TREATMENT OF HISTOLOGICAL MATERIAL FOR THE DETECTION PHOSPHORYLATED AND UNPHOSPHORYLATED ALPHA-SYNUCLEIN

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Parkinsonism is one of the most common human neurodegenerative disorder [20]. During recent years, progress has been made in the differentiation of parkinsonism based on genetic susceptibility to Parkinson's disease (PD) [2], with consideration of influence of various environmental factors [2, 8].

Alpha-synuclein (a-Syn) is the most likely candidate, influencing the course of the pathological process and its distribution. Identification of alpha-synuclein is widely studied with genetic, immunohistochemistry and other techniques.

Alpha-synuclein is a member of the synuclein family – small proteins are made of 120-140 amino acids. Synuclein family includes alpha-, beta-, gamma-synuclein and synoretin (detected in the retina). Alpha-synuclein is widespread in nervous system and making up to 1% of all cytoplasmic proteins of the nervous tissue of human brain [5]. Alpha-synuclein is most studied in detail due to his discovery in Lewy bodies - abnormal aggregates of protein in neurons are detected in PD [10] and other neurodegenerative disorder. This disorder are called synucleinopathies [8]. Mutation in the α-Syn gene associated with one of hereditary forms of PD [14]. In addition, α-Syn is a part of the amyloid plaques in Alzheimer's disease [15]. Important role in the pathology of neurodegenerative disorder is associated with a tendency to form insoluble aggregates [13] that leads to disfunction of neurons. It is assumed that they are formed by a prion-like mechanism [15], when a protein molecule with incorrect conformation promotes to appearance and spread of such molecules from neuron to neuron, which confirmed *in vitro* and *in vivo* studies [9] . Pathological and clinical data suggest that the formation of Lewy bodies and neurodegeneration in Parkinson's disease begins with the structures of the peripheral nervous system and then spreads to the overlying parts of the CNS and affects eventually the substantia nigra and basal nuclei [7].

Despite more than 20 years history of α-Syn study [16, 13] there is not enough data about its normal intracellular localization and functions.

The nuclear and synaptic localization of α-Syn is shown and also found the soluble cytoplasmic and membrane-bound forms, including mitochondrial localization [12]. It is assumed involvement of α-Syn in synaptic transmission [16]. Alpha-synuclein has multiple splice variants that differ in length C-end of the molecule, and is present in various phosphorylated forms, which have big importance in the formation of aggregated forms of the protein [4]. Probably due to the presence of different forms of the protein and its tendency to form insoluble aggregates [4], immunohistochemical data about localization of α-Syn in the nervous system dependent on the antibodies that are used for recognition different epitopes. For example, researchers distinguish in one case predominantly nuclear, in the other case the synaptic localization of α-Syn when they use different antibodies [19]. There are few studies that are generally describe normal distribution of α-Syn in different structures of the human [2,3,6] and laboratory animals nervous system, moreover is shown possible interstrain and species differences [17].

In the literature information on the localization of a-Syn in different structures of the nervous tissue is presented not enough due to properties of the preparation materials to the immunohistochemical and immunofluorescence reactions (the ways of fixing of material, preliminary treatment of the sections etc.) and conducting reactions. In connection with the above in our work the following goal was set.

Objective: to work out the optimal protocol for immunohistochemistry and immunofluorescence reactions to detect phosphorylated alpha-synuclein in the human salivary glands biopsies.

Material and methods

The object of the study were the nerve fibers in the human salivary glands biopsies which were fixed in 4% neutral-buffered formalin, rinsed with tap water. Then one part of the samples was conducted through 15% and 30% sucrose solution and impregnated with medium O.C.T. (Tissue Tek), after that they were frozen and cut in the frozen state with a freezing microtome (Sakura Tissue Tek). Another part of the fixed samples were dehydrated and prepared for filling in paraffin, comparing the two variants of conduits - through chloroform and isopropanol [1]. Paraffin sections from 10 to 40 mkm thick were prepared with sliding microtome Leica SR2000.

In our work, we tested mouse monoclonal antibodies to phosphorylated alpha-synuclein (α-Syn-p129) from two different manufacturers Wako and Biolegend. As secondary antibodies we used antibodies labeled with fluorochromes from Sigma company: sab4600060 (CF555), sab4600045 (CF488). During the work we compared the polymeric detection

system MultiVision TL-012-MARH (Thermofisher) and peroxidase method using EXTRA 3 kit (Sigma).

