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Estimation of autistic children by metallomics analysis

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Clarification of the pathogenesis and treatment of autism spectrum disorders is one of the challenges today. In this study, we examine scalp hair concentrations of 26 trace elements for 1,967 children with autistic disorders (1,553 males and 414 females). Five-hundred and eighty-four (29.7%), 347 (17.6%) and 114 (5.8%) subjects was found deficient in zinc, magnesium and calcium, respectively, and 2.0% or less in the other essential metals. The incidence rate of mineral deficiency was highly observed in infants aged 0-3 year-old. In contrast, 339 (17.2%), 168 (8.5%) and 94 (4.8%) individuals was found suffering from high burden of aluminium, cadmium and lead, and 2.8% or less from mercury and arsenic burden. These findings suggest that infantile zinc- and magnesium-deficiency and/or toxic metal burdens may epigenetically play principal roles as environmental factors in autistic disorders and that metallomics approach may lead to early screening and prevention of the neurodevelopment disorders.

Autism spectrum disorders are a group of neural development disorders characterized by impairments in social interaction and communication, and by the presence of restricted and repetitive behaviours[1,2], and the prevalence of this disease continues to increase up to 1 in 88 children[1–4]. Autism spectrum disorders are known to be highly heritable (~90%), and some related genes have been reported[5,6]. However, the critical genetic determinants are still unclarified[2,4], and the interaction of heritable factors with uncertified lifestyle and environmental factors seem play a significant role in the aetiology. For example, organic mercury had been claimed one of environmental candidates causing autistic disorders[7], but its relationship remains to be established. Recently, epigenetic alteration of gene expression by environmental factors is considered one of key events in the pathogenesis of genetic diseases[8], and some toxic elements such as cadmium and arsenic have been reported to be candidate factors that induce epigenetic disorders[9–13].

Recent great advances in high-sensitive and reliable trace element analysis method using inductively coupled plasma mass spectrometry (ICP-MS) have enabled us to apply it to forensic medical researches and estimating chronic toxic metal burden and mineral deficiency in human body[14,15]. Thus, clinical applications of the reliable hair mineral analysis method with ICP-MS have been tried for investigating the association of some diseases and symptoms with trace element kinetics including toxic metals and essential minerals[16–21].

For the last six years, we have examined the association of toxic metal burdens with autistic disorders and reported that some of the autistic children have suffered from high accumulation of toxic metals such as cadmium, lead or aluminium[22–24]. Recently, we demonstrated the association of infantile zinc deficiency with autism spectrum disorders[25].

In this metallomics analysis study, we have determined human scalp hair concentrations of 26 trace elements for 1,967 children with autistic disorders aged 0–15 years and showed that many of the patients, especially in infants aged 0–3 year-old, are suffering from marginal to severe zinc- and magnesium-deficiency and/or high burdens of several toxic metals such as aluminium, cadmium and lead. These findings suggest that there is a critical term "infantile window" in neurodevelopment and probable for therapy of autistic disorders.

Results

The histogram of hair logarithmic zinc concentrations for 1,967 children with autistic spectrum disorders is shown in Figure 1. The distribution of the zinc concentration was non-symmetric with tailing in lower range, and 584 in 1,967 subjects (29.7%) were found to have lower zinc concentration than –2 S.D. (standard deviation) level of the reference range (86.3–193 ppm; geometric mean = 129 ppm), estimated as zinc deficiency. The incidence rate of zinc deficiency in the age groups of 0–3, 4–9 and 10–15 year-old was estimated 43.5, 28.1 and 3.3% in male and 52.5, 28.7 and 3.5% in female, respectively (Table 1) and a significant correlation of zinc concentration with

