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Therapeutic Strategies and Metal-Induced Oxidative Stress: Application of Synchrotron Radiation Microbeam to Amyotrophic Lateral Sclerosis in the Kii Peninsula of Japan

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A series of extensive gene-environment studies on amyotrophic lateral sclerosis (ALS) and Parkinsonism–dementia complex (PDC) in Guam Island, USA, and the Kii Peninsula of Japan, including Auyu Jakai, West New Guinea, have led us to hypothesize that a prolonged low calcium (Ca) and magnesium (Mg) intake, especially over generation, may cause oxidative stress to motor and nigral neurons by an increased uptake of environment metallic elements, i.e., aluminum (Al), manganese (Mn), and iron (Fe). Otherwise, 5–10% of total ALS cases are familial ALS (fALS), of which 20% of the fALS cases linked to a point mutation of Cu/Zn superoxide dismutase (SOD1). In the vicinity of the Kii Peninsula, about 7% of the ALS cases are also linked to the SOD1 mutation. Using synchrotron radiation (SR) microbeam, conglomerate inclusion (SOD1 aggregates) within a spinal motor neuron of the fALS case in the vicinity revealed a loss of copper (Cu) in contrast to extremely high contents of Zinc (Zn) and Ca. That means an exceptionally low Cu/Zn ratio with an increased Ca content, indicating the abnormalities of the active site of SOD1 protein of the fALS. Furthermore, sALS in the southernmost high incidence areas of the Kii Peninsula showed a low Cu/Zn ratio within a motor neuron, suggesting a fragility of SOD1 proteins. From the perspective of gene–environment interactions, the above two research trends may show a common oxidative stress underlying the neuronal degenerative process of ALS/PDC in the Kii Peninsula of Japan. Therefore, it is a crucial point for the prospect of therapeutic strategy to clarify a role of transition metals in the oxidative process in both ALS/PDC, including ALS elsewhere in the world. This paper reviews a history of the genetic epidemiological studies, especially from the aspect of gene–environment interaction, on ALS/PDC in the Kii and Guam high incidence foci and the results of a series of analytical research on trace metallic elements within neurons of both sALS and fALS cases, especially using a synchrotron radiation (SR) microbeam of Spring-8 and Photon Factory of Japan. The SR microbeam is an ideal X-ray source, which supplies an extremely high brilliance (high-intensity photon) and tunability (energy variability) to investigate trace metallic elements contained in biological specimens at the cellular level, even more without any damages. This research will provide a valuable information about

the mechanism of oxidative stress involved in neuronal cell death in ALS and related neurodegenerative disorders. To elucidate the physicochemical mechanism of the oxidative process in neuronal degeneration, it will shed a new light on the therapeutic strategies for ALS/PDC in near future.

Keywords: amyotrophic lateral sclerosis (ALS), gene-environment interaction, parkinsonism-dementia complex, transition metals, synchrotron radiation microbeam

INTRODUCTION

Based on the genetic epidemiological studies on Western Pacific foci of Guam, USA, Kii Peninsula of Japan and West New Guinea (**Figure 1**), Espinosa et al. (1) identified three major forms of ALS: (1) the sporadic or classical form, (2) the familial and dominant genetic form, and (3) the Western Pacific (Mariana Islands) form. The latter form was first described by Hirano (2, 3) among the Indigenous Chamorros people of Guam, often linked to another unique spectrum of disorder—a parkinsonism–dementia complex (PDC). Subsequently, the ALS/PDC was recognized in residents in the two high incidence areas of Kozagawa focus (Kozagawa, Koza, and Kushimoto towns neighboring Kozagawa river, Wakayama Prefecture) and Hohara (Hohara district in Nansei Town, Mie Prefecture) focus in the southern and eastern parts of the Kii Peninsula of Japan (**Figure 1**) (4) and in the small villages of Auyu and Jakai people of Western New Guinea (5). The excess occurrence of ALS/PDC in these Western Pacific foci had made it the largest and best-known foci of ALS in the world (geographic isolate, initially 50–100 folds of the worldwide average) (6).

To elucidate the causes of the clustering of ALS/PDC, it will provide important clues to understand the causative factors of ALS/PDC. First, this paper presents a brief history of genetic epidemiological studies, especially concerning a gene–environment interaction, on ALS/PDC of the Kii Peninsula and Guam. Second, recent genetic trends of ALS and SOD1 animal models are overviewed. Third, the results of elemental analyses of environmental minerals in the brain and spinal cord tissues of the ALS/PDC autopsy cases and the experimental animal models are presented, using a variety of analytical methods. Specifically, an overly sensitive and high-resolution technique of a synchrotron radiation (SR) microbeam of Spring-8 (Hyogo) and Photon Factory (Tukuba) of Japan was applied at cellular level to elucidate a role of transition metals in the oxidative process of ALS/PDC and related neurodegenerative diseases. Finally, the current trends of the SR microbeam study and the prospect of therapeutic strategy are discussed, especially concerning metal-protein attenuating compounds (MPACs) of transition metals, i.e., Fe, Cu, and Zn.

EPIDEMIOLOGY AND GENE–ENVIRONMENT INTERACTION

Genetic Epidemiological Study

A series of genetic epidemiological studies in the Kii and Guam foci since early 1950s (7, 8) revealed that the genetic penetrance did not exceed 20% (9), suggesting a multifactorial inheritance and emphasizing the involvement of environmental factors. Genetics alone could not account for the clusters of ALS/PDC among Chamorros on Guam and other Western Pacific foci as follows: (1) There appears to be three different ethnic groups involved, (2) there appears to be excess of ALS and PDC among Filipinos who settled on Guam as young adults, and (3) there has been a dramatic decline in incidence with a shift of clinical preponderance from ALS to PDC and a significant increase in age at onset of both diseases during the past 30 years (10, 11).

Regarding the environmental factors of ALS/PDC, there were mainly two hypotheses in the Western Pacific foci of the Kii Peninsula and Guam: (1) an imbalance of environmental minerals, resulting in a long-term Ca and Mg deficiencies (4), and (2) addictions to excitatory amino acids (β-methyl-amino-L-alanine; BMAA) in cycads (12–14). However, the residents in the Kii Peninsula have no habit to eat or take cycads (15) Moreover, mass spectrometry did not constantly detect a significant amount of BMAA (β-N-methylamino-L-alanine) in the brain tissues of Kii ALS (16). The hypothesis (2) was, therefore, ruled out in Japan (15, 16). However, Spencer et al. have recently reported a medical use as Kampo medicine (17) and presents a hypothesis of genotoxic chemicals (methylazoxymethanol, MAM) derived from seed of the cycad plant (18). As for hypothesis (1), the long-term Ca and Mg deficiencies lead to the increase in transition metals, such as Al, Fe, and Cu in the cerebral cortex and spinal cord tissues (5, 19, 20). Yase (21, 22) concluded that there was a unique pattern of low content (or lack) of Ca and Mg with a high content of Al and Mn in in the environmental specimens, including soil, water, and plant obtained from the high incidence foci and hypothesized that prolonged exposure to these environments would cause abnormal metabolism detrimental to motor neurons. Depending on the results, it is finally emphasized the synergistic effect of genetic and environmental factors as "gene-environmental interaction."

