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<th>Lifestyle Changes and Oxidative Stress in a High-incidence Area of Amyotrophic Lateral Sclerosis in the Southwestern Kii Peninsula, Japan</th>
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<td>Author(s)</td>
<td>Kihira, Tameko; Okamoto, Kazushi; Sakurai, Iori; Arakawa, Yuya; Wakayama, Ikuro; Takamiya, Koichi; Okumura, Ryo; Iinuma, Yuhto; Iwai, Keiko; Kokubo, Yasumasa; Yoshida, Sohei</td>
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<tr>
<td>Citation</td>
<td>Internal Medicine (2017), 56(12): 1497-1506</td>
</tr>
<tr>
<td>Issue Date</td>
<td>2017</td>
</tr>
<tr>
<td>URL</td>
<td><a href="http://hdl.handle.net/2433/226546">http://hdl.handle.net/2433/226546</a></td>
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<td>Rights</td>
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<tr>
<td>Type</td>
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Kyoto University
Abstract

Objective Lifestyle changes may play an important role in the incidence reduction and delay of onset age of amyotrophic lateral sclerosis (ALS) in the Koza/Kozagawa/Kushimoto (K) area. The aim of this study was to evaluate recent lifestyle changes in the K area and to investigate the relationships between lifestyle and oxidative stress among the residents.

Methods We conducted a medical checkup for elderly residents in the K area and the control area and evaluated the urinary 8-OHdG levels, cognitive function test scores and metal contents in serum and scalp hair, coupled with a lifestyle questionnaire survey between 2010 and 2015.

Results Recent lifestyle changes among the K residents, including a decrease in the Japanese pickle consumption, increase in fresh vegetable consumption and decrease in farm work, were evaluated in this study. Low consumption of Japanese pickles, high consumption of fresh vegetables, rare farm work and low levels of 8-OHdG/creatinine were all associated with high scores in the cognitive function tests. Frequent farm work and consumption of Japanese pickles was associated with high contents of transition metals, such as Mn, Al and V, in the scalp hair.

Conclusion These lifestyle changes among residents in the K area may be associated with their oxidative stress.

Key words: Kii-ALS, lifestyle change, oxidative stress, Mn, Zn

(DOI: 10.2169/internalmedicine.56.8038)
The medical checkup included physical measurements, a neurological examination, blood (including a measurement of serum Ca, Mg, Zn, Cu, albumin, aspartate aminotransferase (AST), and alkaline phosphatase (ALP)) and urine biochemistry (including a routine dipstick test and measurement of 8-OHdG), and tests of the cognitive function, including a Mini-Mental State Examination (MMSE), Hasegawa Dementia Scale-revised (HDS-R), Frontal assessment battery (FAB) and a scent identification card test (Open Essence, Wako, Japan). The scent identification card test “Open Essence” was reported to be useful as a screening test of the olfactory function (15). Deterioration of the olfactory function is an early sign of cognitive impairment (16). Scalp hair samples from the K residents were collected during the research period between 2010 and 2011, and the metal levels were determined by a neutron activation analysis (NAA) at Kyoto University Research Reactor (KUR) between 2011 and 2012. Details regarding conducting measurements via a NAA at KUR have been previously reported (11).

Based on the questionnaire, we examined the lifestyle characteristics of the K residents compared with the control areas, both in the 1960s and “at present”. Among the K residents, we analyzed the relationships between the lifestyle change and the medical checkup data, including the 8-OHdG/creatinine (8-OHdG/c) and transition metal contents in the scalp hair, with special reference to the oxidative stress.

**Statistical analysis**

The data were expressed as the mean ± standard deviation (SD) or standard error (SE). A p value of <0.05 was considered statistically significant. The software program SPSS version 11 (SPSS, Chicago, IL, USA) was used for all statistical analyses.

**Ethics statement**

This study was approved by the Institutional Ethics Committee of the Kansai University of Health Sciences (10-03, 12-16 and 14-07). All participants provided informed consent and voluntarily agreed to participate in this study.

**Results**

**Lifestyle changes in the K area**

A total of 160 K residents without duplication and 62 control area residents participated in this study and voluntarily received a medical checkup, and 93 of the K residents and 62 control residents answered the questionnaire during the research period. In the K area, the cumulative participant during the 4 medical checkups was 352, and 160 of these 352 persons were unique individuals, so the first-time data of these 160 K residents were used to prevent overlap. We analyzed the relationships between lifestyle and the medical checkup data of the 93 K residents who answered the questionnaire in this study (Table).