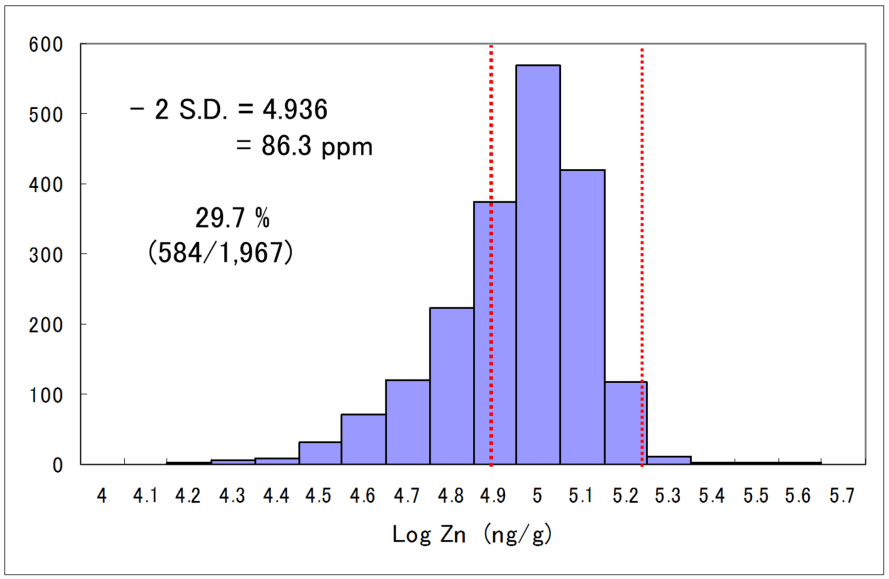

Figure 1 | Histogram of logarithmic zinc concentration in autistic children (N = 1,967). The histogram of scalp hair zinc concentrations for 1,967 children (1,553 males and 414 females) aged 0–15 years is shown in the logarithm. The numbers on the abscissa indicate the logarithms of scalp hair zinc concentrations (ng g^{-1}: ppb). The height of each rectangle represents the frequency in the class interval in logarithmic hair zinc level. Two dotted vertical lines represent the ±2 S.D. levels of the reference range of hair zinc concentrations.

age ($r = 0.367$, $p < 0.0001$) was observed (Fig. 2), indicating that infants are more liable to zinc deficiency than elder children. The minimum zinc concentration of 10.7 ppm detected in a 2-year-old boy corresponded to about 1/12 of the mean reference level. The zinc concentration of only one 0-year old female subject was 173 ppm in the normal range (Fig. 2) and seem be a suspect, because she was suffered from high burdens of aluminium (52.5 ppm), lead (9.1 ppm), iron (12.8 ppm) and copper (134 ppm). There was no significant gender difference in hair zinc concentration and incidence rate of zinc deficiency.

Following to zinc deficiency, magnesium and calcium deficiency was observed in 347 (17.6%) and 114 (5.8%) individuals in the autistic children (Table 2), and for the other essential metals such as iron, chromium, manganese, copper and cobalt, their incidence rates of deficiency were 2.0% or less. The incidence rate of magnesium deficiency in the age groups of 0–3, 4–9 and 10–15 year-old was 27.0, 17.1 and 4.2% in male and 22.9, 12.7 and 4.3% in female subjects, respectively (Table 3), and a significant correlation of magnesium concentration with age ($r = 0.362$, $p < 0.0001$) was observed (Fig. 3), suggesting that infants are also liable to magnesium deficiency than elder children. The minimal magnesium concentration of 3.88 ppm detected in a 2-year-old girl corresponded to almost 1/10 of the mean reference level (39.5 ppm). Calcium deficiency was observed only in lower age groups less than 10 year-old (Table 4).

In contrast, high toxic metal burden of aluminium, cadmium and lead of over their +2 S.D. level was observed in 339 (17.2%), 168 (8.5%) and 94 (4.8%) individuals, and their incidence rates were higher than that of mercury and arsenic (2.8 and 2.6%, respectively) (Table 5). The detected maximal concentration of aluminium, cadmium and lead was 79.4 ppm in a 4-year-old boy, 5.47 ppm in a 5-year-old boy and 24.9 ppm in a 5-year-old girl, respectively, corresponding to 21-, 782- and 57-fold of each mean reference level. The maximal burden level of mercury and arsenic was 36.3 ppm and 1.7 ppm, respectively, corresponding to about 9- and 33-fold of the mean reference level.

Figure 4 shows a typical autistic metallome profile in a 1-year-old boy suffering from severe zinc- and magnesium-deficiency and simultaneous high burdens of cadmium and lead. Another autistic metallome profile with high burdens of aluminium and manganese in a 2-year-old boy is shown in Fig. 5.

Discussion

Zinc is an essential trace element that plays important roles in nucleic acid/protein synthesis, cell replication, tissue growth and repair, especially in pregnant women and infants. Therefore, zinc deficiency is known associated with various pathological conditions, including dysgeusia, delayed wound healing, impaired immunity, retarded growth, and neural-degenerative diseases[26–30].

