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Elevated serum autoantibody against high mobility group box 1 as a potent surrogate biomarker for amyotrophic lateral sclerosis

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a complicate and progressive onset devastating neurodegenerative disease. Its pathogenic mechanisms remain unclear and there is no specific test for diagnosis. For years, researchers have been vigorously searching for biomarkers associated with ALS to assist clinical diagnosis and monitor disease progression. Some specific inflammatory processes in the central nervous system have been reported to participate in the pathogenesis of ALS. As high mobility group box 1 (HMGB1) is elevated in spinal cord tissues of patients with ALS, we hypothesized, therefore, that serum autoantibody against HMGB1 (HMGB1 autoAb) might represent an effective biomarker for ALS. Patients with ALS, Alzheimer's disease, Parkinson's disease, and healthy age-matched control subjects were recruited for this study. ALS group consisted of 61 subjects, the other groups each consisted of forty subjects. We generated a polyclonal antibody against HMGB1 and developed an ELISA-based methodology for screening serum samples of these subjects. All samples were coded for masked comparison. For statistic analyses, two-tailed Student's *t*-test, ANOVA, Bonferroni multiple comparison test, Spearman correlation, and receiver operating characteristic curve were applied. We discovered that the level of HMGB1 autoAb significantly increased in patients with ALS as compared with that of patients with Alzheimer's disease, Parkinson's disease, and healthy control subjects. The differences between all groups were robust even at the early stages of ALS progression. More importantly, higher HMGB1 autoAb level was found in more severe disease status with significant correlation. Our study demonstrates that serum HMGB1 autoAb may serve as a biomarker for the diagnosis of ALS and can be used to monitor disease progression.

Introduction

Amyotrophic lateral sclerosis (ALS) is a relentlessly devastating neurodegenerative disease that is characterized by selective loss of motor neurons within the spinal cord, brainstem, and cerebral cortex (Mitchell and Borasio, 2007). Motor function is increasingly attenuated as the disease progresses, often resulting in complete paralysis and respiratory failure within 2–3 years after diagnosis (Haverkamp et al., 1995; Pratt et al., 2012). The pathogenesis of ALS, however, remains largely unresolved. ALS clearly affects motor circuits within the central nervous system (CNS), but it is now recognized that extra-motor systems are also involved (Dettmers et al., 1993; Hayashi and Kato, 1989; Oey et al., 2002). The annual incidence of ALS is 1–2 per 100,000 (Worms, 2001), with approximately 10% of these patients inheriting a familial form of the disease (Kunst, 2004). The remaining cases represent a sporadic form of ALS, with even less understanding of direct cause.

Unfortunately, a diagnostic test that is specific for ALS has still not been developed. Neurologists conventionally diagnose ALS based on clinical symptoms and electrophysiological data, in general acquired from the techniques available since the late nineteenth century (Brooks et al., 2000; Radunovic et al., 2007). As a result, about 10% of patients with ALS are misdiagnosed (Davenport et al., 1996; Traynor et al., 2000), and the disease is usually not correctly diagnosed until 13 to 18 months after the onset of symptoms (Chio et al., 1999). For many

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years, clinical investigators have been searching for biomarkers that could be used to accurately diagnose ALS during early stages of the disease (Cronin et al., 2006; Henkel et al., 2004; Mitsumoto et al., 2007; Nagata et al., 2007; Ranganathan et al., 2005, 2007; Tanaka et al., 2006). It is hoped that these ALS biomarkers could also be used to monitor disease progression and to assess treatment effectiveness. In addition, identification of disease relevant biomarkers would undoubtedly provide critical insights into the pathogenesis of ALS.

Because ALS is a disease of the CNS, specific biomarkers will likely prove highly effective when directly analyzing either brain tissue or cerebrospinal fluid (CSF) of patients. Obtaining brain tissue for diagnostic purposes, however, is not an acceptable practice within a clinical setting. Even the use of spinal tap procedures to obtain CSF is generally considered too invasive to be used in the follow-up examinations of patients with ALS. Recent proteomic approaches have identified several potential ALS biomarkers, but none of these markers represents a solution that can be effectively implemented in a clinical setting (Ludolph, 2011; Ryberg and Bowser, 2008).

By analyzing CSF samples from patients with ALS, a panel of ALS biomarkers was recently described (Mitchell et al., 2009). This panel includes molecules that promote inflammation and blood vessel formation, which suggests that inflammatory responses are activated within the CNS of patients with ALS. These biomarkers, therefore, may reflect neuroinflammation associated with this degenerative process (Mitchell et al., 2009). Damage associated molecular pattern molecules (DAMPs) are molecules that can initiate and perpetuate immune response in the noninfectious inflammatory processes. DAMPs can be divided into two groups, *i.e.*, intracellular protein group and extracellular matrix protein group. High mobility group box 1 (HMGB1) and heat shock proteins (HSPs) are the two most important members of intracellular DAMPs.

