ORIGINAL ARTICLE

Expression of Mutant Ubiquitin and Proteostasis Impairment in Kii Amyotrophic Lateral Sclerosis/Parkinsonism-Dementia Complex Brains

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Abstract

Kii amyotrophic lateral sclerosis/parkinsonism-dementia complex (ALS/PDC) is a progressive neurodegenerative disorder that is endemic to the Kii peninsula of Japan. The disorder is clinically characterized by a variable combination of parkinsonism, dementia, and motor neuron symptoms. Despite extensive investigations, the etiology and pathogenesis of ALS/PDC remain unclear. At the neuropathological level, Kii ALS/PDC is characterized by neuronal loss and tau-dominant polyproteinopathy. Here, we report the accumulation of several proteins involved in protein homeostasis pathways, that is, the ubiquitin-proteasome system and the autophagy-lysosome pathway, in postmortem brain tissue from a number of Kii ALS/PDC cases (n = 4). Of particular interest is the presence of a mutant ubiquitin protein (UBB^{+1}), which is indicative of disrupted ubiquitin homeostasis. The findings suggest that abnormal protein aggregation is linked to impaired protein homeostasis pathways in Kii ALS/PDC.

Key Words: Autophagy, Kii ALS/PDC, Protein aggregation, Protein quality control, Tauopathy, UBB^{+1}, Ubiquitin-proteasome system, Unfolded protein response.

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INTRODUCTION

The Kii peninsula of Japan is one of 3 high-incidence foci of amyotrophic lateral sclerosis (ALS) and parkinsonism-dementia complex (PDC) in the Western Pacific [1]. Similar foci have been found in the Pacific island of Guam (Marianas Islands) [2, 3] and in West New Guinea [4]. Kii ALS/PDC is a heterogeneous disorder in which patients show motor neuron signs and/or parkinsonian features with dementia. The Western Pacific variant of ALS is clinically indistinguishable from ALS in other parts of the world, but PDC appears to be a unique disease in the endemic areas. At the neuropathological level, Kii ALS/PDC is characterized by tau-dominant polyproteinopathy without abundant senile plaques [5–8].

The cause of Western Pacific ALS/PDC is unclear, although both genetic and environmental factors have been considered. A hexanucleotide repeat expansion with the sequence (guanine)$_4$(cytosine)$_2$ in the *C9ORF72* gene partly accounts for ALS in the Kii peninsula [9]. There is tremendous interest in understanding the etiopathogenesis of Western Pacific ALS/PDC because insight might be gained regarding neurodegenerative diseases found elsewhere [10, 11].