Since 1980s, in the Kozagawa focus, clusters of ALS have gradually disappeared, accompanying emigrants from the high incidence areas in the southernmost part of the Kii Peninsula (23). However, emigrant ALS out of the focus areas was still highly prevalent. Among the emigrant ALS cases, the youngest age at emigration apart from the Kozagawa focus was 3–5 years in the very early childhood with a very long incubation time over 70 years (23, 24). However, ALS did not develop in the second generation of the emigrants from the high incidence areas, including the Kozagawa focus. Similarly,

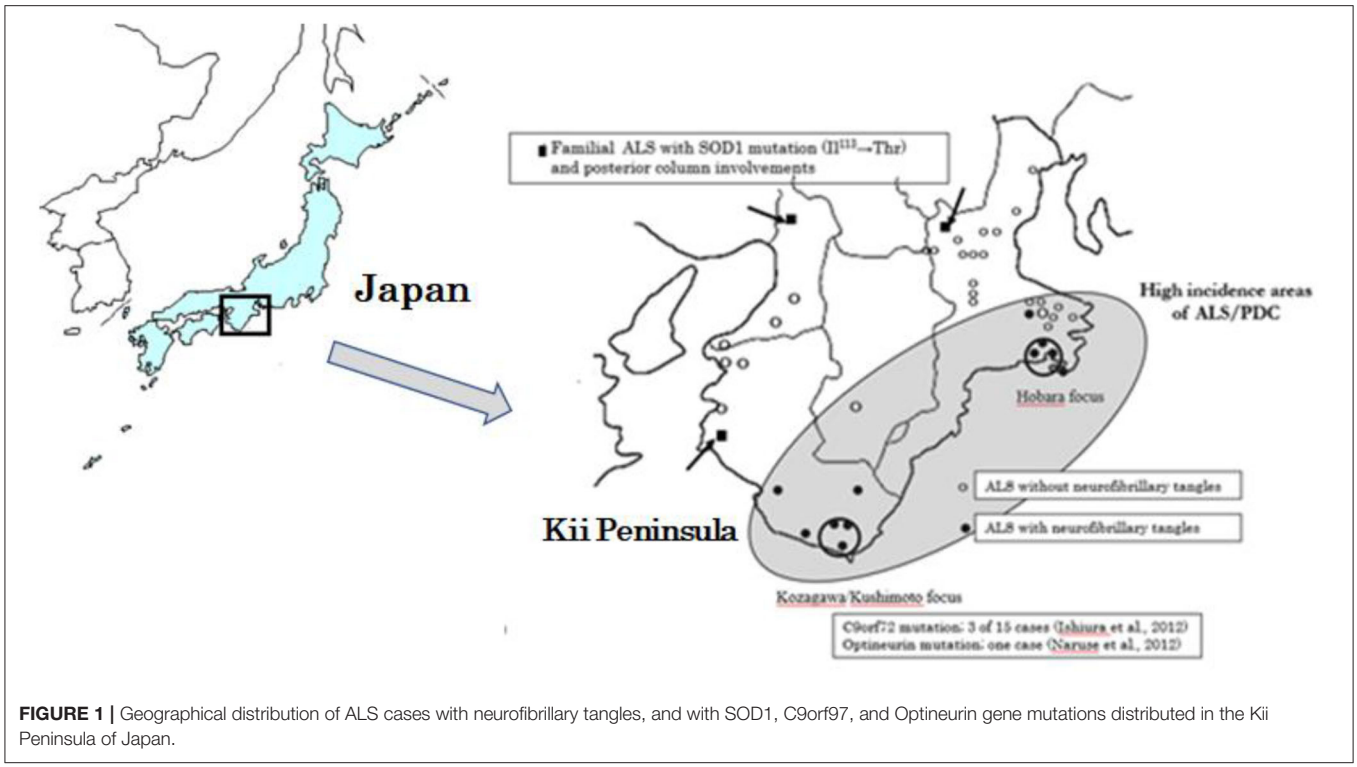

FIGURE 1 | Geographical distribution of ALS cases with neurofibrillary tangles, and with SOD1, C9orf97, and Optineurin gene mutations distributed in the Kii Peninsula of Japan.

in the follow-up study of Chamorros emigrated from Guam to California, the mainland of USA, a high incidence of ALS/PDC was observed in the emigrants who had been raised in Guam during their childhood, but no ALS/PDC was observed in their second generation born in the mainland (25). In contrast, immigrants into both the Kii and Guam foci developed ALS/PDC after a long-term stay in the Kii and Guam foci (23, 26).

Environmental Animal Models

Due to the unique geological and environmental conditions of the Kii Peninsula, we have tried to create animal experimental models. In an experiment animal model of Japanese macaque monkeys reared for a prolonged period in low Ca/Mg and high Al diets, it neuropathologically showed a small diameter of spinal motor neurons with atrophies of nucleus and nucleolus, eventually leading to the decrease in the cell number (27). In other animal models raised under the same conditions, we clarified the shrinkages of the spinal motor neurons with an increased number of spheroids with anti-PHF antibody-positive staining (28–30). It confirmed the accumulation of neurofilaments by induction of abnormally phosphorylated tau protein (31). Interestingly, Mitani et al. (32) pointed out that only low Mg and high Al diet enhanced most likely to absorb Al into the brain of the experimental animals. Oyanagi et al. (33) proved a loss of nigral dopaminergic neurons in the experimental rats raised by only Mg deficiency with high Al diets, especially over the generation from the fetal stage to 1 year of age.

Additionally in the autopsied ALS cases in both Kii and Guam ALS, it is pointed out the presence of multinucleated cells in the cerebellar cortex (4). In sALS, ectopic neurons were also found within the deep white matter of the spinal cord (34). These findings indicated a congenital migration of neurons in the central nervous tissues of ALS under the gene–environment interaction.

