	Igκ-1 chain C region	IGHA1_HUMAN	105	93	3
	Igκ-2 chain C region	IGHA2_HUMAN	101	93	3
8	α -amylase 1	AMY1_HUMAN	784	666	9
	α -amylase 2B	AMY2B_HUMAN	754	666	9
9	α -amylase 1	AMY1_HUMAN	1,110	971	12
	α -amylase 2B	AMY2B_HUMAN	1,080	971	12
10	Serum albumin	ALBU_HUMAN	176	129	4
	α amylase 2B	AMY2B_HUMAN	165	117	4
	Pancreatic α -amylase	AMYP_HUMAN	149	108	2
11	Actin α cardiac muscle 1	ACTC_HUMAN	101	70	2
	Actin, α skeletal muscle	ACTS_HUMAN	100	70	2
12	Zinc- α -2-glycoprotein	ZA2G_HUMAN	167	102	4
	Protein S100-A8	S10A8_HUMAN	167	135	3
13	Zinc- α -2-glycoprotein	ZA2G_HUMAN	124	86	2
	Carbonic anhydrase 6	CAH6_HUMAN	110	91	4
	Protein S100-A8	S10A8_HUMAN	82	51	2
14	α -amylase 2B	AMY2B_HUMAN	776	688	10
15	α -amylase 1	AMY1_HUMAN	504	417	9
16	α -amylase 2B	AMY2B_HUMAN	238	189	5
17	Ig kappa chain C region	IGKC_HUMAN	94	83	2
18	Zymogen granule protein 16 homolog B	YP003_HUMAN	56	31	2
19	α -amylase 2B	AMY2B_HUMAN	172	114	3
20	Lipocalin-1	LCN1_HUMAN	318	258	4
	α -synuclein	SYUA_HUMAN	232	211	4
21	Cystatin-SN	CYTN_HUMAN	494	428	7
22	Cystatin-SN	CYTN_HUMAN	270	216	4
	Cystatin-S	CYTS_HUMAN	184	119	3
	Hemoglobin subunit α	HBA_HUMAN	141	109	3
	Cystatin-SA	CYTT_HUMAN	119	95	2