The proportion of K residents who answered that they frequently ate Japanese pickles (p<0.05) and dried fish (p<0.05) at 20-30 years of age (in the 1960s) was significantly higher than that of the controls at the time. The proportion of K residents who answered that they frequently ate eggs and egg dishes (p<0.05), milk and dairy products (10 points) and meat dishes (12 points) in the 1960s was lower than that of the controls (Fig. 1a). On comparing the responses between the 1960s and present, the percentage of K resi-
and paddy agriculture (from 42% to 3%) in the K area was 10%), fishing (from 32% to 7%), forestry (from 7% to 0%) frequently engaged in heavy physical labor (from 56% to 1960s. The percentages of residents who answered that they 69%) were also increased at present compared with in the 1960s (18%). Similarly, purchasing at supermarkets at present had increased 37 points (55%) from 35% in the 1960s to 8.5% at present. Purchasing fish 1.4% at present, and that of spring water users decreased which was 55 points higher than in the 1960s. The percent-

The population of the K area in 2010 was 22044, and 8333 people were ≥65-years-old according to the Wakayama Prefectural 2010 census.

*: The cumulative number who participated in 4 medical checkups was 352, and 160 of these were unique individuals. The first-time data of these 160 K residents were analyzed.

**: The sample number from the questionnaire respondents is shown.

### Participants in the Study between 2010 and 2015.

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<th>K area</th>
<th>Control</th>
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<tr>
<td>Participants</td>
<td>160*</td>
<td>62</td>
</tr>
<tr>
<td>Respondent to the questionnaire</td>
<td>93</td>
<td>62</td>
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<tr>
<td>Age (mean±S.D.)</td>
<td>76.9±7.7</td>
<td>73.8±6.5</td>
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The MMSE scores were significantly different among the four groups of Japanese pickle consumption. The MMSE scores of the K residents who answered that they rarely ate Japanese pickles both in the 1960s and at present (↔ group) were significantly higher than those who frequently ate them in both the 1960s and at present (f→f group, p<0.05) and those who frequently ate them in the 1960s but rarely at present (f→r group, p<0.05), and tended to be higher than those of the ↔ group. In contrast, the MMSE scores of the f→f group tended to be lower than those of the f→r group (Fig. 2a). The FAB scores were significantly different among the four groups, and the scores of the ↔ group were higher than those of the f→f group (p<0.05, Fig. 2b). The scent identification card test scores (scent scores) were not significantly different among the four groups (Fig. 2c).

The urinary levels of 8-OHdG/c were measured in the K residents who did not have smoking habits, overt kidney disease or liver disease in the medical checkup. The 8-OHdG/c levels of the ↔ group tended to be lower than those of the f→f and f→r groups, but they were not significantly different among the four groups (Fig. 2d). The hair Mn and V contents were significantly different among the four groups. The hair Mn contents of the ↔ group were lower than those of the f→f group (Fig. 2e). The hair V contents of the ↔ group were lower than those of the f→f group (p<0.05, Fig. 2f).

### Relationships between the changes in the consumption of fresh vegetables and cognitive test scores, 8-OHdG/c levels and hair metal contents among K residents

The MMSE scores were not significantly different among the four groups of consumption of fresh vegetables. How-
ever, on comparing the $r\rightarrow r$ group and $r\rightarrow f$ group, the MMSE scores of the $r\rightarrow r$ group were significantly lower than those of the $r\rightarrow f$ group ($p<0.05$, Fig. 3a). The FAB scores were not significantly different among the four groups. On comparing the $r\rightarrow r$ group and $r\rightarrow f$ group, the FAB scores tended to be lower in the $r\rightarrow r$ group than in the $r\rightarrow f$ group ($p=0.056$) (Fig. 3b). The scent scores were significantly different among the four groups, and the scores of the $r\rightarrow r$ group tended to be higher than those of the $r\rightarrow f$ group. Furthermore, on comparing the $f\rightarrow f$ group and the $f\rightarrow r$ group, the scent scores of the $f\rightarrow f$ group tended to be higher than those of the $f\rightarrow r$ group ($p=0.058$) (Fig. 3c). The 8-OHdG/c levels were significantly different among the four groups, and the levels of the $f\rightarrow f$ group were significantly lower than those of the $f\rightarrow r$ group ($p<0.05$) (Fig. 3d). The 8-OHdG/c levels of the $f\rightarrow r$ group were significantly higher than those of the $r\rightarrow r$ group. The hair Mn and V contents were not significantly different among the four groups (Fig. 3e and f).