Table 1 | Incidence rate of zinc deficiency and the minimum level in autistic children. The number and incidence rate of individuals with zinc deficiency (lower than −2 S.D.) in 1,967 autistic children (1,553 males and 414 females) and the minimum zinc concentration in each age group are tabled

			Number of Cases	Rate (%) of	Minimum
	Age	No.	with Zn Deficiency	Zn Deficiency	(ppm)
Male	0–3 year-old	N = 577	251	43.5	10.7
	4–9 year-old	N = 736	207	28.1	21.3
	10–15 year-old	N = 240	8	3.3	66.1
Female	0–3 year-old	N = 118	62	52.5	17.3
	4–9 year-old	N = 181	52	28.7	35.7
	10–15 year-old	N = 115	4	3.5	72.4

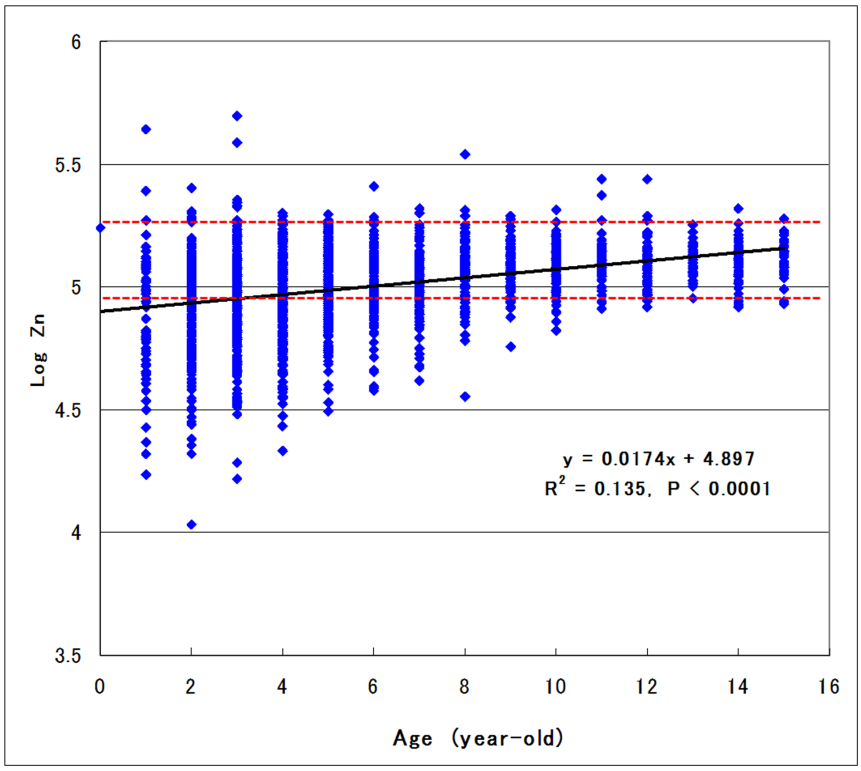

Figure 2 | Relation of logarithmic zinc concentration with age in autistic children. The association of hair logarithmic zinc concentration with age in autistic children (N = 1,967) is shown. Each point represents the corresponding age and logarithmic zinc concentration of the individual child. Two dotted horizontal lines represent the ±2 S.D. levels of the reference range (86.3–193 ppm) of hair zinc concentrations. A significant relation of the zinc concentration with age ($r = 0.367$, $p < 0.0001$) in the autistic children is shown.

Recently we reported that many infants with autistic disorders are suffered from marginal to severe zinc deficiency, suggesting considerable relationship of infantile zinc deficiency with autism[25]. In the present study, we have determined scalp hair concentrations of 26 trace elements for 1,967 subjects diagnosed with autism spectrum disorders and investigated their association with mineral disorders.

The histogram of logarithmic zinc concentration in the autistic children showed a non-symmetric profile with a marked tailing in lower range, indicating that about 30% of the autistic children were estimated as zinc deficiency (Fig. 1). In particular, nearly one half (male: 43.5%; female: 52.5%) of the infant group aged 0–3 year-old were found to suffer from marginal to severe zinc deficiency (Table 1). Thus, the lowest mean zinc concentration was observed in the infant group aged 0–3 year-old in both genders (male: 87 ppm; female: 81 ppm), and a high significant correlation of the zinc concentration with age was observed in the autistic children (Fig. 2). These findings suggest that infants are liable to zinc deficiency because they need larger amount of zinc (per kg body weight) for their development and growth. In addition, age-related changes in the production of metallothionein proteins and differences in glutathione levels may be associated with the age-related differences in zinc requirements[31]. There was little gender difference in zinc deficiency rate and also in hair zinc concentration (Table 1).