ALS GENETICS AND SOD1 ANIMAL MODEL

Genetically, about 5–10% of cases of ALS are a familial form (fALS), and the remainder is a sporadic form (sALS); they are clinically indistinguishable, but genetically possible to differentiate. Recently, genetically identified subtypes of fALS increased in number. In 1993, missense mutations in Cu, Zn superoxide dismutase (*SOD1*) have first reported and accounted for ∼20% of fALS (35, 36). After 2008, there continuously found out *TARDBP* (37, 38) and *FUS/TLS* (39, 40) causative genes. Since 2011, the discovery of *C9orf72* causative gene (41, 42), the most frequent causative gene of fALS in the Europe and the United States, has greatly changed the research a landscape of ALS genetics, as a founder effect.

Over 200 different Cu/ZnSOD (SOD1) gene abnormalities have been found in sALS and fALS (35, 36). Motor neuron degeneration was demonstrated in transgenic mice using mutant Cu/ZnSOD cDNA (43). However, knockout mice with the Cu/ZnSOD (SOD1) gene do not develop the disease (44). Only mice expressing mutant SOD1 show neuronal degeneration, indicating "gain of function" rather than "loss of function" (45). In terms of pathogenesis, the following theories have been

proposed: 1) peroxynitrite production, 2) oxidative stress by free Cu, 3) aggregation of mutant SOD1, and 4) pathological glycation of mutant Cu/ZnSOD (SOD1). However, the developments of recent genetic studies of fALS, based on the causative gene analyses, are mainly directed toward 1) disturbance of proteostasis, 2) abnormal RNA metabolism, and 3) axonal pathology and cytoskeletal abnormalities (45, 46).

In the Kii Peninsula of Japan, optineurin and C9orf72 causative genes have been found only in the Kozagawa focus (47, 48). In the vicinity of the Kii Peninsula foci, SOD1 abnormalities have also been reported, as in the other Japanese areas (49). Except for the Kozagawa focus, none of the causative genes have been found in the Hobara focus, including in the other Western Pacific high incidence foci of Guam. In this context, the Kozagawa area of the Kii Peninsula occupies genetically a unique feature even in the Western Pacific foci.

From the border of the Kii Peninsula foci, Yoshimasu et al. (50) reported a first case of fALS neuropathologically examined and showed conglomerate inclusions within a motor neuron and posterior column involvements. There was a SOD1 gene analysis on 23 ALS cases (three FALS and 20 sALS cases) from the Kii Peninsula and its vicinity (**Figure 1**) (51). The two of the sALS cases, outside of the Kozagawa and Hohara foci, showed a Ile113Thr point mutation of exon4. Kokubo et al. (52) neuropathologically examined one of the two cases and found loss of spinal motor neurons, conglomerate inclusions, and posterior column involvements. Incidentally, the Ile113Thr point mutations were reported from various families with an exceptionally low penetrance. Neuropathological examinations revealed a massive accumulation of neurofilaments in spinal motor neurons, globus pallidus, substantia nigra, nucleus pellucida, and inferior olivary nucleus in large amounts (53, 54).

TRACE ELEMENTAL ANALYSIS OF KII AND GUAM ALS/PDC

To examine the effects of trace elements, metal dynamics in the CNS tissues of ALS/PDC were analyzed by a variety of methods: X-ray microanalysis (XMA), particle-induced X-ray emission analysis (PIXE), and electron energy loss spectrometry (EELS).

XMA and PIXE Analyses

The XMA imagines of the spinal cord tissues obtained from autopsy cases of the Kii ALS showed that trace elements such as Al, Ca, and Mn were found to be deposited alternately along the blood supply of intraspinal arteries (55–57). Specifically, these metallic elements deposited along the anterior spinal arteries alternatively in the left or right side of the anterior horn tissues, finally to motor neurons. It indicated that the deposition of trace elements was a phenomenon occurred under hyperparathyroidism due to the long-term deficiency of Ca and Mg intake.

The PIXE analysis showed that increased Al in the spinal cord and frontal cortex tissues obtained from the Guam and Kii ALS/PDC autopsy cases, together with Ca and transition metals of Fe, Mn, Fe, Ti, and V (58). The Al and Ca contents were significantly negatively correlated with ages at onset, and only Ca content was significantly positively correlated with the duration of the illness. Finally, the X-ray powder diffraction and infra-absorption spectrometry revealed that Ca was finally deposited as Ca-hydroxyapatite in the cerebral cortex and spinal cord of ALS (59). Based on these findings, Yase (60) proposed the hypothesis of metal-induced calcifying degeneration as a pathogenesis of ALS, referring to Selye's theory of calciphylaxis (61).

EELS Study of Ultrastructural Localization of Aluminum

Furthermore, the ultrastructural distribution of Al within a lumbar motor neuron of ALS was analyzed by electron energy loss spectrometry (EELS) (62). Al was localized in nucleoli, nuclei, and rough endoplasmic reticulum, which are rich in phosphorylated nucleic acid components. In addition, Al was found in a Bunina body, which is a pathognomonic intracellular inclusion of ALS. Multivariate analysis of the spinal cord of ALS revealed that the contents of Al and Fe were significantly associated with the frequencies of early pathological changes of chromatolysis and Bunia bodies of spinal motor neurons (63). Al^{3+} has similar characteristics to Fe^{3+} and acts as a prooxidant, inducing reactive oxygen species (ROS) production. Al is non-redox active metal and Al^{3+} firmly binds to metal-binding amino acids (rich in *His, Tyr,* and *Arg*) and phosphorylated amino acids (64). Thus, Al may preferentially bind to nucleic acids and cause a progressive inhibition of the protein synthesis of rRNA and the transcription or gene modulation of DNA (65).

Application of Synchrotron Radiation Microbeam to ALS/PDC

To elucidate the physicochemical mechanism of oxidative stress, the chemical states of transition metals such as Cu, Zn, and Fe in the aggregates of mutant Cu/ZnSOD (SOD1) proteins (49, 52) within a spinal motor neuron of autopsy fALS cases were analyzed (**Figure 2**), using synchrotron radiation (SR) microbeams at Spring-8 and Photon Factory (**Figure 3**) (66, 67). X-ray fluorescence spectroscopy (SRXRF) using a SR microbeam is non-destructive and extremely sensitive, and its characteristics are suitable for trace elemental analysis within a single neuron and for chemical state analysis of transition metals, providing important information for elucidating the physicochemical properties during the oxidative process (66, 67). Furthermore, SRXRF can image a microdistribution of elements and detect a chemical shift (valency changes) of transition metals in the oxidative process, resulting in cellular death (68).