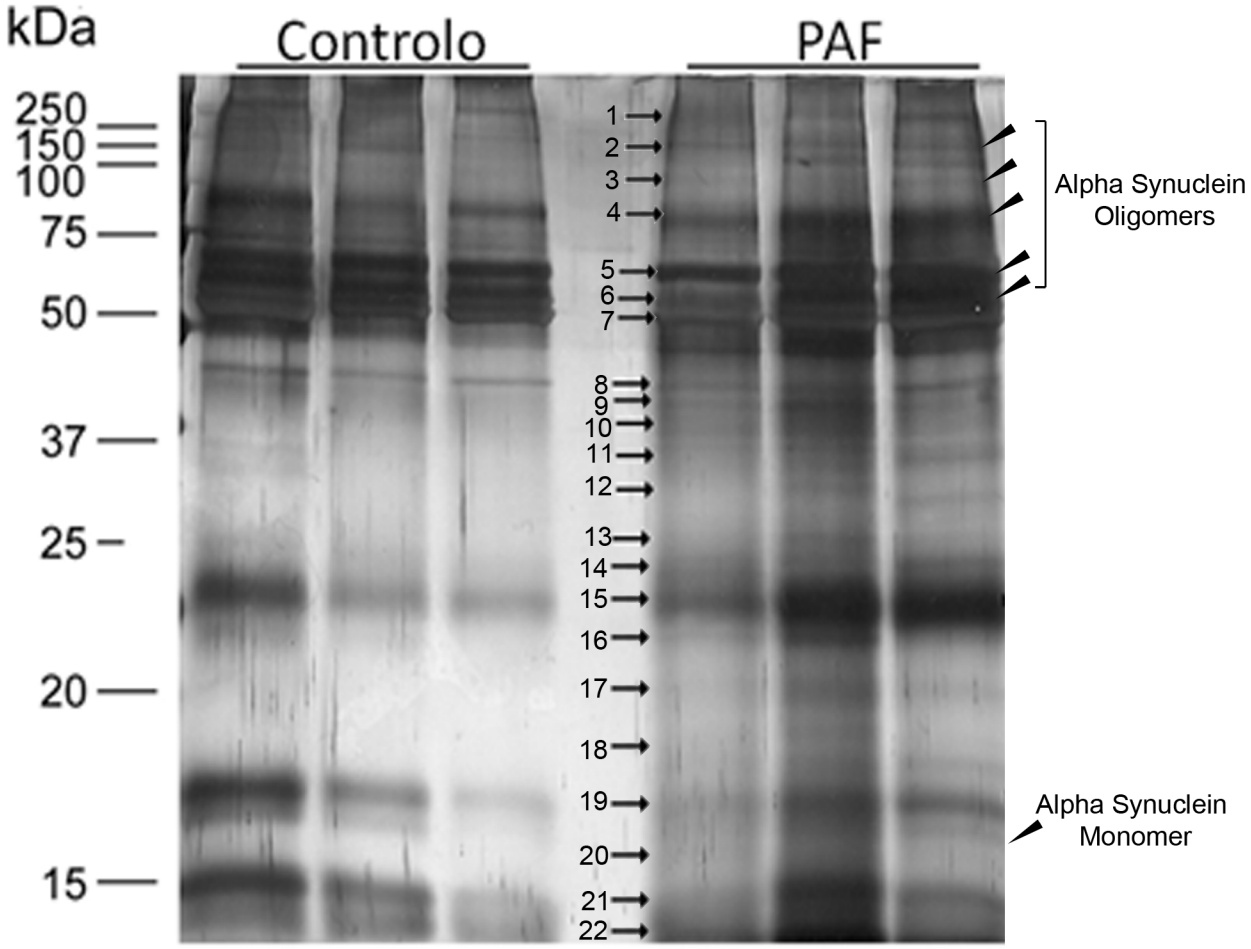
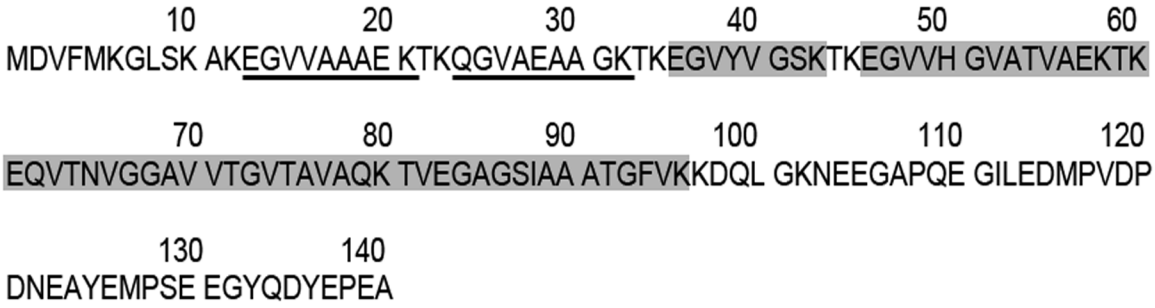


Figure 1 - SDS-PAGE of salivary proteins. Molecular markers are represented on the left-hand side of the SDS-PAGE gel image. We characterized the saliva profile by using the 1D Phoretix software to assign discreet protein bands in the profiles obtained. These results are in agreement with visual observation of the gel pattern. Despite slight inter-individual variations in whole saliva composition, 22 protein bands (from 1 to 22) were consistently present in all saliva protein profiles. The protein bands in which α-synuclein was identified are assigned on the right-hand side.

