The autophagic marker p62 highlights Alzheimer type II astrocytes in metabolic/hepatic encephalopathy

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Metabolic/hepatic encephalopathy is neuropathologically characterized by the presence of Alzheimer type II astrocytes (AA II) with large and clear nuclear morphology. To date, there is no good immunohistochemical marker to better identify these cells. Here, we assessed cases of hepatic encephalopathy of different etiologies by immunohistochemistry using an anti-p62 antibody. We observed peripheral or diffuse nuclear staining of variable intensity in AA II in all cases but not in normal controls or reactive astrocytes. We conclude that p62 is a useful immunohistochemical marker for the identification of AA II and may be helpful for the neuropathological diagnosis of metabolic/hepatic encephalopathy in difficult or equivocal cases.

Key words: Alzheimer type II astrocytes, astrogliopathy, hepatic encephalopathy, metabolic encephalopathy, p62.

INTRODUCTION

The presence of so-called Alzheimer type II astrocytes (AA II) in the human brain usually reflects a metabolic disturbance caused by renal or, more frequently, hepatic dysfunction. Indeed, hepatic encephalopathy has been associated with the presence of AA II in the gray matter of different brain regions, usually involving the deep cortical layers, basal ganglia, and pontine nuclei.1 It is, therefore, considered to reflect a gliopathy.

AA II are characterized by a larger nucleus than in resting or reactive fibrillary or gemistocytic astrocytes, a clear chromatin (Fig. 1G), and scarce cellular processes. They are frequently forming pairs: doublets, or even triplets1 (Fig. 1H). However, AA II may be difficult to visualize on sections stained with hematoxylin and eosin (HE), showing a spectrum of nuclear changes, from slight enlargement and chromatin loosening to a completely clear or empty appearance of the nucleus with a well-defined membrane rim and peripheral dot-like condensation (Fig. 1I, arrow). There is currently no good marker to specifically identify AA II: they are characteristically not or are poorly stained by glial fibrillary acidic protein (GFAP) immunohistochemistry, while they can be depicted using anti-S-100 protein antibodies.1,2 However, S-100 protein is not a specific marker of astrocytes and labels most glioneuronal elements.

Because the neuropathological diagnosis of metabolic encephalopathy may be difficult in its early or less severe disease stages, the application of a reliable immunohistochemical marker would be helpful to objectively support this diagnosis in the routine diagnostic or experimental settings.

Through staining of postmortem brains for other diagnostic purposes, we observed an intense staining of AA II nuclei using p62 immunohistochemistry in patients with metabolic encephalopathy. This observation prompted us to systematically assess glial p62 immunoreactivity in hepatic encephalopathy of different etiologies and to compare the staining pattern with other conditions characterized by prominent reactive gliosis.
MATERIALS AND METHODS

Postmortem brains were selected from the archives of the Institute of Neurology of the Medical University of Vienna and the Neurological Tissue Bank of the IDIBAPS Biobank in Barcelona. The use of brain tissue for research was approved by the respective institutional ethics committees and conforms to the provisions of the Declaration of Helsinki.