In a case of fALS with a mutant SOD1 protein (Ile113Thr point mutation) (53), the pre-edge peak (∼8.983 Kev) below the Cu-K absorption edge was not detected by photo reduction (**Figure 4**) (66, 67). It indicated the planar triangular structure (Cu-*His*-Zn imidazolate linkage) in the active center of SOD1 protein was impaired before photoreduction (69). The Cu contents in the motor neurons of fALS cases were

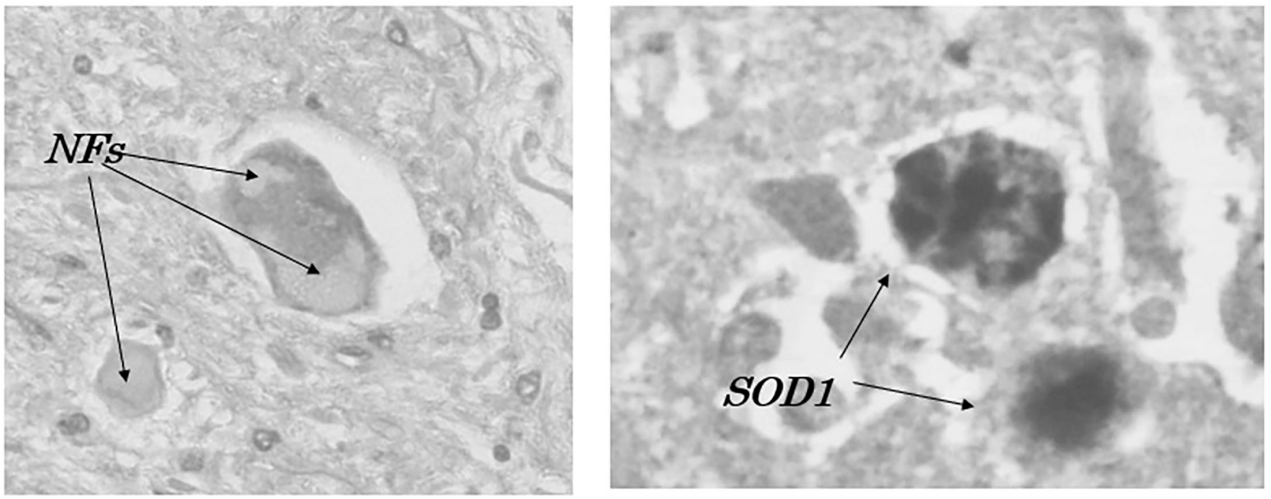

FIGURE 2 | Accumulation of neurofilaments (NFs) and SOD1-immunoreactive products (SOD1) in a patient with familial amyotrophic lateral sclerosis with I113T SOD1 mutation (52).

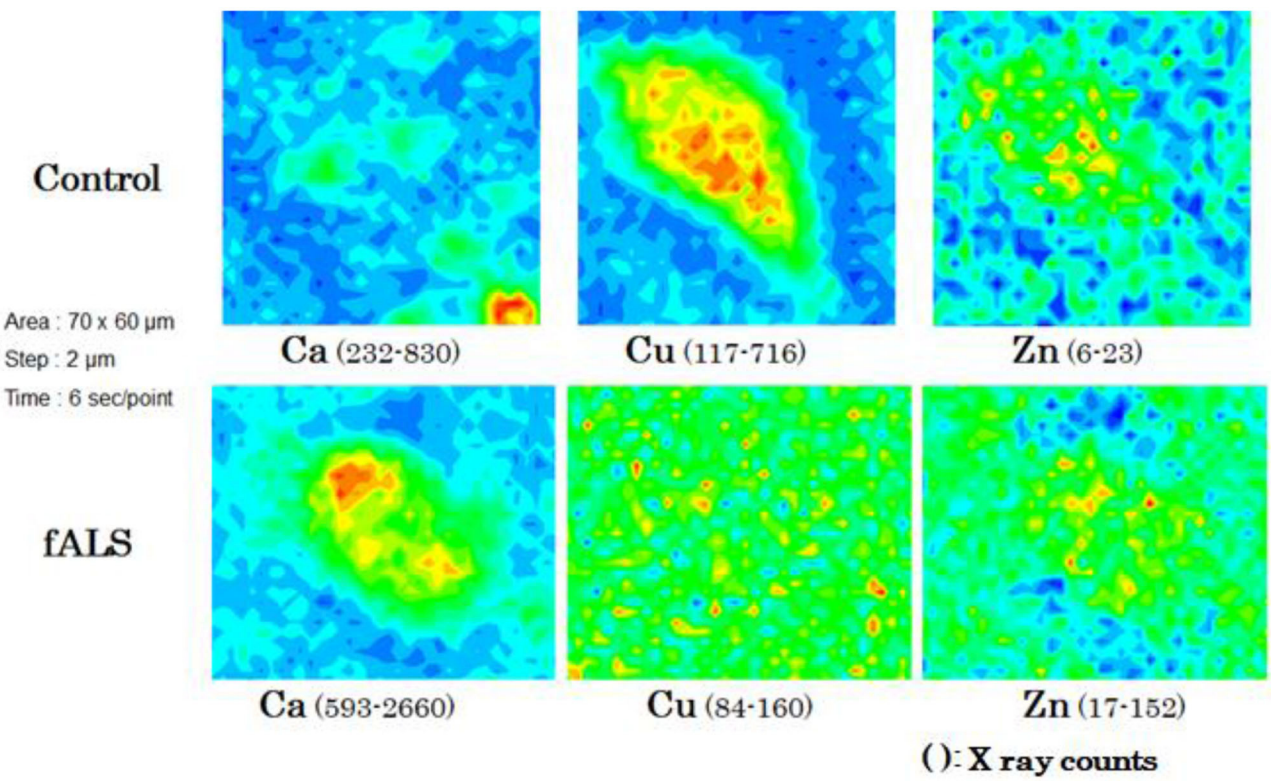

FIGURE 3 | Chemical mappings of Ca, Cu, and Zn of a single motor neuron obtained from fALS and control cases.

extremely low, whereas the Zn contents were extremely high, as compared to those of the sALS cases and the controls. Comparing with the Cu/Zn ratios of the control neurons (1.03±0.24), the Cu/Zn ratio of sALS neurons was about half (0.5±0.24) and the Cu/Zn ratio of fALS neurons was extremely low (0.12±0.08). Overall, it was expressed as the equation;