We previously demonstrated the presence of ubiquitin-B^{+1} (UBB^{+1}) protein in Guam PDC brains [12, 13]. UBB^{+1} is a frameshift mutant of ubiquitin that is generated by molecular
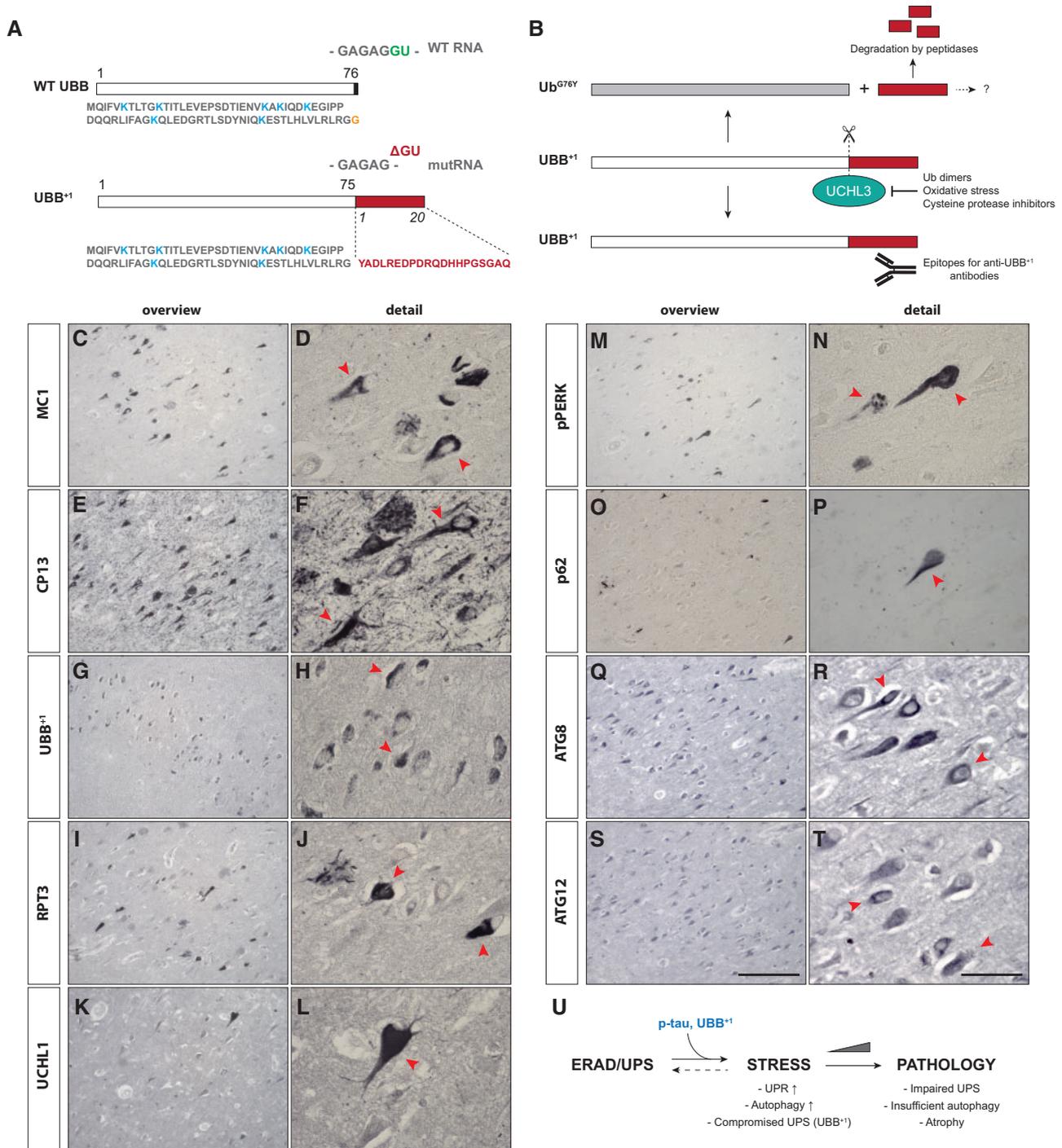
FIGURE 1. Immunohistochemical detection of mutant ubiquitin (UBB[+1]) and protein homeostasis machinery in Kii amyotrophic lateral sclerosis/parkinsonism-dementia complex (ALS/PDC) brains. **(A)** UBB[+1] is a frameshift mutant of ubiquitin that is generated by molecular misreading, a type of transcriptional mutagenesis. Occasional dinucleotide deletions in Ubiquitin-B (UBB) mRNA lead to the formation of a dysfunctional ubiquitin protein that inhibits the ubiquitin-proteasome system (UPS). **(B)** UBB[+1] contains an abnormal C-terminal extension that can be recognized by specific antibodies. UBB[+1] can be truncated from its C-terminus by deubiquitinating enzymes (DUBs), that is, ubiquitin carboxyl-terminal hydrolase L3 (UCHL3), resulting in another dysfunctional ubiquitin (Ub[G76Y]) (23) (M.H. Glickman, personal communication). Preventing truncation results in increased UBB[+1] toxicity, hinting at a protective effect of DUB cleavage (34). Inhibition of DUBs, for example, by oxidative stress, prevents UBB[+1] truncation. **(C–F)** Immunostaining for MC1 (Tau[5–15/312–322] conformational antibody) and CP13 (tau phospho-epitope Ser202) reveals abundant neurofibrillary tangles (NFTs) in Kii ALS/PDC brain. **(G, H)** UBB[+1] can also be detected in cytoplasmic inclusions in ALS/PDC brain sections. Furthermore, specific components of the UPS, that is, the proteasomal AAA-

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TABLE 1. Overview of the Subjects

Subject	Phenotype	Disease Duration	Age of Death (years)	Gender	Brain Weight (g)	Postmortem Delay (hour)	Cause of Death	Braak Stage	APOE Status	C9ORF72 Status
Kii-1	ALS + PDC	8 years 6 months	60	F	960	6	Suffocation	4+α	23	Normal
Kii-2	PDC + pyramidal tract sign	13 years 11 months	71	F	875	1.5	Respiratory failure	3+α	22	Normal
Kii-3	ALS	5 years 6 months	63	F	1275	5	Respiratory failure	3+α	ND	Normal
Kii-4	ALS	12 years 9 months	73	F	1175	9	Pneumonia	3	34	Normal
Kii-5*	ALS	13 years 4 months	70	F	1190	5.5	Respiratory failure	3	33	Normal
Control	–	–	51	M	1518	6	Liposarcoma, ileus	0	43	ND