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Formalin-fixed, paraffin-embedded tissue blocks from the frontal, temporal and occipital cortices, anterior and posterior basal ganglia, thalamus, midbrain, pons and cerebellum were selected, and 5-μm-thick sections were stained with hematoxylin and eosin (HE) and Luxol fast blue (LFB) and HE (LFB-HE), where the presence of AA II was assessed as present/absent (Table 1). One region per case with obvious AA II was first stained by immunohistochemistry using a commercial...
| Order | Age | Gender | Cause of hepatic/renal damage | Metabolic encephalopathy severity HE | Neuropathological findings | Suspected Alzheimer II astrocytes on HE stained sections |
|-------|-----|--------|-------------------------------|-------------------------------------|-----------------------------|---------------------------------------------------------|
|       |     |        |                               |                                     |                             | Frontal | Cingulum | Parietal | Temporal | Occipital | Hippocampus | BBGG | Amygdala | Thalamus | Midbrain | Pons | Medulla oblat | dentate |
| 1     | 79  | m      | Hepatitis C and liver cirrhosis | Prominent                           | 1) Metabolic encephalopathy; 2) Incidental LB pathology (Braak II) + mild CAA | + + + + + + + + + + + + + |
| 2     | 77  | m      | Hepatitis C                    | Moderate                            | 1) Metabolic encephalopathy; 2) Alzheimer's disease neuropathologic changes A3B3C2; 3) Incidental LB pathology olfactory bulb only | + + + + + + + + + + + + + |
| 3     | 63  | f      | Hepatitis B, liver cirrhosis   | Moderate                            | 1) Metabolic encephalopathy; 2) Acute ischemic stroke | + + + + + + + + + + + + + |
| 4     | 85  | f      | Alcohol abuse, chronic renal insufficiency | Moderate | 1) Metabolic encephalopathy; 2) PART (Braak II) + mild CAA; 3) LATE 3/+ 3/+ 2/+ 3/+ 1/+ 0/s 2/+ 0/s 1/+ 2/+ 0/+ 0/+ 0/+ 0/+ 0/+ |
| 5     | 71  | f      | Alcohol abuse, liver cirrhosis, chronic renal insufficiency | Moderate | 1) Metabolic encephalopathy; 2) Mild cerebellar atrophy; 3) PART (Braak II) + mild CAA | + + + + + + + + + + + + + |
| 6     | 59  | f      | Alcohol abuse, liver cirrhosis | Moderate                            | 1) Metabolic encephalopathy; 2) Acute Wernicke encephalopathy; 3) SVD | + + + + + + + + + + + + + |
| 7     | 59  | m      | Alcohol abuse, liver cirrhosis, hepatocarcinoma | Prominent | 1) Metabolic encephalopathy | + + + + + + + + + + + + + |
| 8     | 65  | m      | Liver cirrhosis                | Prominent                           | 1) Metabolic encephalopathy; 2) Morel cortical laminar sclerosis; 3) Focal subarachnoid bleeding | + + + + + + + + + + + + + |
| 9     | 70  | f      | Primary biliary cirrhosis      | Prominent                           | 1) Metabolic encephalopathy with focal spongy polioencephalopathy | + + + + + + + + + + + + + |
| 10    | 64  | f      | Autoimmune hepatitis, liver cirrhosis | Prominent | 1) Metabolic encephalopathy; 2) Posthypoxic, posterior encephalopathy and bilateral hippocampal sclerosis | + + + + + + + + + + + + + |