**Relationships between the frequency of farm work and cognitive function test scores, 8-OHdG/c levels and hair Mn and Al contents among K residents**

The MMSE scores were not significantly different among the four groups of farm work frequency. On comparing between the $f\rightarrow f$ group and the $r\rightarrow r$ group, the MMSE scores of the $f\rightarrow f$ group tended to be lower than those of the $r\rightarrow r$ group ($p=0.07$, Fig. 4a). The FAB scores and the scent scores were not significantly different among the four groups (Fig. 4b and c). On comparing the $r\rightarrow r$ group and the $f\rightarrow f$ group, the scent scores of the $r\rightarrow r$ group were lower than those of the $f\rightarrow f$ group ($p<0.05$, Fig. 4c). There were no significant differences in the 8-OHdG/c levels among the four groups. On comparing the $r\rightarrow f$ group and the $f\rightarrow r$ group, the 8-OHdG/c levels of the $r\rightarrow f$ group tended to be lower than those of the $f\rightarrow r$ group ($p=0.06$, Fig. 4d). The hair Mn contents were not significantly different among the four groups. On comparing the $r\rightarrow r$ group and the $f\rightarrow f$ group, the hair Mn contents of the $r\rightarrow r$ group were lower than those of the $f\rightarrow f$ group ($p=0.02$) and tended to be lower than those of the $r\rightarrow f$ group ($p=0.04$, Fig. 4e). The hair Al contents were significantly different among the
The relationships between the changes in the consumption of Japanese pickles and cognitive function test scores, 8-OHdG/c levels and hair Mn and V contents. The labels of the x bars from Figs. 2 to 4 are as follows: r\rightarrow r: rarely in the 1960s and rarely at present, r\rightarrow f: rarely in the 1960s and frequently at present, f\rightarrow f: frequently in the 1960s and frequently at present, f\rightarrow r: frequently in the 1960s and rarely at present. The MMSE scores of the r\rightarrow r group were significantly higher than those of the f\rightarrow f and the f\rightarrow r groups (a). The FAB score of the r\rightarrow r group were higher than those of the f\rightarrow f group (b). The scent scores were not significantly different among the four groups (c). The 8-OHdG/c levels (ng/mg of creatinine) were not significantly different among the four groups (d). The hair Mn contents (μg/g wet weight) of the r\rightarrow r group were lower than those of the f\rightarrow f group (e). The hair V contents (ng/g wet weight) of the r\rightarrow r group were significantly lower than those of the f\rightarrow f groups (f). *: \textit{p}<0.05, ns: not significant. The plot and bar show the mean value and standard error, respectively.

Relationships between the cognitive function test scores by age and lifestyle at present among K residents

The relationship between the frequency of Japanese pickle consumption at present and the cognitive function test scores was analyzed by age. Given that the cognitive function test scores were negatively correlated with age, the scores were analyzed for the following age groups: young-old group, \(\leq 69\) years old (n=13); middle-old group (mid-old), 70-79 years old (n=37); old-old group, \(\geq 80\) years old (n=43). The K residents in the mid-old group who answered that they frequently ate Japanese pickles at present (f group) had lower scores in MMSE (p<0.05, Fig. 5a) and FAB (p<0.05, Fig. 5b) than those who rarely ate Japanese pickles at present (r group). The K residents who answered that they frequently ate Japanese pickles at present showed higher contents of Mn (p<0.05, Fig. 5c) and V (p<0.05, Fig. 5d) in the scalp hair than those who rarely ate Japanese pickles at present.

The relationship between the frequency of fresh vegetable consumption at present and the cognitive function test scores was analyzed by age. In the old-old group, the MMSE scores of the K residents who answered that they frequently ate fresh vegetables at present (f group) were higher than in those who rarely ate fresh vegetables at present (r group) (p<0.01, Fig. 5c). The scent scores of the K residents in the young-old and old-old groups who answered that they frequently ate fresh vegetables at present were higher than in those who rarely ate fresh vegetables at present (p<0.05 respectively, Fig. 5f). The hair Mn and V contents of the K residents who answered that they frequently ate fresh vegetables at present were not markedly different from those who rarely ate fresh vegetables at present (Fig. 5g and h).