A



B

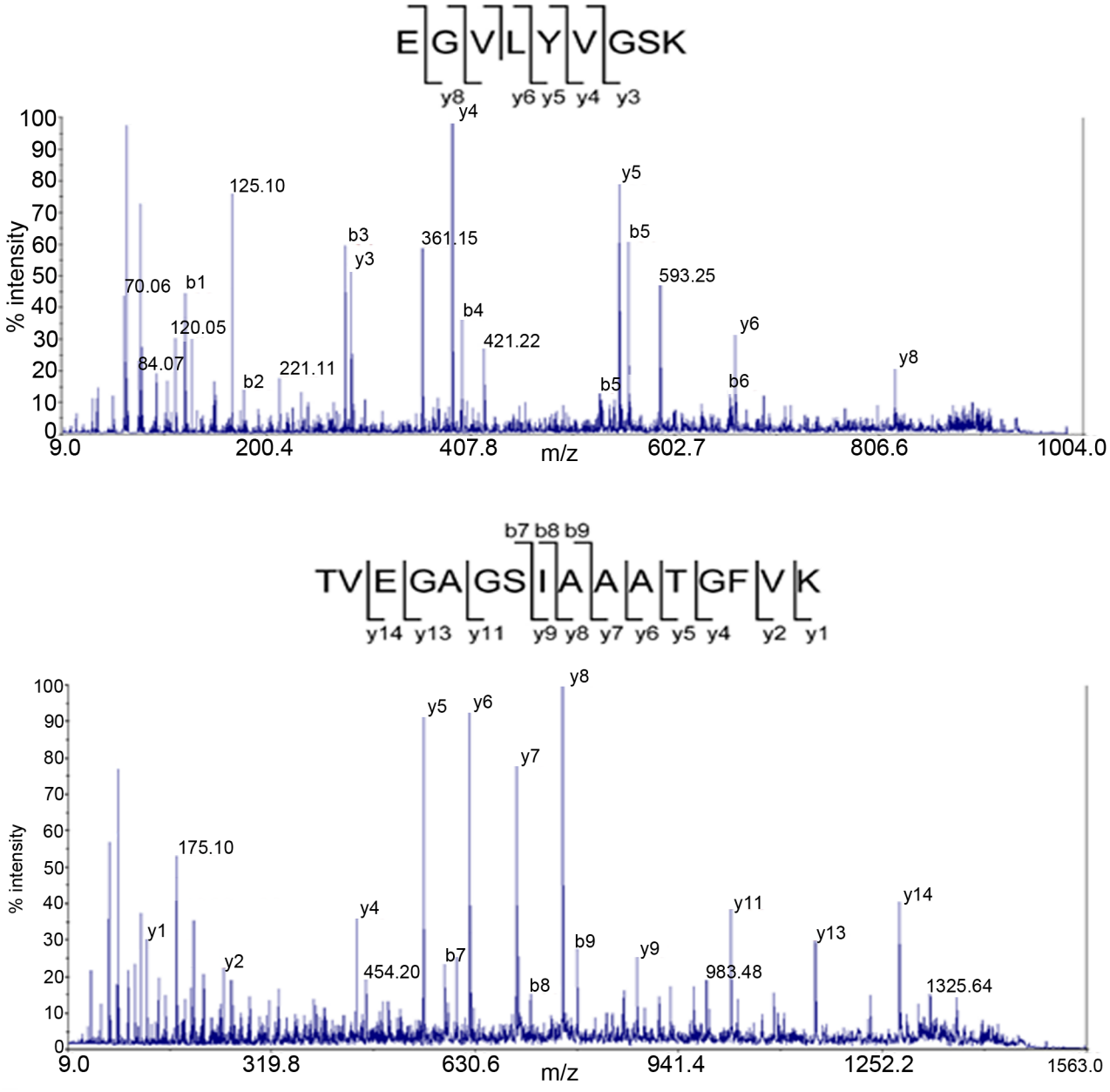


Figure 2– (A) Sequence coverage of α-synuclein identified by tandem MS. MSMS confirmed peptides are highlighted in grey. Underlined peptides were detected by MS only. (B) Example of two CID-MS/MS spectra of the α-synuclein peptide 951.563 with the sequence EGVLYVGSK and the α-synuclein peptide 1478.850, with the sequence TVEGAGSIAAATGFVK.