(Continues)
| Order | Age | Gender | Cause of hepatic/renal damage | Metabolic encephalopathy severity HE | Neuropathological findings | Suspected Alzheimer II astrocytes on HE stained sections |
|-------|-----|--------|-------------------------------|-----------------------------------|---------------------------|--------------------------------------------------------|
|       |     |        |                               |                                   |                           | Frontal | Cingulum | Parietal | Temporal | Occipital | Hippocampus | BRGG | Amygdala | Thalamus | Midbrain | Pons | Medulla | obl | obl | dentate |
| 11    | 84  | f      | Hepatocellular carcinoma      | Mild                              | 1) Metabolic encephalopathy; 2) PART (Braak IV); 3) Incidental LB pathology (Braak 2) | +       | +        | +        | +        | +         | +           | +    | +        | +         | +        | + | +       |    |     |         |
| 12    | 58  | m      | Hepatocellular carcinoma     | Prominent                         | 1) Metabolic encephalopathy; 2) AgD Saito I | +       | +        | +        | +        | +         | +           | +    | +        | +         | +        | + | +       |    |     |         |
| 13    | 51  | m      | Metastatic unknown primary tumor with subtotal liver destruction | Prominent                         | 1) Metabolic encephalopathy; 2) Pontine micro-metastasis carcinoma | +       | +        | +        | +        | +         | +           | +    | +        | +         | +        | + | +       |    |     |         |
| 14    | 81  | f      | Metastatic pancreas carcinoma, liver necrosis | Moderate                           | 1) Metabolic encephalopathy; 2) Mild ARP (Braak II, CERAD B) | +       | +        | +        | +        | +         | +           | +    | +        | +         | +        | + | +       |    |     |         |
| 15    | 85  | m      | Metastatic prostate carcinoma including liver | Mild                              | 1) Metabolic encephalopathy; 2) Subdural hemorrhage; 3) Acute hypoxic-ischemic neuronal damage in pons and cerebellum | +       | –        | –        | –        | +         | +           | +    | +        | –         | +        | + | +       |    |     |         |
| 16    | 78  | f      | Metastatic colon carcinoma including liver | Mild                              | 1) Metabolic encephalopathy; 2) Mild ARP (Braak I, CERAD A) | +       | +        | –        | +        | +         | –           | –    | –        | +         | +        | + | +       |    |     |         |
| 17    | 81  | f      | B-cell lymphoma diffuse, acute renal and hepatic failure | Moderate                           | 1) Metabolic encephalopathy; 2) Lymphomatosis meningea | +       | +        | +        | +        | +         | +           | +    | +        | +         | +        | + | +       |    |     |         |
| 18    | 68  | m      | Sepsis                        | Moderate                           | 1) Metabolic encephalopathy; 2) PART (Braak II) | +       | –        | –        | –        | +         | +           | +    | +        | +         | +        | + | +       |    |     |         |
| 19    | 52  | m      | Sepsis, cardiopulmonary reanimation | Moderate                           | 1) Metabolic encephalopathy; 2) posthypoxic-postischemic encephalopathy | +       | –        | –        | –        | +         | +           | +    | +        | +         | +        | + | +       |    |     |         |
| 20    | 15  | f      | Sepsis, vasculitis c-ANCA     | Moderate                           | 1) Metabolic encephalopathy; 2) Multiple microbleeds | +       | +        | +        | +        | +         | +           | +    | +        | +         | +        | + | +       |    |     |         |
| 21    | 84  | m      | Sepsis, renal insufficiency  | Mild                               | 1) Metabolic encephalopathy; 2) SVD; 3) PART (Braak I) | +       | –        | +        | +        | +         | –           | –    | +        | –         | +        | + | +       |    |     |         |
| 22    | 0.5 | f      | Sepsis; pulmonary transplantation, fungal sepsis, pulmonary fibrosis, hepato-splenomegaly and hepatic steatosis | Prominent                          | 1) Metabolic encephalopathy; 2) Diffuse gliosis | +       | –        | –        | –        | –         | +           | +    | –        | –         | +        | + | +       |    |     |         |

(Continues)
| Order | Age | Gender | Cause of hepatic/renal damage | Metabolic encephalopathy severity HE | Neuropathological findings | Suspected Alzheimer II astrocytes on HE stained sections |
|-------|-----|--------|-------------------------------|-----------------------------------|----------------------------|---------------------------------------------------|
| 23 | 69 | m | Sepsis, cor pulmonale, hepatic steatosis | Mild | 1) Metabolic encephalopathy - Wernicke encephalopathy; 2) Incidental LB pathology (Braak 3; 3) PART (Braak II) | + + + + + + + + + + + + + |
| 24 | 7 | m | Sepsis, cardial transplantation, fungal pneumonia and sepsis, acute liver necrosis | Prominent | 1) Metabolic encephalopathy; 2) Hypoxic-ischemic damage with cortical necrosis; 3) Fungal microabscesses | + + + + + + + + + + + + + |
| 25 | 64 | m | Pericardial tamponade, congestive liver | Mild | 1) Metabolic encephalopathy; 2) Mild acute hypoxic-ischemic neuronal damage; 3) PART (Braak I) | + + + + + + + + + + + + + |
| 26 | 69 | f | Cardiac and hepatic fibrosis, anemia perniciosa, colitis ulcerosa | Mild | 1) Metabolic encephalopathy; 2) Arteriolosclerosis and status cribrosus; 3) PART (Braak I) | + + + + + + + + + + + + + |
| 27 | 81 | f | Cardial insufficiency | Moderate | 1) Metabolic encephalopathy; 2) PART (Braak II) | + + + + + + + + + + + + + |
| 28 | 1 | m | Cardial malformation with insufficiency and hepatosplenomegaly | Prominent | 1) Metabolic or hypoxic? encephalopathy; 2) malformation: Dandy-Walker like and agenesis of olfactorius | + − + − + + − + + + + + + + |
| 29 | 74 | f | Suprarenal insufficiency; insulinnoma, multiple complications | Mild | 1) Metabolic encephalopathy with Wernicke-like changes and Morel cortical laminar sclerosis; 2) Mild ARP (Braak I, CERAD B) | + + + + + + − + + + + + + |
| 30 | 85 | f | Hepatic insufficiency, unknown origin | Moderate | 1) Metabolic encephalopathy; 2) PART (Braak II; 3) Old infarct | + − + − + + + + + + + + + |
| 31 | 2 | m | Mitochondrial encephalopathy - Leigh syndrome; hepatomegaly | Moderate | 1) Mitochondrial encephalopathy consistent with Leigh-syndrome | − + + − − + + − + + + + |