For disclosure of epitope was performed pretreatment, using different methods of treatment sections: heating in a microwave oven (600W, 10 min), with an acidic and an alkaline buffer solution (citrate buffer, pH 6.0 and tris-EDTA buffer, pH 9.0-9.2) and concentrated formic acid.

Results

In association to the hypothesis of peripheral onset Parkinson's disease [7] in the human salivary glands biopsies we estimated specificity of antibodies from different manufacturers to identify a-Syn in peripheral nerves innervating salivary glands and efficiency of different methods of unmasking antigen contained in the crosslinks of proteins after fixation.

We found that unphosphorylated and phosphorylated a-Syn had a similar localization and detected in salivary gland tissue both in large bundles of nerve fibers and in individual fibers. However, expression of phosphorylated α-Syn (α-Syn-p129) was significantly lower and usually detected in large bundles of nerve fibers both along the course of the whole fiber and in the form of clumps or clusters of dots. In patients with PD were found α-Syn-positive fibers in 5 cases out of 8, along with that α-Syn-p129-immunopositive nerve fibers found in 6 cases out of 8 [3].

When we worked with fixed material during preparation samples for filling in paraffin the best results have been shown with the use of isopropyl alcohol than chloroform (see Table 1). In comparing paraffin and frozen sections we preffered the first, because in frozen section a hight level of non-specific staining of the salivary gland by using antibodies conjugated to alkaline phosphatase (MultiVision TL-012-MARH, Thermofisher) is observed. In comparing sets of detection TL-012-MARH (Thermofisher) and EXTRA 3 kit (Sigma) it was found that the polymer set shows greater sensitivity and reproducibility.

Condidtions of antigen's pretreatment significantly influenced on the binding of antibodies manufactured by Wako, while antibodies manufactured by Biolegend was less sensitive to the pretreatment methods for material. If antigen's pretreatment is not performed the antibodies both manufacturers showed weak immunoreactivity. In comparing sections were treated with citrate buffer (pH = 6.0) and Tris-EDTA buffer (pH = 9.0-9.2) we found that antigen's pretreatment in acidic medium was more effective than the alkaline, and antibodies manufactured by Wako showed the best results.

Treatment of sections with formic acid showed that it is a potent solubilizing agent and enhances detection of α-Syn, it enhances detection of amyloid and prion protein

according to the literature [11, 18]. Short time treatment with formic acid (1-2 minutes) showed the result was similar to another which observed after heat treatment. In comparing the time of treatment with formic acid we observed minimal differences for antibodies manufactured by Biolegend, and for antibodies manufactured by Wako optimal time was at least 10 minutes and the results were significantly better (Fig. 1) than in the treatment with citrate buffer. Important to note, after treatment with formic acid sections must be rinsed with tap water during 20 minutes, disregard of these conditions leads to the formation crystals in the sections.

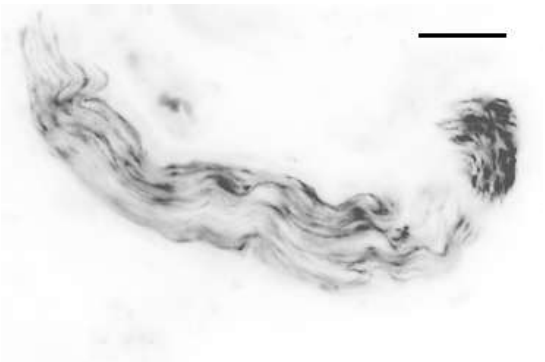

Figure 1. Nerve fiber in the human salivary glands biopsies.
Image is inverted. It was obtained by immunofluorescence method. The length 10 mkm.

When working with the system of detection MultiVision TL-012-MARH, it is better to perform a joint detection in one section α-Syn-p129 and neuronal enzyme, in our case neuronal enzyme was tyrosine hydroxylase. It is necessary for reliable detection of phosphorylated alpha-synuclein in nerve fibers and exclude nonspecific staining associated with the endogenous alkaline phosphatase activity (served as an enzyme label for the secondary antibodies). Co-localization α-Syn-p129 and tyrosine hydroxylase is an indication of the accumulation of α-synuclein in the adrenergic nerve fibers.

Table 1. Original immunohistochemistry methods to identify alpha-synuclein in paraffin sections of the human salivary gland biopsies.