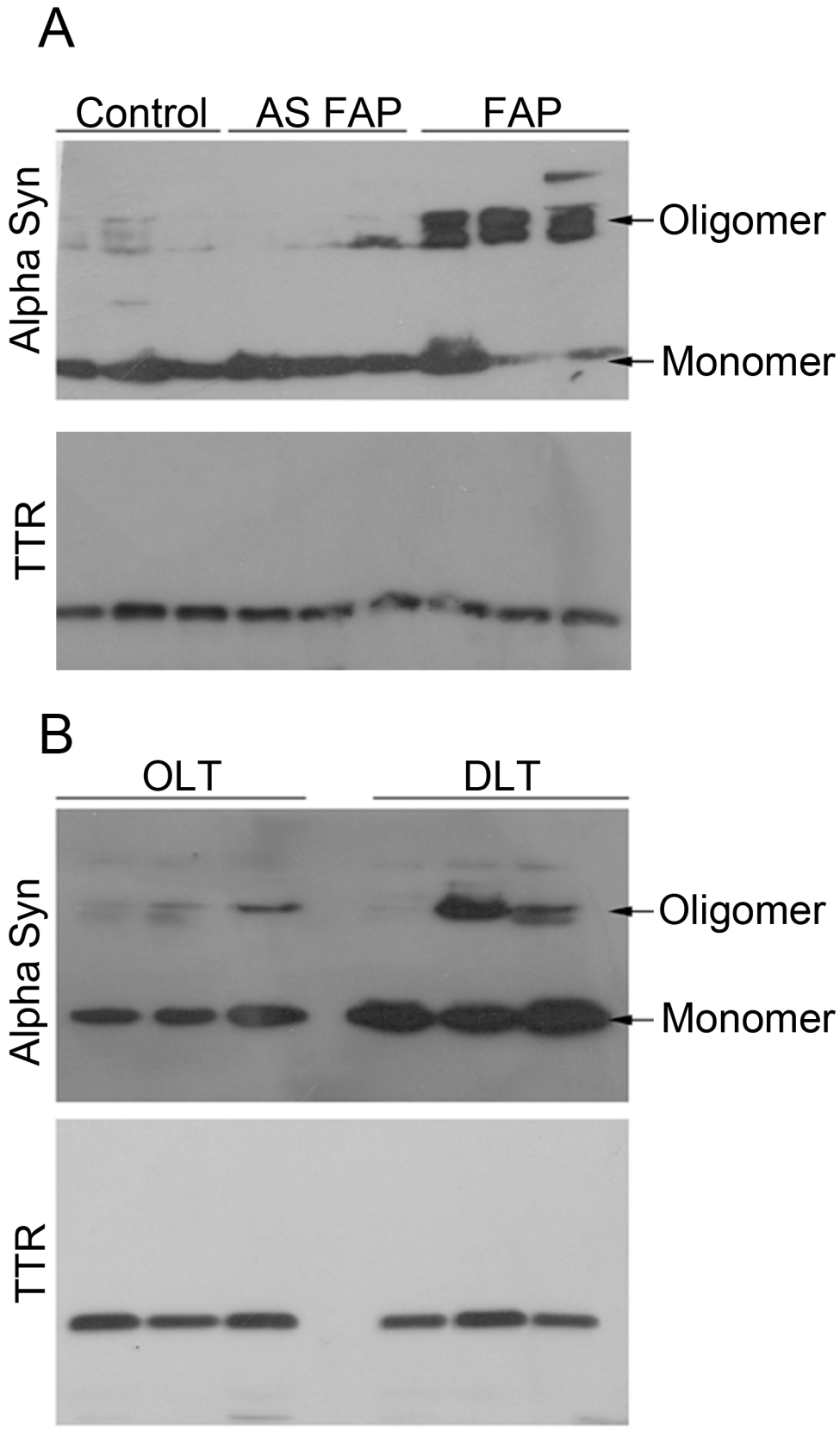


Figure 3 - (A) Western blot analysis of saliva from control, asymptomatic TTR V30M carriers and FAP subjects for α-synuclein detection. (B) Saliva western blot analysis from OLT and DLT individuals for α-synuclein and TTR detection.