Next to zinc deficiency, a considerable number of autistic children were found to be deficient in magnesium and calcium, and no significant deficiency was observed for the other essential metals (Table 2). The incidence rate of magnesium deficiency in the age group of 0–3 and 4–9 year-old was 27.0 and 17.1% in male, and 22.9 and 12.7% in female subjects, respectively, suggesting that about one fourth of the infant group are suffered from a simultaneous deficiency of zinc and magnesium. Compared to magnesium, significant deficiency in calcium was observed only in the lower age groups (Table 4). These findings indicate that infantile autistic children have a character liable to deficiency in zinc and magnesium.

Table 2 | Incidence rate of mineral deficiency in autistic children. The number and incidence rate of individuals with mineral deficiency (lower than −2 S.D.) in 1,967 autistic children (1,553 males and 414 females) are tabled

Mineral	Number of Cases with Deficiency	Rate (%) of Deficiency
Zn	584	29.7
Mg	347	17.6
Ca	114	5.8
Co	40	2.0
Fe	17	0.9
Cr	12	0.6
Mn	4	0.2
Cu	4	0.2

Table 3 | Incidence rate of magnesium deficiency and the minimum level in autistic children. The number and incidence rate of individuals with magnesium deficiency (lower than −2 S.D.) in 1,967 autistic children (1,553 males and 414 females) and the minimum magnesium concentration in each age group are tabled

	Age	Number of Cases with Mg Deficiency	Rate (%) of Mg Deficiency	Minimum (ppm)
Male	0–3 year–old	156	27.0	3.92
	4–9 year–old	126	17.1	4.19
	10–15 year–old	10	4.2	5.31
Female	0–3 year–old	27	22.9	3.88
	4–9 year–old	23	12.7	4.75
	10–15 year–old	5	4.3	6.27

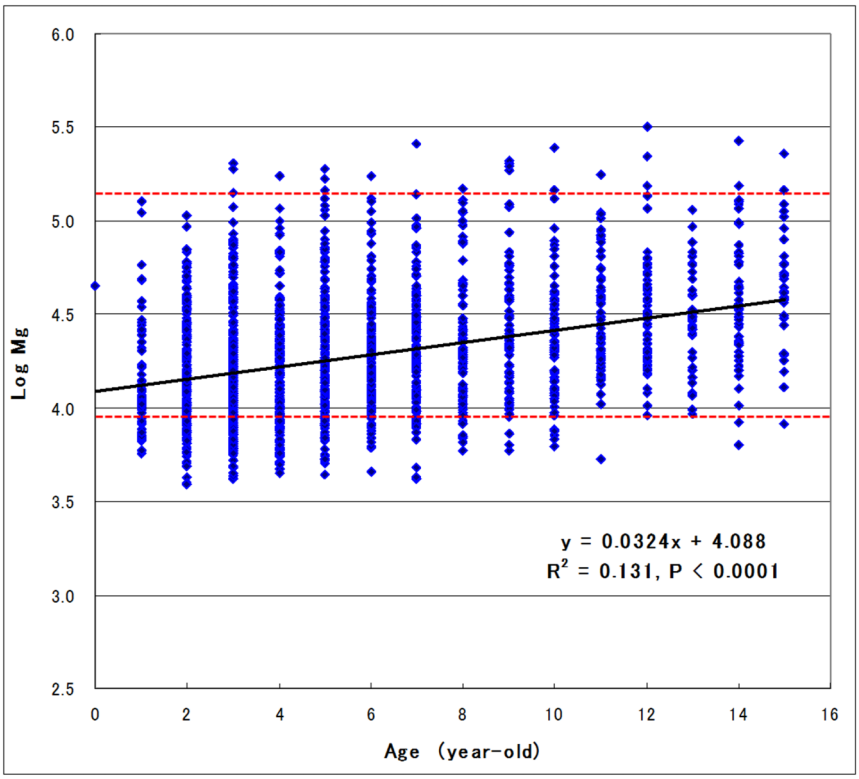

Figure 3 | Relation of logarithmic magnesium concentration with age in autistic children. The association of hair logarithmic magnesium concentration with age in autistic children (N = 1,967) is shown. Each point represents the corresponding age and logarithmic magnesium concentration of the individual child. The dotted horizontal lines represent the ±2 S.D. levels of the reference range (9.6–163 ppm) of hair magnesium concentrations. A significant relation of the magnesium concentration with age ($r = 0.362$, $p < 0.0001$) in the autistic children is shown.