HMGB1, one important intracellular DAMP, is a non-histone architectural protein located within the nucleus. Passive release of HMGB1 from the nucleus represents a signal of cell injury, which triggers the release of proinflammatory factors and thus activates cellular processes that stimulate tissue regeneration (Ulloa and Messmer, 2006). Although no different expression pattern is observed in spinal cord tissue of SOD1-G93A transgenic mice as compared with controls (Lo Coco et al., 2007), the levels of HMGB1 and other proteins involved in HMGB1 function, *e.g.*, toll-like receptor 2 (Tlr-2), Tlr-4, and serine/threonine kinase 30 (ortholog of the human advanced glycosylation end product-specific receptor, AGER), are elevated in spinal cord tissues of patients with ALS (Casula et al., 2011; Lo Coco et al., 2007). In addition to HMGB1, HSPs are discussed in the progress of protective effects on ALS. HSPs are typically cytoprotective, and their levels increase when motor neurons encounter excessive oxidative stress (Kalmar et al., 2002; Mailhos et al., 1993). Histochemical analyses of tissue samples from patients with ALS have revealed high levels of HSPs within motor neuron inclusion bodies. Similar results have been observed in a mouse model of ALS (SOD1-G93A), which expresses a mutant form of human superoxide dismutase 1 (SOD1) (Pratt et al., 2012; Watanabe et al., 2001). In SOD1-G93A transgenic mice, the chaperone activity of HSPs diminishes within the lumbar spinal cord but increases or remains unchanged within tissues that are not clinically affected (Bruening et al., 1999).

As HMGB1 and HSPs are intracellular proteins, they are released extracellularly upon cellular injury or activation (Abdulahad et al., 2011; Tsan, 2011). We aimed, therefore, to investigate whether autoantibodies (autoAbs) against HMGB1 and HSPs could be produced in the chronic pathological processes of ALS, and whether serum levels of these autoAbs could be used as biomarkers for ALS. To test this theory, we measured the serum levels of autoAbs against HMGB1, HSP60, and HSP70 in patients with ALS, Alzheimer's disease (AD), Parkinson's disease (PD), and age-matched healthy control subjects. Correlation factors including age, disease duration, and disease severity of patients with ALS were statistically analyzed. Taken together, serum levels of selective autoAbs could be used to effectively diagnose ALS and to monitor disease progression.

Materials and methods

Subjects and samples

Fifty-three patients with sporadic ALS (34 males, 19 females) and eight patients with familial ALS (4 males, 4 females) were recruited for this study at the Motor Neuron Disease Center of Taipei City Hospital in Taiwan. As such, the ratio of sporadic to familial ALS cases was 6.6 to 1. All patients with ALS were diagnosed as definite ALS (according to the El Escorial criteria) by more than two experienced neurologists (Brooks et al., 2000; Radunovic et al., 2007). The clinical severity of each patient was measured using the revised ALS functional rating scale (ALSFRS-R) (Cedarbaum et al., 1999). The age of onset for ALS was defined as the time when the first motor symptom was noticed, according to a thorough review of patient's disease course. Disease duration was then calculated based on the age of onset. Within the 61 patients with ALS, 20 had received tracheotomy ventilation, close to the rate of approximately 30% patients with ALS receiving tracheotomy in Taiwan. According to the demographic information of patients with ALS (Table 1), the cohort in this study was representative.

Forty patients that were clinically diagnosed with AD, as confirmed *via* brain computed tomography, and 40 patients that were clinically diagnosed with PD, as confirmed *via* brain computed tomography and 99mTc-TRODAT-1 single-photon emission computed tomography, were recruited from the neurological outpatient clinic of the same hospital. Forty age-matched healthy control subjects (23 males, 17 females) were recruited from the Health Centre of the same hospital. None of the healthy control individuals had evidence of any major neurodegenerative disease. Informed consents were obtained before the samples were collected, and the Institutional Review Board of Taipei City Hospital approved all the procedures of this study. All participants in this study were Han Taiwanese. Venous blood (10 mL) was collected from each participant using yellow-stopper clot accelerating tubes. All subjects were free of acute infection and any acute or chronic stress condition at least 2 weeks prior the time the blood was sampled. Tubes containing venous blood were centrifuged at 3000 $\times g$ for 20 min at 4 °C to harvest the serum. Serum samples were then immediately separated into 1-mL aliquots and stored at −30 °C for further testing.

Enzyme-linked immunosorbent assay

The autoAbs against HMGB1, HSP60, and HSP70 were quantified using an enzyme-linked immunosorbent assay (ELISA). To measure the concentration of HMGB1 autoAb we developed a sandwich ELISA. A rabbit polyclonal antibody against HMGB1 was generated by LTK BioLaboratories (TaoYuan, Taiwan) and purified using a Montage® Ab purification kit (Millipore, Billerica, MA). For specificity tests, recombinant D-amino acid oxidase (DAO) and D-amino acid oxidase activator (DAOA, also known as G72 protein) were produced and purified. Proteins were recognized using the following procedures (rabbit IgG served as a negative control). Recombinant proteins (100 ng each of

Table 1
Patient demographics and clinical characteristics.

	N	Age (years)[a]			Gender (M/F)	ALSFRS-R, mean (SD)	Duration (months), mean (SD)
		Mean (SD)	Range	Median			
HCS	40	60 (17)	30–87	65	23/17	–	–
AD	40	81.3 (7.8)	54–93	81	18/22	–	–
PD	40	75.5 (9.1)	53–90	75	21/19	–	–
ALS	61	61 (13)	30–86	58	38/23	17 (14)	23 (21)

N: total number of individuals; M: male; F: female; HCS: healthy control subjects.
[a] Age when the blood sample was taken.