ND, no data; α, excess over Braak staging.
*Kii-5 was only included for experiments described in Supplementary Data Figure S2.

misreading, a type of transcriptional mutagenesis (14) (Fig. 1A). UBB^{+1} is a dose-dependent inhibitor of the ubiquitin-proteasome system (UPS), the major pathway for intracellular proteolysis, and accumulation of UBB^{+1} in cells is a marker for impairment of the UPS (15) (Fig. 1B). It has been reported that UBB^{+1} accumulates in the neuropathological hallmarks of several tauopathies (e.g., Alzheimer disease [AD] and Down syndrome) and in polyglutamine repeat disorders (14–16). Abnormalities in protein homeostasis (proteostasis) are implicated in the pathogenesis of a number of neurodegenerative conditions and it was hypothesized that accumulation of UBB^{+1} may also be observed in Kii ALS/PDC brains. To evaluate expression of UBB^{+1} and specific components of the proteostasis network, immunohistochemical analyses were performed on Kii ALS/PDC postmortem brain tissue.

MATERIALS AND METHODS

Ethics Approval

This study was approved by the ethics committee of Mie University (Approval No. 2592).

Immunohistochemistry on Human Postmortem Brain Tissue

Formalin-fixed paraffin-embedded brain tissue sections (6 μm thick) from several Kii ALS/PDC cases and a nonneurological control (Table 1) were immunostained using standard procedures. Briefly, formalin-fixed paraffin-embedded sections were deparaffinized in xylene and rehydrated in a descending alcohol series. Sections were then treated with formic acid for 1 hour, followed by washes in tap water for

30 minutes. Sections were incubated with primary antibodies for 1 hour at room temperature (RT) followed by overnight incubation at 4°C. Primary antibodies included mouse anti-MC1 (1:200, Dr Peter Davies, Albert Einstein College of Medicine), mouse anti-CP13 (1:200, Dr P. Davies), rabbit anti-UBB^{+1} (Ubi2a, 1:400, Dr F.W. van Leeuwen, Maastricht University) (specificity confirmed in [15]), rabbit anti-PSMC4/RPT3 (1:400, Biomol, Plymouth Meeting, PA), rabbit anti-PGP9.5/UCHL1 (1:500, Biomol), rabbit anti-PGP9.5/UCHL1 (1:500, Millipore, Temecula, CA), rabbit anti-p-EIF2AK3/PERK (1:400, Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-SQSTM1/p62 (1:400, Biomol), rabbit anti-ATG8 (1:500, Dr A. Iwata, Stanford University), rabbit anti-ATG12 (1:500, Dr A. Iwata), and rabbit anti-Iba1 (1:1000, Wako, Richmond, VA). All antibodies were diluted in SuMi buffer (50 mM Tris, 150 mM NaCl, 0.25% gelatin, and 0.5% Triton X-100, pH 7.6). Next, sections were rinsed in TBS and incubated with biotinylated horse anti-mouse or horse anti-rabbit antibodies (1:400) (Vector Laboratories, Burlingame, CA) for 1 hour at RT. After incubation with secondary antibodies, sections were rinsed in TBS and incubated with avidin-biotin-peroxidase complex (1:800) (Elite ABC kit; Vector Laboratories) for 1 hour at RT. After final washes in TBS, sections were reacted with 0.5 mg/mL 3,3′-diaminobenzidine (Merck, Darmstadt, Germany) containing 0.2% nickel ammonium sulfate and 0.01% hydrogen peroxide (H_2O_2; Merck) in TBS (pH 7.6). Reactions were stopped in distilled water. Stained sections were dehydrated in an ascending alcohol series, cleared in xylene, and coverslipped with Entellan (Merck). AD patient brain sections were used as positive controls in all experiments.

Immunohistochemical staining was scored semiquantitatively (−, negative; +, mild; ++, moderate; +++, strong). Average scores from 3 independent examinations of complete

FIGURE 1. Continued
ATPase subunit RPT3(/S6b) and the DUB ubiquitin carboxyl-terminal hydrolase L1 (UCHL1), are present in disease-associated aggregates **(I–L)**. **(M, N)** Immunoreactivity for phosphorylated protein kinase R (PKR)-like endoplasmic reticulum (ER) kinase (pPERK) (phospho-epitope at Thr981) implies activation of the unfolded protein response in ALS/PDC brain. **(O–T)** Accumulation of the proteins p62, ATG8, and ATG12 in cytoplasm suggests disturbances in the autophagy-lysosomal pathway. **(U)** Hypothetical sequence of events leading to proteostasis collapse in ALS/PDC. ERAD, ER-associated degradation. Representative photomicrographs of hippocampal sections (Sommer's sector) are shown. Arrowheads indicate distinct immunoreactive structures. Scale bars: overview = 200 μm; detail = 50 μm.