The relationship between the frequency of farm work at present and the cognitive function test scores was analyzed by age. The K residents in the old-old group who answered that they frequently do farm work at present (f group) had lower MMSE scores than those who rarely do farm work at present (r group) (p<0.05, Fig. 5i), and the residents in the mid-old group who answered that they frequently do farm work at present showed lower scent scores than those who rarely do farm work at present (p<0.05, Fig. 5j). The K resi-
students who answered that they frequently do farm work at present exhibited higher Mn (p<0.05, Fig. 5k) and Al contents (p<0.01, Fig. 5l) in their scalp hair than those who rarely do farm work at present. 

**Relationships between the cognitive function test scores and oxidative stress among K residents**

The mean value of 8-OHdG/c in the total K residents was 11.8 ng/mg of creatinine (SD: 5.2). The values of 8-OHdG/c did not correlate with age, body mass index, blood pressure or pulse and showed mild negative correlations with serum Zn levels (r: -0.30, p<0.05), HDS-R scores (r: -0.30, p<0.05) and MMSE scores (r: -0.28, p<0.05). The K residents were categorized by the mean value of their 8-OHdG/c levels into either a high 8-OHdG/c group (≥11.8 ng/mg creatinine) or a low 8-OHdG/c group (<11.8 ng/mg creatinine). The HDS-R scores of the low 8-OHdG/c group were significantly higher than those of the high 8-OHdG/c group in the young-old group (p<0.05) and tended to be higher than those of the high 8-OHdG/c group in the mid-old group (p<0.05) and the young-old group (p<0.05).

**Discussion and Conclusion**

In this study, we found that recent lifestyle changes among the K residents were characterized by a decrease in the Japanese pickle consumption, an increase in the fresh vegetable consumption, a well-balanced diet, globalized food acquisition and a decrease in the frequency of heavy agricultural labor. Recent trends in diet changes in the control areas obtained from our questionnaire were consistent with the literature (17, 18). Therefore, we consider that the answers from the residents about their lifestyle at present and in the 1960s, when they were 20-30 years old, reflected reasonable trends in change, although memory bias may ex-
The consumption of Japanese pickles and salted dried fish with rice among K residents was significantly higher than in the controls, while the consumption of egg dishes and meat dishes was significantly lower, indicating that the diet of the K residents in the 1960s was high in carbohydrates and salt and low in fat, protein and minerals. These trends were also found in our previous study between 2004 and 2005 in the Kozagawa area, as evaluated by a self-administered food frequency questionnaire (21). A high intake of carbohydrates and low intake of fat has been associated with an increase in the odds of having ALS (22), and the diet of the K residents in the 1960s obtained by the present study was similar to the eating habits seen in patients with sporadic ALS three years prior to disease onset (22).

The K residents with an increased frequency of fresh vegetable consumption at present compared with the 1960s showed higher cognitive function test scores than those who continued rare consumption without change. The K residents with a decreased frequency of fresh vegetable consumption at present compared with the 1960s showed higher levels of oxidative stress markes of oxidative stress, 8-OHdG/c levels and hair Mn and Al contents. The MMSE scores were not significantly different among the four groups. On comparing the f→f group and the r→r group, the MMSE scores of the f→f group tended to be lower than those of the r→r group (a). The FAB scores were not significantly different among the four groups (b). The scent scores were not significantly different among the four groups. On comparing the r→r group and the f→f group, the scent scores of the r→r group were higher than those of the f→f group (c). The 8-OHdG/c levels were not significantly different among the four groups. On comparing the r→r group and the f→f group, the 8-OHdG/c levels of the r→r group tended to be lower than those of the f→f group (d). The hair Mn contents were not significantly different among the four groups. On comparing the r→r group and the f→f group, the hair Mn contents of the r→r group were lower than those of the f→f group and tended to be lower than those of the r→f group (e). The hair Al contents of the r→r group were lower than those of the f→f and the f→r groups (f). *: p<0.05, **: p<0.01, &: p=0.07, β: p=0.02 , ψ: p=0.06, γ: p=0.02, #: p=0.04, ns: not significant. The plot and bar show the mean value and standard error, respectively.
8-OHdG/c than those who continued frequent consumption. Furthermore, the K residents who answered that they frequently ate fresh vegetables at present showed higher scores of MMSE and scent identification card test than rare consumers. Fresh vegetables exert various antioxidant effects on cells through vitamins, carotenoids, phytochemicals, phenolics, flavonoids and minerals as well as other essential components for metabolism (23). The K residents with a decreased frequency of fresh vegetable consumption or who rarely ate fresh vegetables at present may have low protection against oxidative stress, and this may be associated with lower scores on cognitive function tests.