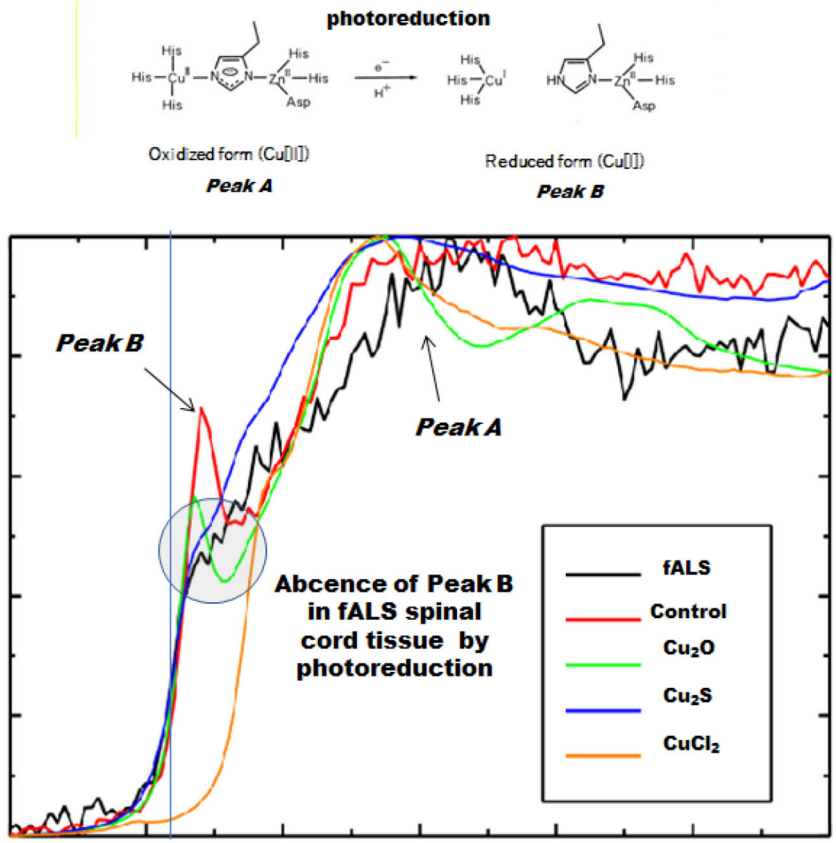

FIGURE 4 | Spectra near the Cu-K absorption edge (XANES). Loss of the pre-edge peak B (~8.983 keV) by photoreduction below the Cu-K absorption edge in fALS-1 but not in control-1.

ln(Ca)=0.944–0.92*Cu/Zn (n = 118, r= 0.690, R^2 = 0.476, $p < 0.0001$, **Figures 5**, **6**).

On the other hand, the XRF study of one of the sALS cases revealed the highest content of Fe as compared to those of the fALS and control cases. In XAFS study, the chemical state of Fe was found to be shifted from divalent (Fe^{2+}) to trivalent (Fe^{3+}) as compared to that of the control and fALS cases (**Figure 7**). The changing of chemical state of Fe might also indicate an important participatory role of Fe in the oxidative process in the sALS case (68, 70), leading to the fragility of SOD1 protein with low Cu/Zn ratio in sALS other than genetic factors.

On the other hand, excess Zn may promote nitration of tyrosine (71) and strongly bound to neurofilament L (NFL) (72), resulting in the neuronal death with the formation of intracytoplasmic neurofibrillary aggregations (conglomerate inclusions). Otherwise, Ca2+ is one of the key regulators of cell survival and it can induce ER stress-mediated apoptosis in various conditions (73). Excess Fe within a motor neuron enhances ER stress and inhibits intracellular Ca^{2+} signaling and ER Ca^{2+} pumps (74), leading to neuronal death.

Analyses of Iron in Parkinsonism–Dementia Complex and Parkinson's Disease

In Guam, the incidence of ALS has been drastically declined since 1970s and in contrast, that of PDC has gradually decreased, clinically transforming to a mild form of late onset dementia (Mariana dementia) (75, 76). Parkinsonism–dementia complex (PDC), which was previously highly prevalent in the ALS foci of Guam and the Kii Peninsula, was pathologically characterized by extensive Alzheimer's fibrillary changes in the cerebral cortex and brainstem, as well as spinal motor neurons. In PDC, neuronal death with Alzheimer's fibrillary changes is observed in the substantia nigra of the midbrain (2, 3, 77). Here, we compared the role of Fe in the oxidative stress death of nigral neurons in Guam PDC and PD in Japan, using SR microbeam (66–68).

To elucidate a role of Fe in oxidative stress underlying the pathogenesis of PDC and PD, distributions of Fe within a nigral neuron were analyzed using synchrotron radiation (SR) microbeam (66–68, 70). In the X-ray fluorescence (XRF) spectroscopic study, excess accumulations of Fe were found within the melanized neurons, free neuromelanin (NM) granules, and NM aggregates phagocytosed in glial cells of the substantia

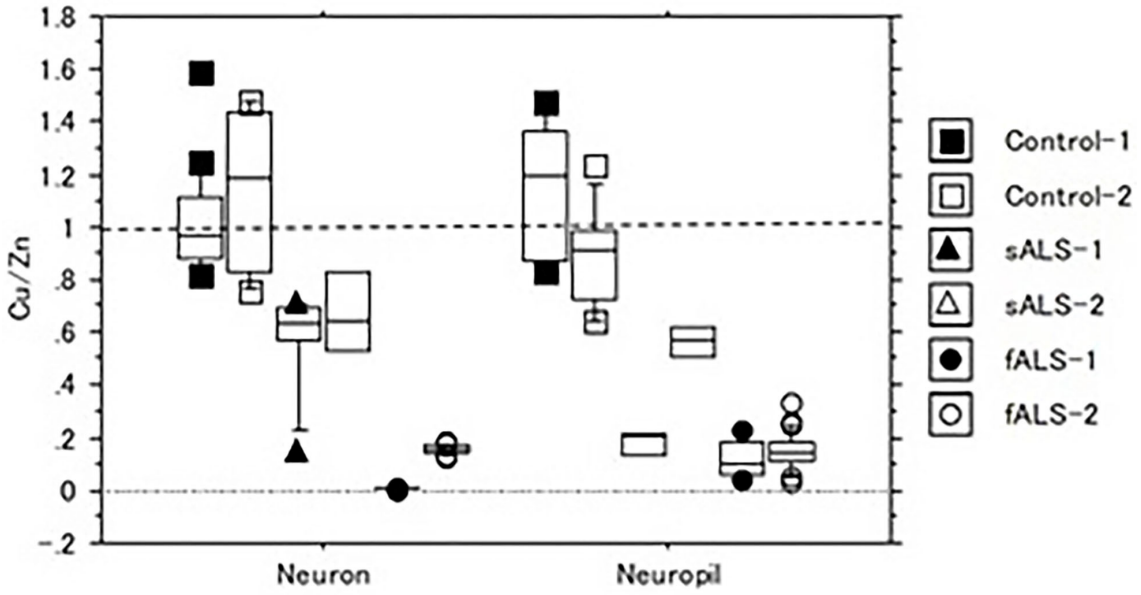

FIGURE 5 | The Cu/Zn ratio of neuron and neuropil in lumbar spinal cord tissues of sALS, fALS and control cases.