TABLE 2. Summary of the Neuropathological Findings

Subject	MC1	CP13	UBB^{+1}	RPT3	UCHL1	pPERK	p62	ATG8	ATG12
Kii-1	(++)	(+++)	(++)	(++)	(++)	(++)	(+)	(++)	(++)
Kii-2	(+++)	(+++)	(+++)	(+++)	(+++)	(+++)	(++)	(+++)	(+++)
Kii-3	(++)	(++)	(+)	(++)	(++)	(++)	(+)	(++)	(+)
Kii-4	(+)	(+)	(+)	(++)	(+)	(++)	(+++)	(+)	(+)
Control	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)	(−)

Kii-1, Kii-2, hippocampus; Kii-3, Kii-4, frontal and temporal lobes.

sections were used. Slides were imaged using a Zeiss Axio Imager.M2 microscope equipped with an AxioCam ICc 3 camera.

RESULTS

To explore whether UBB^{+1} and other proteins involved in proteostasis mechanisms accumulate in Kii ALS/PDC patient brains, immunohistochemical analyses were carried out on postmortem brain tissue from a number of Kii ALS/PDC cases (n = 4) (Table 1). Kii ALS/PDC sections showed numerous neurofibrillary tangles (NFTs) (Fig. 1C–F), in agreement with previous observations (5–8). Importantly, immunohistochemistry revealed marked cytoplasmic deposition of UBB^{+1} in neurons of the hippocampus and frontal/temporal lobe of Kii ALS/PDC cases (Table 2). UBB^{+1} was specifically present in NFT-like inclusions in all evaluated sections (Fig. 1G, H). Interestingly, reactivity for UBB^{+1} was also observed in glial cells, that is, astrocytes (Supplementary Data Fig. S1A). In addition, particular components of the UPS, that is, the proteasomal AAA-ATPase subunit RPT3(/S6b) and the deubiquitinating enzyme (DUB) ubiquitin carboxyl-terminal hydrolase L1 (UCHL1), were detected in protein aggregates in Kii ALS/PDC sections (Fig. 1I–L). In contrast, no reactivity for NFTs, UBB^{+1}, and the specified UPS components was observed in brain sections from a nonneurological control (Table 2).

Another protein that was found to be present in aggregates in Kii ALS/PDC brain sections is phosphorylated protein kinase R (PKR)-like endoplasmic reticulum (ER) kinase (pPERK) (Table 2). pPERK is a marker for activation of the unfolded protein response (UPR), a stress response pathway that reacts to disturbances in ER homeostasis (17). Immunoreactivity for pPERK was evident in tangle-like inclusions and in granular structures (Fig. 1M, N). The granular structures that were seen may represent granulovacuolar degeneration (GVD) bodies (18) (Supplementary Data Fig. S1B). pPERK was not detected in control brain (Table 2).

Lastly, immunostaining experiments were performed for the ubiquitin-binding protein p62, ATG8, and ATG12. All of these proteins are involved in the autophagy-lysosomal pathway (ALP), another key homeostatic mechanism in cells (19). All tested Kii ALS/PDC sections contained aggregates that stained positive for p62, ATG8, and ATG12 proteins, indicating abnormalities in the autophagy pathway (Fig. 1O–T). Control brain did not show reactivity for any of these proteins (Table 2).

DISCUSSION

In the present study, we demonstrated that UBB^{+1}, a frameshift mutant of ubiquitin and dose-dependent inhibitor of the UPS, is expressed in Kii ALS/PDC brains. Specifically, UBB^{+1} was shown to be present in NFT-like structures in ALS/PDC brain sections. This staining pattern is intriguing, because disturbances of the UPS have been strongly associated with tau pathology in previous work (15, 20). Accumulation of UBB^{+1} and tauopathy might be mechanistically linked and it would be interesting, for example, to test whether UBB^{+1} can modify the aggregation and cytotoxicity of tau in experimental models. Of note, UBB^{+1} was also identified in astrocytes in Kii ALS/PDC sections. Such UBB^{+1}-positive astrocytes were previously observed in Guam PDC brains (12). The significance of these glial inclusions is unclear, but similarities between granular or fuzzy astrocytes in ALS/PDC and aging-related tau astrogliopathy have been noticed (7, 21). It was surmised that ALS/PDC might actually represent a kind of accelerated aging (7), and perhaps involves a glial senescence-like mechanism. This hypothesis could be tested in future studies by measuring the expression of senescence markers in patient brains. Preliminary analysis did not indicate clear changes in microglia density and morphology; although this needs to be examined further (Supplementary Data Fig. S2).