There are numerous studies with the same theme reporting nutritional status and mineral deficiencies in autistic children[32–36]. However, the conclusions of their studies, in which the restricted subject age range (over 3 or 5 year-old) and number of minerals were examined, were not consistent, and the critical environmental factors remained to be established. In this metallomics analysis study for the 1,967 autistic children aged 0–15 year-old, we were able to demonstrate not only the critical epigenetic factor (zinc- and magnesium-deficiency and/or high burdens of aluminium, cadmium and lead) but also the presence of another critical factor, "infantile window" in neurodevelopment and for therapy probably.

Arnold et al.[37] reported that mean serum zinc levels were significantly lower in both autism and ADHD groups, and that serum zinc level correlated inversely with parent- and teacher-rated inattention in ADHD children. Furthermore, it is reported that zinc treatment was significantly superior to placebo in reducing symptoms of hyperactivity, impulsivity and impaired socialization in ADHD patients[38,39]. Other preliminary human study showed that many children with ADHD have lower zinc concentration in comparison to healthy children and that zinc supplement as an adjunct to methylphenidate has favourable effects in the treatment of ADHD children, pointing to the possible association of zinc deficiency and ADHD pathophysiology[40].

Kozielec et al.[41] have reported that in hyperactive 116 children with ADHD, magnesium deficiency was found in the 95% of the subjects, most frequently in hair (77.6%), next in red-blood cells (58.6%) and in blood serum (33.6%). Furthermore, they reported that a significant decrease of hyperactivity and increase in hair magnesium contents has been achieved in the group of ADHD children given six months of magnesium supplementation[42]. Mousain-Bosc et al.[43] also reported that 52 hyper-excitable children have low intra-erythrocyte magnesium levels with normal serum magnesium values and that magnesium/vitamin B6 supplementation can restore the erythrocyte magnesium levels to normal and improve their abnormal behaviours. They also reported that thirty three children with clinical symptoms of pervasive developmental disorder or autism (PDD) exhibit significantly lower red blood cell magnesium values and that the combination therapy with magnesium/vitamin B6 for six months

Table 4 | Incidence rate of calcium deficiency and the minimum level in autistic children. The number and incidence rate of individuals with calcium deficiency (lower than −2 S.D.) in 1,967 autistic children (1,553 males and 414 females) and the minimum calcium concentration in each age group are tabled

	Age	Number of Cases with Ca Deficiency	Rate (%) of Ca Deficiency	Minimum (ppm)
Male	0–3 year–old	60	10.4	70.1
	4–9 year–old	45	6.1	67.5
	10–15 year–old	1	0.4	95.5
Female	0–3 year–old	4	3.4	88.2
	4–9 year–old	3	1.7	95.4
	10–15 year–old	1	0.9	90.2

Table 5 | Incidence rate of high toxic metal burden and the maximum level in autistic children. The number and incidence rate of individuals with high toxic metal burden (higher than +2 S.D.) in 1,967 autistic children (1,553 males and 414 females) and the maximum concentration are tabled

Toxic Metal	Number of Cases with High Burden	Rate (%) of High Burden	Maximum (ppm)	Ratio to Reference
Al	339	17.2	79.4	21.1
Cd	168	8.5	5.5	782.0
Pb	94	4.8	24.9	57.4
Hg	56	2.8	36.3	9.3
As	52	2.6	1.7	33.5

improved significantly PDD symptoms in 23/33 children ($p < 0.0001$) with concomitant increases in intra-erythrocyte magnesium values[44].

These findings are consistent with a gradient overarching disorder hypothesis for autism spectrum disorders and ADHD[45] and indicate that infantile mineral deficiency in zinc and magnesium plays epigenetically principal roles as environmental factors in the pathogenesis of these neurodevelopment disorders, suggesting that the supplementation of deficient minerals during the critical "infantile window" may be useful for treatment and prevention of these diseases.

Recently, dietary restriction-induced zinc deficiency has been reported to up-regulate intestinal zinc-importer (ZIP4) and induce the increase in ZIP4 protein located to the plasma membrane of enterocytes[46,47]. This adoptive response to zinc deficiency is known to lead to increase in the risk of high-uptake of toxic metals such as cadmium and lead[48]. Thus, infants with zinc deficiency are liable to increased risk of absorbing high amount of toxic metals and retaining them in their bodies, as shown in Table 5 and Fig. 4. These findings suggest that the increased toxic metal burdens attendant on zinc deficiency may also epigenetically contribute to the pathogenesis of these diseases.