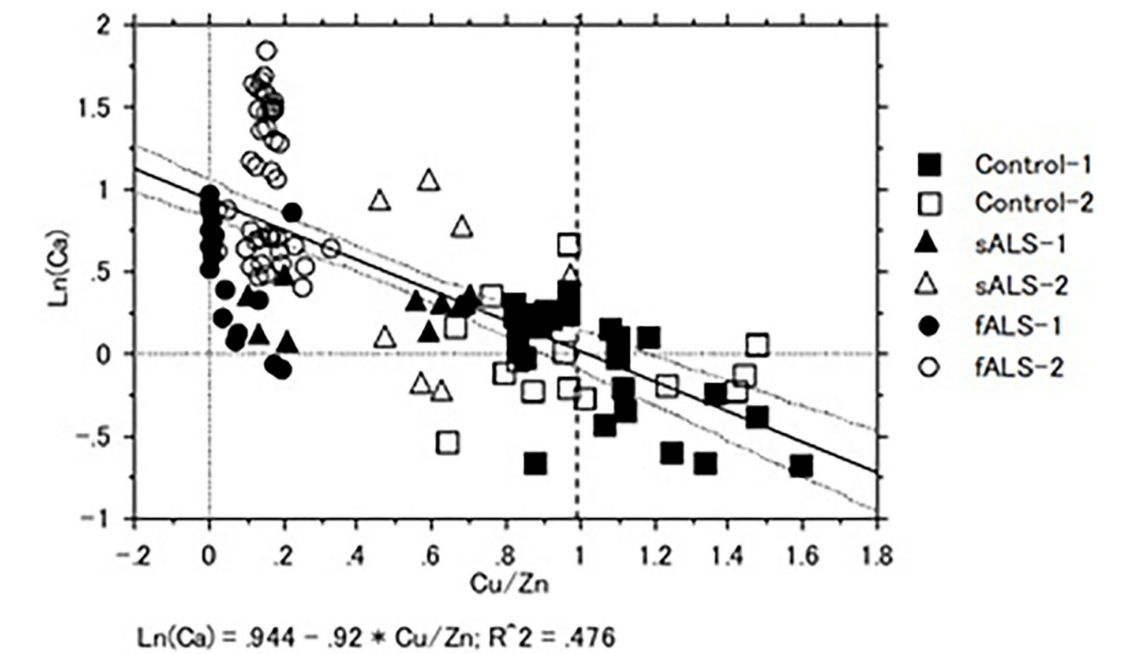

FIGURE 6 | Correlation of Cu/Zn ratios and Ca contents in the lumber spinal cord tissues of sALS, fALS and control cases. Overall, Ln (Ca) = 0.944–0.92*Cu/Zn (n = 118, $r = 0.690$, $R^2 = 0.476$, $p < 0.0001$).

nigra of both PDC and PD. X-ray absorption near-edge structure (XANES) analyses of PD revealed that the chemical state of Fe in the melanized neurons and free NM aggregates or phagocytosed NM aggregates in glial cells shifted from Fe^{2+} to Fe^{3+}, according to the progression of oxidative process (**Figure 8**), associated with a pre-edge peak at Fe K-edge due to a 1s → 3d transition, which indicated a breaking of inversion symmetry around the Fe site (66). However, in the PDC and control, the melanized

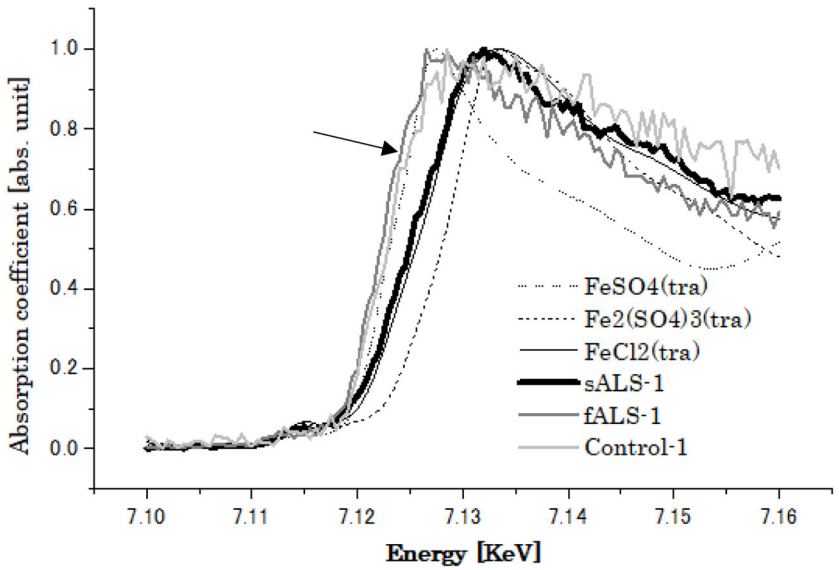

FIGURE 7 | XANES spectra of Fe obtained from of lumbar spinal cords of Control, sALS and fALS cases with SOD1 mutation (indicated by arrow).