In case 4, a detailed anatomical mapping of p62 immunoreactivity in relation to the presence of AA II was performed (0, negative; 1, sparse stained glial nuclei; 2, moderate density of stained glial nuclei; 3, high density of stained nuclei; s, single; +, present; −, absent; n.a, not available. ARP, Alzheimer’s disease-related pathology; CAA, amyloid angiopathy; LATE, limbic age-related TDP43 encephalopathy; LB, Lewy body; PART, primary age-related tauopathy; SVD, small vessel disease.)
monoclonal anti-p62 antibody (clone 3/p62 ligand, dilution 1:500; BD-Transduction Laboratories, Franklin Lakes, NJ, USA). Then, selected cases of hepatic diseases (Table 2) were immunostained for p62 in the frontal cortex, basal ganglia, and pons, and in one case, a detailed mapping of p62 distribution was performed (Table 1). Antigen retrieval was performed by boiling the sections in citrate buffer at pH 6.0 for 20 min. The immunoreaction was visualized by the polymer-immuno-complex method using an Envision System kit (Dako, Glostrup, Denmark), and 3,3′-diaminobenzidine was used as chromogen. For double immunofluorescence labeling, the anti-p62 antibody was combined with antibodies against S-100 protein (rabbit polyclonal, dilution 1:2000; Dako), GFAP (rabbit polyclonal, dilution 1:5000; Dako), and tubulin polymerization-promoting protein (TPPP/p25 (rabbit polyclonal, dilution 1:2000; non-commercial). It has been shown that TPPP is mainly expressed in differentiated oligodendrocytes of the central nervous system (CNS),3 After blocking of autofluorescence with Sudan Black B, antibody binding immunoreactivities was visualized with secondary antibodies such as anti-mouse IgG conjugated with Alexa Fluor488 (Thermo Fisher Scientific, Waltham, MA, USA) at a dilution of 1:800 and anti-rabbit IgG conjugated with Cy3 (Thermo Fisher Scientific) at a dilution of 1:1000.

We selected 31 cases with hepatic encephalopathy of viral (Hepatitis C), neoplastic (hepatocarcinoma and liver metastasis), alcoholic (liver cirrhosis), systemic (sepsis), and mitochondrial (Leigh syndrome) origins (Table 1). For additional comparison of the immunostaining pattern, we assessed different pathologies, including subacute stage of cerebral infarction with prominent reactive gliosis, as well as different neurodegenerative diseases with variable degrees of chronic reactive gliosis, including Alzheimer’s disease, corticobasal degeneration, progressive supranuclear palsy, Parkinson’s disease, frontotemporal lobar degeneration with inclusions immunoreactive for transactivation response DNA-binding protein 43 kDa (TDP-43), and Creutzfeldt-Jakob disease (one case each), as well as one normal brain.

Details of cases with hepatic/metabolic encephalopathy are shown in Table 1.

### RESULTS

Nuclear p62 staining was detected in enlarged glial cells of the gray matter that were consistent with AA II on HE-stained sections (Fig. 1J–L). This immunopositivity was observed in all cases with hepatic encephalopathy of different etiologies, except for some of septic origin. Nuclear staining for p62 in AA II was particularly intense in a case of mitochondrial encephalopathy (Leigh syndrome) (Fig. 1M–O). Double immunofluorescence revealed p62-positive nuclei in some delicate GFAP-positive (Fig. 2A) and diffuse S-100 protein-positive cells (Fig. 2B) but not in TPPP/p25-positive oligodendrocytes (Fig. 2C), thus supporting the astrocytic nature of the cells.