In addition to UBB^{+1}, specific components of the UPS, that is, the proteasomal ATPase subunit RPT3 and the DUB UCHL1, were found in aggregates in Kii ALS/PDC brains. Aggregate structures containing these proteins were also observed in Guam PDC cases (12, 13). Accumulation of the neuron-specific DUB UCHL1 is of particular interest, because UBB^{+1} has been reported to be a DUB inhibitor in vitro (22). UCHL1 normally hydrolyzes small C-terminal adducts of ubiquitin to generate ubiquitin monomers, but appears to be unable to truncate UBB^{+1} (23) (M.H. Glickman, personal communication) (Fig. 1B). We speculate that an interaction effect between UBB^{+1} and select DUBs, like UCHL1, might contribute to abnormalities in the UPS and neurodegeneration.

Furthermore, we provided evidence for activation of the UPR (PERK arm) in Kii ALS/PDC brains. The UPR is a cellular stress response pathway that, analogous to the heat shock response (an important regulator of nuclear and cytoplasmic proteostasis), is involved in homeostatic regulation in the ER (17). The activated UPR represses global protein translation and induces expression of effector genes that allow cells to cope with stress (e.g., via stimulation of autophagy) or, alternatively, induces cell death when the level of stress cannot be

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handled. pPERK, an activated ER stress sensor, was frequently located in tangle-like structures. Intriguingly, pPERK-positive granules were noted in the evaluated sections and may reflect GVD bodies (18). Evidence for increased pPERK and GVD has also been found in Guam PDC brains (13, 24). It has recently been established that GVD bodies are neuron-selective lysosomal structures that can be induced by intracellular tau pathology (25, 26). Strikingly, activated necrosome components could be detected in GVD bodies in AD neurons and it was hypothesized that the observed lesions might represent a delayed type of necroptosis (27). It remains to be determined whether necrosome components can also be identified in granular bodies in ALS/PDC brain. Longitudinal single-cell analysis of NFT formation and its relationship to GVD would assist in clarifying a mechanistic link.

Finally, we revealed that multiple proteins of the ALP, that is, p62, ATG8, and ATG12, accumulate in Kii ALS/PDC brains. Again, immunoreactivity for these proteins was previously observed in Guam PDC patient brains (13). The ALP plays crucial roles in several physiological processes (e.g., protein and organelle turnover), and deficiencies in the ALP can lead to disease (19). ATG8 and ATG12 are involved in vesicle expansion and completion in the autophagic process, and the ubiquitin-binding protein p62 is an adaptor that acts at the intersection between the UPS and autophagy (19). Inclusions containing another adaptor protein involved in autophagy and vesicle trafficking, that is, optineurin, were detected in Kii ALS/PDC tissue as well (28). Excitingly, tau pathology could be reduced by pharmacological activation of lysosomes in mice, suggesting that the ALP may represent a therapeutic target in tauopathies (29).

It is important to stress that altered protein degradation pathways, and in particular expression of UBB^{+1}, are not limited to the diseases mentioned hereinbefore. Other tauopathies, including argyrophilic grain disease, show similar alterations (15, 30). This feature is in contrast with the inclusions found in synucleinopathies (15), except for cases with mixed tau and α-synuclein pathology (31). Moreover, these alterations are not limited to the nervous system; similar pathology is also encountered in striated muscle in myofibrillar myopathies and related conditions (32, 33). These findings illustrate the role of UBB^{+1} in a heterogeneous group of disorders encompassing diverse pathologies and clinical manifestations.

In summary, we showed that UBB^{+1} and several components of proteostasis pathways are accumulated in disease-associated aggregates in both Kii ALS and Kii PDC brains. This indicates a potential role for impairment of the proteostasis network in the pathogenesis of ALS/PDC. The results also support a similar pathogenesis of Kii and Guam ALS/PDC variants. Proteostasis mechanisms critically contribute to the cellular response to toxic proteins and pathogenic protein seeding. Therefore, improved understanding of proteostasis dysfunction in neurodegenerative disease may offer clues for therapeutic interventions that prevent or reverse aggregation. Kii ALS and Kii PDC also show TDP-43 and α-synuclein pathology in brain and spinal cord (7). It will be interesting to investigate the relation of impaired proteostasis to Kii ALS/PDC multiple proteinopathy in more detail. Double-labeling experiments to show colocalization of different abnormal proteins in

aggregates and the addition of more cases would strengthen the significance of these findings.

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