Antibodies	Variant of histological conduit	Restoration of the antigen	Detection system	Primary antibodies dilution	Evaluation of results
Pan-synuclein (Sigma)	Chloroform	Citrate buffer pH=6.0	IF	1:200	-
			EXTRA-3	1:200	+
			MultiVision	1:200	+
		Formic acid ~5 min	IF	1:200	+
			EXTRA-3	1:200	+
			MultiVision	1:200	++

	Isopropyl alcohol	Citrate buffer pH=6.0	IF	1:200	+
			EXTRA-3	1:200	+
			MultiVision	1:200	+
		Tris-EDTA pH=9.2	IF	1:200	-
			EXTRA-3	1:200	-
			MultiVision	1:200	-
		Formic acid 1 min	IF	1:200	+
			EXTRA-3	1:200	+
			MultiVision	1:200	++
		Formic acid 5 min	IF	1:200	++
			EXTRA-3	1:200	++
				1:400	++
			TL-012-MARH	1:200	+++
				1:400	+++
		Formic acid 10 min	IF	1:200	+++
			EXTRA-3	1:200	+++
			MultiVision	1:200	+++
		Formic acid 20 min	IF	1:200	+++
			EXTRA-3	1:200	+++
			MultiVision	1:200	+++
α-synuclein S-129 (Biolegend)	Isopropyl alcohol	Citrate buffer pH=6.0	IF	1:500	+
			EXTRA-3	1:500	-
			MultiVision	1:500	+
		Tris-EDTA pH=9.2	IF	1:500	+
			EXTRA-3	1:500	-
			MultiVision	1:500	-
		Formic acid 1 min	IF	1:500	++
			EXTRA-3	1:500	+
			MultiVision	1:500	++
		Formic acid 5 min	IF	1:500	+++
			EXTRA-3	1:500	++
			MultiVision	1:500	+++
		Formic acid 10 min	IF	1:500	+++
			EXTRA-3	1:500	+++
			MultiVision	1:500	+++
			IF	1:1000	+++
			EXTRA-3	1:1000	+++
			MultiVision	1:1000	+++
		Formic acid 20 min	IF	1:500	+++
			EXTRA-3	1:500	+++
			MultiVision	1:500	+++
	Chloroform	Citrate buffer pH=6.0	EXTRA-3	1:500	-
			MultiVision	1:500	+
		Tris-EDTA pH=9.2	EXTRA-3	1:500	-
			MultiVision	1:500	-
		Formic acid 10 min	EXTRA-3	1:500	+++
			MultiVision	1:500	+++
α-synuclein S-129 (Wako)	Isopropyl alcohol	Citrate buffer pH=6.0	IF	1:1000	+
			EXTRA-3	1:1000	-
			MultiVision	1:1000	+

		Tris-EDTA pH=9.2	IF	1:1000	-
			EXTRA-3	1:1000	-
			MultiVision	1:1000	-
		Formic acid 1 min	IF	1:1000	+
			EXTRA-3	1:1000	+
			MultiVision	1:1000	++
		Formic acid 5 min	IF	1:1000	++
			EXTRA-3	1:1000	++
			MultiVision	1:1000	+++
		Formic acid 10 min	IF	1:1000	+++
			EXTRA-3	1:1000	+++
			MultiVision	1:1000	+++
			IF	1:1000	+++
			EXTRA-3	1:2000	++
			MultiVision	1:2000	+++
		Formic acid 20 min	IF	1:1000	+++
			EXTRA-3	1:1000	+++
			MultiVision	1:1000	+++
	Chloroform	Citrate buffer pH=6.0	EXTRA-3	1:1000	-
			MultiVision	1:1000	+
		Tris-EDTA pH=9.2	EXTRA-3	1:1000	-
			MultiVision	1:1000	-
		Formic acid 10 min	EXTRA-3	1:1000	+++
			MultiVision	1:1000	+++

Comments: Pan-synuclein – unphosphorylated form of alpha-synuclein; α-synuclein S-129 – phosphorylated form of alpha-synuclein at S-129*;* MultiVision – MultiVision Polymer Detection System TL-012-MARH (Thermofisher); EXTRA-3 – EXTRA-3 KIT (Sigma); + pale staining, antigen located in cluster of large blood vessels predominantly; ++ medium staining, antigen located in bundle of nerve fibers innervating blood vessels of different caliber; +++ intense staining, bundles of nerve fibers of different caliber identified.

Thus, the unphosphorylated and phosphorylated α-Syn had a similar localization and detected in salivary gland tissue in large bundle of nerve fibers and in separate fibers. However, expression of phosphorylated α-Syn was significantly lower and usually detected in large bundle of nerve fibers both along the course of the whole fiber and in the form of clumps or clusters of dots.

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