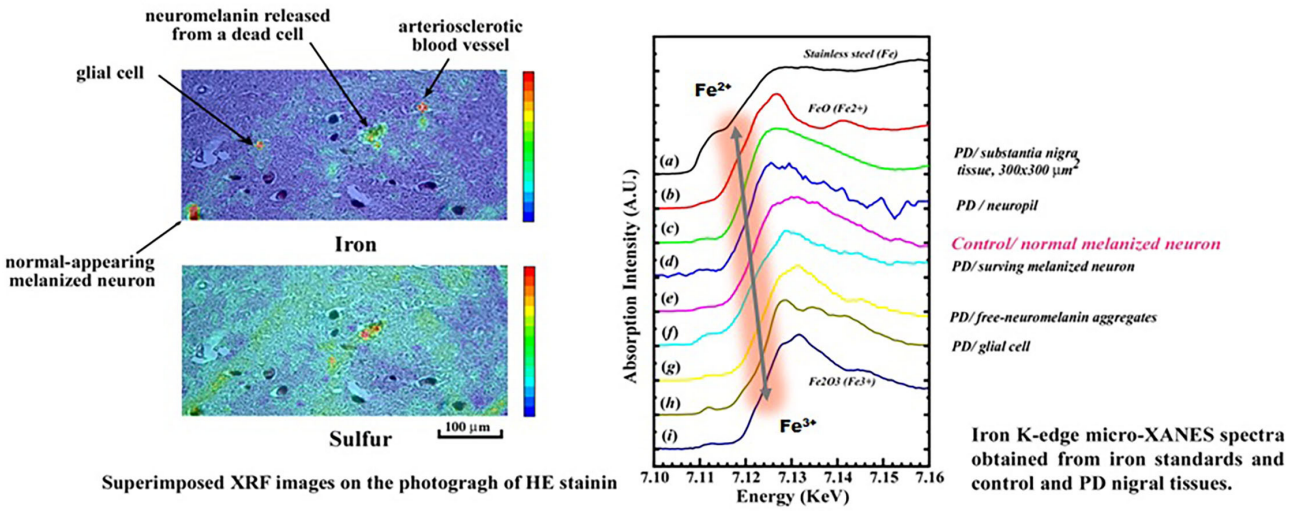

FIGURE 8 | Chemical shifts of Fe from Fe^{2+} to Fe^{3+} in the neuromelanin complex during the Oxidative process of nigral cell degeneration in Parkinson's disease (PD).

neurons and free MN aggregates showed mixed states of Fe^{2+} and Fe^{3+} without any pre-edge peak in the spectra. Together with the results, it confirmed that the Fe in the PD nigral neurons was shifted to the trivalent state as "masked iron" (not easily ionized), which does not easily show iron staining properties, and formed a unique tightly bound Fe-NM complex to the membrane proteins (78–80) and not easily ionized. The NM aggregates in PD may be protectively associated with a long-term course of PD. These results suggested that the changes in distribution and chemical states of Fe may play a crucial role in the oxidative process of PDC, but in the different ways other than PD (68).

NEW RESEARCH TRENDS AND THERAPEUTIC STRATEGY

Causative Genes and Oxidative Stress by Transition Metals

Recently, it has been pointed out that oxidative stress caused by reactive oxygen species (ROS) is involved in neuronal cell death in neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis (81). Intracellular redox reactions involve the transfer of electrons, producing ROS with unpaired electrons. Transition metals, such as iron (Fe) and copper (Cu), have unpaired

electrons and change their electronic states relatively easily, generating ROS *via* the Fenton and Haber–Weiss reactions (82), but the detailed mechanism of Fenton's reactions is not fully understood (83). Anyhow, oxidative stress may be deeply involved in the degenerative process of neurons in these neurodegenerative diseases, and the elucidation of such an oxidative mechanism is a crucial point for therapeutic approaches to neurodegenerative diseases.

Using 62Cu-ATSM in positron emission tomography (PET), a significant increase in Fe accumulation in the motor and motor-related cortices has been clinically observed in a group of patients with ALS, and oxidative stress and abnormal mitochondrial function have been pointed out as the pathogenic mechanisms (84). On the other hand, a transactive DNA-binding protein of 43 kDa (TDP-43) has been identified as a major component of intracellular inclusions in spinal motor neurons and ubiquitin-positive inclusions in frontotemporal lobar degeneration with ubiquitinated inclusions (FTLD-U) in sporadic ALS. TDP-43, an RNA-binding protein, has been identified as a major component of the neuronal inclusion bodies in frontotemporal lobar degeneration with ubiquitinated inclusions (FTLD-U), which is recently considered a pathological hallmark in the diagnosis of ALS (85). Interestingly, it is reported that TDP-43 protein is co-localized with Bunina body, which has been considered as a pathognomonic hallmark of ALS (86, 87). In a mouse model with a genetic abnormality of TDP-43 protein (A315T), the aggregation site of TDP-43 protein is enriched in transition metals such as Cu, Zn, and Mn, which may be involved in oxidative stress and neuroinflammation (88, 89). C9orf72 repeat elongation is said to cause mitochondrial dysfunction, oxidative stress, and DNA damage (90). The SR microbeam techniques may provide an important information to elucidate the coordination structure of TDP-43 proteins of ALS-FTD in future.

In addition, it was reported that the cause of the FTD/ALS ancestry linked to chromosome 9 was due to an abnormal expansion of GGGCC repeat sequence in the intron of the C9orf72 gene (41, 90). In Europe and the United States, this repeat extension was found to be the most common cause of familial and sporadic ALS, accompanied by TDP-43 pathology. However, the frequency of the C9or72 repeat expansion is low in Asia. In Japan, it is extremely low, that is, accounting for less than 2.6% of familial ALS and 0.2% of solitary ALS (48, 49, 91) However, the C9orf72 repeat expansion has been energetically investigated in both foci of the Kii Peninsula, and abnormal repeat expansion was frequently observed 20%, three of 15 patients with ALS in the Kozagawa focus (neighboring the Koza river in the Wakayama Prefecture in the southernmost part of the Kii Peninsula) (48). Paradoxically, no C9orf72 repeat expansion has been observed in the other Hohara focus (Nansei Town in the Mie Prefecture), where familial cases are over 70%, as compared to very few in the Kozagawa focus (4). It is supposed to be a founder effect. Even more, in the Kozagawa focus, a ALS case of young onset with long-term survival has been confirmed optineurin gene mutation (47). Therefore, even if gene mutations are involved, causative genes are heterogenous in the Kozagawa focus. The paradox and discrepancy of gene mutations between the Kozagawa and Hohara foci have remained to be elucidated, about 200 Km apart from each other in the Kii Peninsula of Japan.

Therapeutic Strategy of Metal-Protein Attenuating Compounds

Recently, clioquinol (CQ) has been considered as a moderate chelator/ionophore, only which can across a blood–brain barrier, for the metal-protein attenuating compounds of transition metals, i.e., Fe, Cu, and Zn (92–94). Based on such functions of CQ, researchers began using it for therapeutic purposes in a variety of experimental mouse models and clinical trials, such as Alzheimer's disease (AD) and Parkinson's disease (PD). In transgenic AD mouse models, it was observed that CQ reduced amyloid plaque burden and improved cognitive functions (95). Next, CQ was applied to a human phase II clinical trial for patients with AD (96). The results suggested that CQ prevented cognitive deterioration and reduced plasma Aβ-42 levels. After warning given by the Tabira (97) concerning the SMON tragedy from Japan (2001), the CQ therapy for AD was changed to a derivative of PBT-2 (as a second-generation 8-hydroxyquinoline) (98, 99). However, it was reported that the chelating properties of CQ depleted copper and increased lethality in amyloid precursor protein transgenic mouse (100). On the other hand, it was demonstrated that iron chelation by ferritin transgene or the metal chelator of CQ protected against the neurotoxicity of 1-methyl-4-phenyl-1,2,3,6-tetra-pyridine (MTPT) in mice (101), which was biologically transformed to MPP+ by oxidation and induced that was parkinsonism clinically like PD (102). However, CQ is cytotoxic as a transition metal ionophore of such as Cu, Fe, and/or Zn and induces mitochondrial swelling and loss of mitochondrial membrane potential (103), acting as a potential anticancer agent (104). Moreover, CQ inhibits superoxide dismutase-1 (SOD-1) activity and enhances reactive oxygen (ROS) production, eventually leading to apoptosis in neuronal cells (105). Hence, the clinical efficacy of both CQ and PBT-2 therapies has also remained a matter of controversy due to incomplete understanding of the underlying physicochemical mechanism of oxidative stress in neurodegenerative diseases.