In cortical areas, AA II were best identified in deep layers. In severely affected areas, laminar microvacuolation of the neuropil in deep layers could be observed at a low magnification (arrows in Fig. 1A, B) and at a higher magnification (Fig. 1D, E). Here, abundant p62-positive nuclei were identified at a low magnification (arrows in Fig. 1C) and at a higher magnification (Fig. 1F). When AA II showed the characteristic enlarged nuclei with clear chromatin, immunoreactivity was enhanced along the nuclear membrane and in the small punctate condensations (Fig. 1L). In cells with less obvious nuclear change, immunoreactivity was more diffuse.

The distribution and intensity of p62 immunoreactivity in AA II nuclei was not homogeneous among different brain areas of the same patient and between patients. The strongest signal was generally observed in cortical areas and was lower in the basal ganglia and pontine nuclei, but this was not uniform (Table 2). There were cases

### Table 2  p62 Immunoreactivity in the frontal cortex, basal ganglia, and pons in selected patients with different ages, etiologies, and formalin fixation times

| Order | Age | Weeks in formalin | Hepatic pathology | Glial p62 nuclear immunoreactivity |
|-------|-----|-------------------|-------------------|-----------------------------------|
|       |     |                   |                   | Frontal cortex | Basal ganglia | Pons |
| 1     | 79  | 1                 | Hepatitis C/cirrhosis | 0/+             | 1/+           | 0/s |
| 4     | 85  | 1                 | Alcohol abuse/cirrhosis | 3/+             | 3/+           | 0/s |
| 22    | 0.5 | 2                 | Hepatic steatosis | 1/+             | 2/+           | 1/+ |
| 20    | 15  | 2                 | Sepsis, vasculitis | 2/+             | 0/+           | 2/+ |
| 19    | 52  | 3                 | Sepsis | 0/+             | 0/+           | 0/+ |
| 13    | 51  | 4                 | Metastasis | 2/+             | 2/+           | 0/+ |
| 31    | 2   | 4                 | Mitochondrial disorder/Leigh syndrome | 0/-             | 3/+           | 2/+ |
| 14    | 81  | > 4               | Metastasis | 3/+             | 3/+           | 0/+ |
| 7     | 59  | 6                 | Alcohol abuse/cirrhosis/hepatocellular carcinoma | 3/+             | 3/+           | 2/+ |
| 11    | 84  | 6                 | Hepatocellular carcinoma | 1/+             | 0/+           | 0/+ |
| 17    | 58  | 14                | Acute hepatic and renal failure, B cell lymphoma | 0/+             | 0/+           | 0/+ |
| 28    | 1   | > 25              | Cardiac malformation, hepatosplenomegaly | 3/+             | 0/+           | 0/+ |
| 21    | 84  | n.a.              | Sepsis | 0/+             | 0/+           | 0/+ |

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(e.g. case 4) showing a patchy distribution of immunoreactivity in the basal ganglia and cerebral cortex. In rare cases, cells that were considered to be AA II on HE-stained sections were not or were only faintly immunoreactive for p62. Inversely, some cases with p62-positive nuclei were not always clearly identifiable as AA II: for example, in the case of Leigh syndrome (Fig.1M–O) where immunopositivity filled the whole nucleus, in contrast to other cases with peripheral nuclear immunostaining. Bergmann’s glia also showed relatively prominent p62 nuclear staining in one case of metabolic encephalopathy that had no prominent Purkinje cell loss (case 30). In contrast, other pathologies associated with Bergmann gliosis remained negative. Aquaporin 4 immunoreactivity was undetectable in AA II.

No immunostaining in glial nuclei was observed in the control and most neurodegenerative conditions with reactive astrogliosis, except for one case of corticobasal degeneration. In that case, p62-positive nuclei corresponded to those of tau-positive astrocytic plaques on adjacent tissue sections. Moreover, one case of suacute stage cerebral infarction showed moderate nuclear immunoreactivity of large reactive astrocytes and also of some “eosinophilic neurons.” There were no differences in staining intensity that could be related to fixation time (Table 2) or postmortem delay (data not shown). No data on ammonia levels were available.

