Annals of Diagnostic Pathology 32 (2018) 10–16

Contents lists available at ScienceDirect

Annals of Diagnostic Pathology

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

Increased expression of importin-β, exportin-5 and nuclear transportable proteins in Alzheimer's disease aids anatomic pathologists in its diagnosis

Gerard Nuovo[a,b,*], Vicky Amann[b], James Williams[b], Paige Vandiver[b], Maria Quinonez[b], Paolo Fadda[a], Bernard Paniccia[b], Louisa Mezache[a], Adel Mikhail[b]

[a] Ohio State University Comprehensive Cancer Center, Columbus, OH, United States
[b] GNOME Diagnostic Laboratory, Powell, OH 43065, United States

ARTICLE INFO

Keywords:
Co-expression
Importin
Exportin
Alzheimer's disease

ABSTRACT

Understanding the metabolic profile of neurons with the hyperphosphorylated tau protein characteristic of Alzheimer's disease is essential to unraveling new potential therapies and diagnostics for the surgical pathologist. We stratified 75 brain tissues from Alzheimer's disease into hyperphosphorylated tau positive or negative and did co-expression analyses and qRTPCR for importin-β and exportin-5 plus several bcl2 family members and compared the data to controls, Down's dementia and Parkinson's disease. There was a significant increase in the expression of importin-β and exportin-5 in Alzheimer's disease relative to the three other categories (each p value < 0.0001) where each protein co-localized with hyperphosphorylated tau. Both apoptotic and anti-apoptotic proteins were each significantly increased in Alzheimer's disease relative to the three other groups. Neurons with hyperphosphorylated tau in Alzheimer's disease have the profile of metabolically active cells including increased exportin-5 and importin-β mRNA and proteins which indicates that immunohistochemistry testing of these proteins may aid the surgical pathologist in making a definitive diagnosis.

1. Introduction

Many proteins and RNAs require transit through the nuclear core complex either by diffusion or, for proteins > 40 kDa, through a GTPase dependent mechanism. Importins and exportins are the main proteins that chaperone macromolecules through the complex [1-9]. Importins have two subunits, α and β, with importin-β capable of transporting molecules alone. Exportin-5 plays a key role in oncogenesis [10-12].

The role of dysregulation of importins and exportins has best been detailed in oncogenesis. Elevated exportin-5 expression correlates with poor survival in colorectal cancer and its knockout in cell lines limits tumor cell growth [10]. Both importin and exportin levels are increased in hepatocellular carcinoma and correlate with increased tumor aggressiveness [11]. The tumor suppressor p53 has been shown to reduce levels of importin and exportin in hepatocellular carcinoma models and, in this way, reduce the tumor's aggressiveness [12].

Much less is known regarding the dysregulation of importin and exportin expression in neurodegenerative diseases. Increased importin-α expression has been shown in Alzheimer's disease in cells with Hirano bodies (actin-aggregates) [9]. Hyperphosphorylated tau protein, the classic marker of neurofibrillary tangles in Alzheimer's and Down's dementia, binds to and distorts the nuclear pore complex and, thus, may alter nuclear trafficking [6]. Abnormal cellular localization of proteins is the more typical finding related to nuclear trafficking in neurodegenerative diseases [1-9]. For example, Nrf2 (activator of the antioxidant response element) is primarily nuclear in normal neurons but mostly cytoplasmic in Alzheimer's disease neurons and nuclear in Parkinson's disease [7]. Abnormal cytoplasmic localization of NTF2 (nuclear transport factor 2) has been described in hippocampal neurons in Alzheimer's disease [6]. Both importin α and β show reduced nuclear and increased cytoplasmic localization in the anterior motor neurons of amyotrophic lateral sclerosis [1].

The bcl2 family includes apoptotic (bcl2, bclX, MCL1) and anti-apoptotic proteins (PUMA, NOXA, BIM, BAD) whose balance may determine whether the bcl2 family direct modulators of apoptosis (BAK, BAX) induce cell death [13-17]. Nuclear trafficking of the bcl2 family members and caspase-3 is correlated with apoptosis [13-16].

Metabolically active cells, such as cancer cells, often display dysregulated nuclear trafficking evidenced by increased exportin and/or importin levels [10-12,16]. Also, such cells may show increased levels of bcl2 family members such as MCL1 [10-12] that block programmed cell death.

The purpose of this manuscript was to compare the expression of

* Corresponding author at: Phylogeny Lab, 1476 Manning Parkway, Powell, OH 43065, United States.
E-mail address: nuovo.1@osu.edu (G. Nuovo).

http://dx.doi.org/10.1016/j.anndiagpath.2017.08.003

importin-β and exportin-5 in Alzheimer's versus controls, Down's syndrome dementia, and Parkinson's disease, and to correlate this with the hyperphosphorylated tau protein.

2. Materials and methods

2.1. Tissues

Formalin fixed, paraffin embedded tissues were available from the files of Folio (Alzheimer's and Parkinson's disease) and from the consult files of one of us (GJN, Down's dementia). The age, specific brain region of the tissue, and BRAAK scoring were available. For Alzheimer's disease, 23 formalin fixed, paraffin embedded tissues samples from 11 separate donors that included cortex and hippocampus were available from people who had BRAAK scores of III or IV. Twenty seven formalin fixed, paraffin embedded tissues sections from age matched controls from the cortex and hippocampus served as the negative controls. For Down's dementia, 11 tissues were available from 3 donors that ranged in age from 34 to 42 years old.