Future in Application of Synchrotron Microbeam Techniques

From a physicochemical point of view, this paper presents a history of genetic epidemiological studies of ALS/PDC on Guam and the Kii Peninsula, which is focused on a role of environmental metallic elements, such as Ca, Mg, Al, Fe, Cu, and Zn, in the oxidative process of neuronal degeneration. Therefore, it is very important to regulate ionic homeostasis for therapeutic strategy to prevent the development and progression of neurodegenerative diseases (106).

A rapid development of synchrotron radiation (SR)-based studies in the recent decades provides us non-destructive analyses, chemical state analyses, and imaging distribution of the elements at a single cellular level (sensitivities in $< 10^{-6}$ g [mg/Kg] and spatial resolution 2–10 μm) (107).

For the therapeutic strategies, it is very important to elucidate a role of transition metals in the physicochemical mechanisms of oxidative stress on neurons, leading to the conformational changes in disease-related proteins, such as α-synuclein (Parkinson's disease, PD), amyloid β-peptide (Alzheimer's disease, AD), TDP-43 (amyotrophic lateral sclerosis/frontotemporal lobar degeneration, ALS/FTLD and frontotemporal lobar degeneration, FTLD), and so on (108). Recently, Fourier transformed infrared spectroscopy (FTIR) using SR microbeam has been applied to analyze secondary structure of β-amyloid deposits in Alzheimer's disease (109) and the intracytoplasmic β-sheet-rich structures of Lewy bodies in Parkinson's disease (110, 111). FTIR provides chemical information of tissue components such as proteins, lipids, nucleic acids, and carbohydrates and very sensitive to protein secondary structure, such as α-helical, β-sheet, and extended coil proteins (109–112). In near future, the combination of SXRF and FTIRM analyses will be a powerful tool to examine the misfolding of disease-specific proteins and accumulation of transition metals in various neurodegenerative diseases (113).

The application and development of SR microbeams in the fields of medicine and biology is expected to bring a new stage of future biomedical research and therapeutic strategies of ALS/PDC and other neurodegenerative diseases.

CONCLUSION: THERAPEUTIC STRATEGY AND PREVENTION

Before World War II in Guam, the Chamorros used rainwater from tin roofs and ground surface water as a drinking water, containing extremely low contents of Ca and Mg. During 1970s, some 60 deep wells were begun to drill through the limestone formation to the water lens in the ground (average hardness of 250–300 ppm $CaCO_3$). However, until 1977, there was not enough water supply for the south (less than 25 ppm $CaCO_3$), where the incidence of ALS/PDC had been the highest (114). In the 1950s, the incidence of ALS and PDC on the island of Guam was much higher than those observed in the continental USA (115). From the late 1960s to the early 1980s, the incidence of both diseases has declined to the rates only slightly higher than that of elsewhere in the world (116). Concurrently, the leading cause of adult deaths in Guam has shift from ALS/PDC to cardiovascular and cerebral diseases and complications from type II diabetes, reaching the disease patterns of Westernized society (117).

On the other hand, after 1980s, the clustering of ALS/PDC in the Kii Peninsula has gradually disappeared by westernization of the lifestyle and socioeconomic changes, as well as Guam (118, 119). Recent environmental studies in the Kozagawa focus in the southernmost part of the Kii Peninsula of Japan have revealed; 1) high levels of Mn and Al in the soils, 2) markedly low levels of Ca, Mg, and Zn in the drinking water, 3) lower Ca and Zn levels in serum and higher urinary levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG, an oxidative stress maker of DNA), and 3) high Cu/Zn ratio (a marker of oxidative stress) and intact PTH in serum of the patients with ALS and also some residents (119, 120). Furthermore, the spinal cord tissues and scalp hair of Kii ALS contained a high level of transition metals, i.e., Mn, V, and/or Ti (58, 121).

In 1975, the water source in Oshima (population: 1,217 in 2009, 9.9 Km^2), a small island opposite to the top of the mainland of the Kii Peninsula, was changed from the Kozagawa river, in which the Ca and Mg contents were extremely low. Before 1975, the Oshima residents had taken a drinking water from wells and small rivers, rich in Ca and Mg and other minerals. Fujita et al. (122) compared the Ca metabolism of Oshima residents with that of Kozagawa residents. The Kozagawa residents in the mountainous area had thinner bone cortex and a higher frequency of lumbar spondylosis. The Oshima residents were mainly engaged in fishing and probably consumed abundant Ca from fish and shellfish. In Oshima, ALS had not been observed until 1999. However, three ALS cases appeared during the cross-sectional study from 2000 to 2009, which indicates that a long-term low intake of Ca and Mg over 25 years. It may play an important role in developing ALS among the Oshima residents (123).

Together with the results of extensive genetic environmental studies of both Kii and Guam ALS/PDC, it suggested that the lifestyle changes due to westernization in the recent years might exert a protective effect against the oxidative stress, finally leading to the decline or disappearance of ALS/PDC from both foci. On the public health prevention, it is important to correct environmental factors for protecting against the oxidative stress; 1) correcting low Ca and high Mn levels in drinking water and daily food, 2) improving the Cu/Zn ratio in serum, and 3) avoiding too much intake foods rich in transition metals, i.e., Al, V, and Ti, such as dried whole sardines, horse mackerels, and so on.

Currently, the only approved drugs for ALS are riluzole (glutamate antagonist) taken orally and edaravone (free radical scavenger) given intravenously. However, none of these drugs can ameliorate the progression of the disease (124). The importance of free radicals in the oxidative process of both sALS and fALS is an issue to be elucidate near future.

Globally and domestically, medical applications of SR-radiation microbeam are still very few, and their powerful analytical capabilities are not fully recognized. This technique has the advantage that they can be applied to biological specimens, such as autopsy cases, tissue specimens such as transgenic mice, cultured cells, and iPS cells in a living state without any damage in the air. By combining both XRF-XAFS and FTIR methods, it will provide a useful technique for translational interdisciplinary researches of drug discovery, using an iPS cell model of ALS in future (125).

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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