The K residents in the 1960s were mainly engaged in farming, fishing, paddy agriculture and forestry. The number of residents in the K area engaged in agricultural and fishing work has decreased to one fifth of that in the 1960s, which was comparable to the demographic data of Kushimoto-cho (24, 25). The products of agricultural pesticides have been found to be dose-dependently associated with ALS in men (26). The decrease in the frequency of agricultural work and an eventual decrease in the usage of agricultural pesticides in the K area may have played a role in the decrease in the ALS incidence in this area.

Figure 5. The relationships between the cognitive function test scores by age and lifestyle at present. For the frequency of Japanese pickle consumption, the MMSE scores of the f group were lower than those of the r group in the mid-old group (70-79 years old) (a). The FAB scores of the f group were lower than those of the r group in the mid-old group (b). The hair Mn (c) and V contents (d) of the f group were higher than those of the r group. For the frequency of fresh vegetable consumption, the MMSE scores of the f group were higher than those of the r group in the old-old group (≥80 years old) (e). The scent scores of the f groups were higher than those of the r groups in the young-old (≤69 years old) and old-old groups (f). The hair Mn and V contents were not markedly different between the f and r groups (g and h). For the frequency of farm work, the MMSE scores of the f group were lower than those of the r group in the old-old group (i). The scent scores of the f group were lower than those of the r group in the mid-old group (j). The Mn and Al contents of the f group were higher than those of the r group (k and l). The plot and bar show the mean value and standard error, respectively. △ and f: frequently at present, □ and r: rarely at present. *: p<0.05, **: p<0.01, ns: not significant.
and at present tended to have higher MMSE scores and the scent identification card test scores and had lower contents of Al and Mn in their scalp hair than those who continued to frequently do such work. The K residents who frequently ate Japanese pickles also had higher contents of Mn and V in their scalp hair than those who rarely ate them. Mn, Al and V are widely distributed in the environment, and the levels of Mn and Al in the soil of the K area have been reported to be high (3, 9). Although the mechanisms are not clear, long-term exposure to the soil during farm work and the frequent consumption of Japanese pickles may have been associated with an accumulation of these transition metals in the scalp hair of the K residents. Transition metals exert potent toxic effects on neurons (27, 28) and are associated with a decline in the cognitive ability as determined by the MMSE (29). Given these findings, we speculate that an accumulation of Mn, V and Al may have played a role in the increasing oxidative stress on neurons leading to the deterioration of age-associated cognitive function in vulnerable people of the K area.

In the Hohara area, another high-incidence area of ALS/PDC, Iwami et al. studied the metal contents in the environment between 1989 and 1991 and found that the Mg contents in the drinking water were low and the Mn and Al contents in the food were high via the 24-h total food duplicate method (30). Kuzuhara reported that the epidemiological and clinical features of ALS/PDC in the Hohara area have recently changed, and some unknown environmental factors are speculated to modulate the disease process, some of which may be genetic (7). In this study, we found that the lifestyle of the K residents was recently changed, and we suggest that the lifestyle changes may be associated with the decrease in the oxidative stress and the high scores in the cognitive function test among K residents. The relationships between the lifestyle changes and the epidemiological changes of ALS in the K area should be further investigated in the future.

The limitations of this study are that the questionnaire used was based on recall of present and past events, and that the sample size was small. The relationships between lifestyle/environment and the risk of ALS need to be examined long-term among both the residents and patients with ALS in the high-incidence areas. In the present study, however, our findings suggest that the recent lifestyle changes in the K area may have helped reduce the oxidative stress on neurons, suggesting that environmental/lifestyle changes may be useful for identifying new therapeutic strategies for not only K-ALS but also sporadic and familial ALS.

The authors state that they have no Conflict of Interest (COI).

Acknowledgement

The authors especially thank the late Mr. Yukihiro Nakano of the Research Reactor Institute, Kyoto University, for his helpful advice, and Prof. Shigeki Kuzuhara of Suzuki University of Medical Science, Prof. Fumio Yoshimasu, Prof. Yoshiro Yase, Prof. Sachiko Wada, Ms. Yuka Sakurai and Ms. Satomi Morinaga of Kansai University of Health Sciences for their helpful advice, cooperation and generous encouragement.

This work was supported by Grants-in-Aid from the Research Committee of Muro Disease (Chairman: Dr. Yasumasa Kokubo), the Ministry of Health, Labour and Welfare of Japan, and a Grant-in-Aid for Scientific Research of Japan (No. 22590967 and No. 16590511). A part of this research was reported at the 23rd and 24th International Symposia on ALS/MND and at the 54th Annual Meeting of the Japanese Society of Neurology.

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