**DISCUSSION**

We assessed the immunohistochemical expression of p62 in glial cells in different brain diseases and observed prominent nuclear staining of AA II in metabolic/hepatic encephalopathy, including astrocytes with no typical “clear” morphology. This was not observed in reactive astrocytes of most chronic neurodegenerative diseases, except for one case of corticobasal degeneration and subacute stage cerebral infarction, where nuclei of astrocytic plaques and large reactive astrocytes, respectively, were moderately labeled.

This observation suggests p62 as a very useful neuropathological marker of metabolic gliosis, particularly in hepatic encephalopathy.

Immunohistochemistry using anti-p62 antibodies has proved very useful in the study of neurodegenerative diseases, as it is commonly found in neuronal cytoplasmic or nuclear inclusions (e.g. Alzheimer’s disease, frontotemporal lobar degenerations, Lewy body diseases, or trinucleotide repeat disorders such as Huntington’s disease). The presence of p62 is also a useful predictor of C9orf72 expansion mutation when accumulated in granular neurons of the cerebellar cortex or hippocampal neurons.4,5

p62 or sequestosome-1 is a protein encoded by SQSTM1 and is thought to target protein aggregates for lysosomal degradation, by binding to ubiquitinated proteins, among other functions.6–8 It is, therefore, considered to be an indicator of autophagic degradative activity. p62 itself is also degraded by autophagy. When autophagy is induced, it remains at low levels in the cell, while it accumulates when autophagy is deficient. It is also involved in protein aggregation, as shown for several proteinopathies associated with neurodegenerative conditions.9

Hepatic/metabolic encephalopathy has been reported to underlie several complex metabolic alterations,10 including mitochondrial dysfunction in astrocytes due to increased ammonia levels in blood and in the brain,11,12 among others. Moreover, experimental studies have shown an involvement of mitophagy and autophagy13 in the pathogenesis of hepatic encephalopathy. In particular, treatment of cultured rat astrocytes with low concentrations of NH4Cl induced autophagy, while with higher concentrations from 2 mM onwards, NH4Cl inhibited autophagy in astrocytes in a time- and dose-dependent manner.12 These findings may provide one explanation for why high ammonia levels can induce the accumulation of p62 through inhibition of autophagy. In addition, exposure of astrocytes to ammonia also induces...
astrocytic swelling, which can be exacerbated by cytokines/inflammatory mediators. Some experimental studies have also shown that increased plasma membrane aquaporin 4 levels contribute to the astrocytic swelling/brain edema in hepatic encephalopathy. We found no increased immunoreactivity for aquaporin 4 in AA II. However, the detailed mechanism of peripheral and diffuse nuclear staining for p62 in hepatic/metabolic encephalopathy remains to be elucidated.

We observed a somewhat uneven distribution of p62-positive AA II within the same brain area and between different brain areas and cases. It could be postulated that this might be related to levels of ammonia and/or duration or even to the cause of hepatic damage, reflecting an evolutive process of metabolic alterations of astrocytes. While it is generally considered that ammonia levels are positively related to the severity of hepatic encephalopathy, they are not always determinant as other factors may exacerbate it, and they do not necessarily influence patient management. Unfortunately, we do not have enough data on ammonia levels or details on the duration of hepatic disease. Moreover, there was no particular difference in staining intensity depending on the etiology of liver damage, and it was apparently also not influenced by postmortem delay or formalin fixation time.

In summary, the postmortem neuropathological diagnosis of metabolic/hepatic encephalopathy has been somewhat subjective, not always unequivocal diagnosis and can represent a challenge, particularly in less obvious stages. Even if not absolutely specific, we consider p62 as a useful immunohistochemical marker to visualize AA II. It can improve and objectify the identification of metabolic encephalopathy/gliopathy in postmortem brain tissue, in supplementation of classical HE staining features. Why p62 accumulates in the nucleus is, however, still unclear and deserves further investigation, particularly to better understand metabolic disturbances of astrocytes and their relationship with autophagy.

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**DISCLOSURE**

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