Frozen, unfixed tissues were also available from the files of Folio for Alzheimer's and Parkinson's disease and normal tissues. For the Alzheimer's disease samples, 54 separate tissues primarily from the cortex and hippocampus were available from 6 donors who had BRAAK scores of III or IV. For the Parkinson's disease samples, 16 tissues were available from 5 donors that were primarily from the substantia nigra or midbrain. For each sample from people with Alzheimer's or Parkinson's disease, cryostat sections (5 μm each) were obtained and fixed in formalin. In this way, immunohistochemistry for hyperphosphorylated tau protein (Alzheimer's disease) or a-synuclein (Parkinson's disease) allowed the segregation of these samples into either hyperphosphorylated tau protein positive or negative (Alzheimer's disease) or Lewy body positive or negative (Parkinson's disease).

2.2. Nucleic acid and protein isolation

Tissue samples (150 μm, adjacent to the cryostat sections) collected in RNAlater®-ICE (*Ambion*, AM7030) were stored at − 20 °C for a minimum of 16 h. The standard protocol included lysis Buffer Q (RNA/DNA/Protein Purification Plus Kit: *Norgen Biotek*) containing 10 μl/ml β-mercaptoethanol, homogenization using a motorized Pestle Motor Mixer with nuclease free pestles and microfuge tubes (Midwest Scientific, # A0001), then a 25-gauge needle syringe homogenization, and sequential purification of RNA/DNA/Protein using the RNA/DNA/Protein Purification Plus Kit (*Norgen Biotek*, # 47700).

2.3. qRTPCR

RNA (425 ng) was retro-transcripted using the High Capacity cDNA Reverse Transcription kit (Life Technologies) according to the manufacturer's protocol. The comparative real-time PCR was performed in triplicate, including no-template controls and analyzed using QuantStudio 12K Flex Real-Time PCR System. The Ct Average of each triplicate was used to perform the relative quantification analysis. RNA input was normalized using Human GAPDH as the reference gene and the relative expression was calculated using the comparative Ct method.

2.4. In situ hybridization

Our in situ hybridization protocol has been previously published [17-19]. In brief, in situ hybridization for MCL1 mRNA was done using LNA modified oligomers that were 5′ tagged with digoxigenin.

2.5. Immunohistochemistry

Our immunohistochemistry protocol has been previously published. The tissues were tested for the following antigens: p53, importin-β, exportin-5, hyperphosphorylated tau protein, bclX, or α-synuclein (ABCAM), MCL1, PUMA, and NOXA (Enzo Life Sciences). The analyses were done on the automated Leica Bond platform with the modification that we used the Enzo Life Sciences peroxidase conjugate (catalogue # ADI-950-113-0100).

2.6. Co-expression analysis

Co-expression analyses were done using the Nuance system (CRI) as previously published [17-19]. In brief, a given tissue was tested for two different antigens using fast red and DAB as the chromogens. The results were then analyzed by the Nuance and InForm systems in which each chromogenic signal is separated, converted to a fluorescence based signal, then mixed to determine what percentage of cells were expressing the two proteins of interest.

2.7. Statistical analyses

Statistical analysis was done using the InStat Statistical Analysis Software (version 3.36) and a paired *t*-test (also referred to as a "repeated measure *t*-test"). The null hypothesis was rejected if the significance level was below 5%.

3. Results

3.1. Importin-β and exportin-5 protein in normal brain tissue

Two centimeters normal brain formalin fixed, paraffin embedded tissues from the cortex, midbrain, and hippocampus were analyzed by immunohistochemistry to determine the baseline of importin-β and exportin-5 expression. Importin-β protein was detectable in 11/27 (41%) of the normal brain tissues as compared to 6/27 (22%) that had detectable exportin-5 protein in adjacent sections. Over 80% of the positive cells were neurons based on their cytologic features and the two proteins showed equivalent distribution patterns in serial sections (Fig. 1). Co-expression analyses demonstrated that majority of positive cells for importin-β and exportin-5 were neurons based on both NeuN and pyruvate dehydrogenase co-localization and most neurons expressing importin-β also expressed exportin-5 (Fig. 1). Importin-β localized to the nuclear membrane whereas exportin-5 showed a cytoplasmic based signal (Fig. 1).

3.2. Importin-β and exportin-5 protein in brain tissue from neurodegenerative diseases

Brain tissues from cases of neurodegenerative diseases were tested and scored blinded to the clinical information. The key finding was that the expression of both importin-β and exportin-5 was significantly higher in the Alzheimer's disease tissues when compared to the controls, Down's dementia tissues, or Parkinson's disease tissues (each case $p < 0.001$). The importin-β protein in Alzheimer's disease showed an intense pan-nuclear signal whereas exportin-5 had both a cytoplasmic and nuclear component that was stronger than the controls (Fig. 2). Table 1 indicates that the expression pattern of importin-β and exportin-5 was the same as the controls in Down's dementia (Fig. 2). Similarly, note that the data for exportin-5 was equivalent in the Parkinson's disease tissues to the normal brain samples. There was an increase in the number of positive cases expressing importin-β in Parkinson's disease versus controls (58% versus 41%) but it did not quite reach statistical significance. The signal for importin-β in Parkinson's disease was pan-nuclear and more intense than in the controls (Fig. 2).