Maternal cigarette smoking has been reported to be associated with lower zinc and higher cadmium and lead concentrations in their neonates[49]. During pregnancy and lactation, these toxic metals accumulated in the maternal bone tissues are co-transferred with calcium to foetal and new-born bodies through activated bone-resorption[49–52].

Eklund and Oskarsson[53] reported that soy-based formulas contain approximately six times more cadmium than cow's milk formulas and cereal-based formulas have 4–21 times higher levels. Thus, the dietary intake of the toxic metal in the children fed on infant formulas and weaning foods may be high in comparison with breast milk-fed infants.

For mercury and arsenic, the maximum burden level of 9.3- and 33.5-fold of the reference level (Table 5) may also epigenetically play some pathogenic roles in the respective autistic individuals, even though their incidence rate was 2.8% or less.

In summary, this metallomics study demonstrates that many of autistic infants have been suffered from marginal to severe zinc- and magnesium-deficiency and/or high toxic metal burdens of aluminium, cadmium, lead and so on. These findings suggest that infantile mineral deficiency and/or toxic metal burdens may epigenetically play principal roles in the pathogenesis of autism spectrum

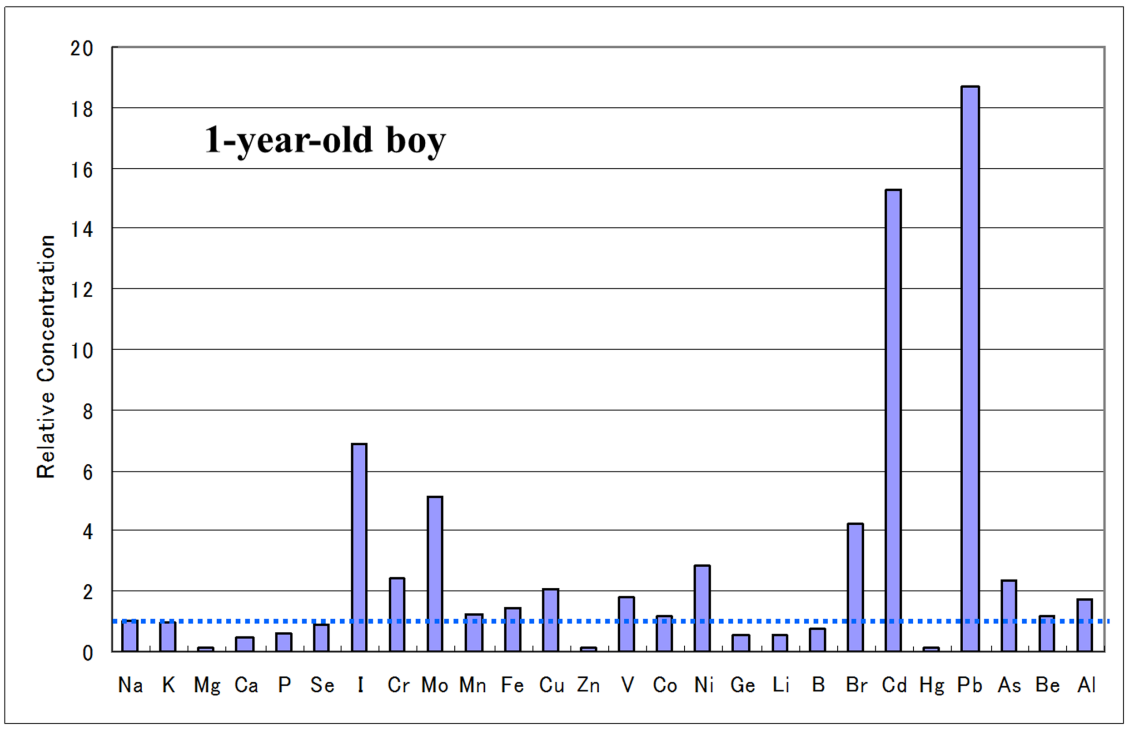

Figure 4 | Metallome profile of an autistic child with high cadmium and lead burdens. A typical metallome profile of 1-year-old boy diagnosed with autism is shown, exhibiting severe zinc- and magnesium-deficiency and simultaneous high burdens with cadmium (107 ppb) and lead (8.11 ppm). Each bar represents the relative concentration of the respective trace element in his scalp hair specimen. The dotted horizontal line at 1.0 represents the reference control level of each trace element.

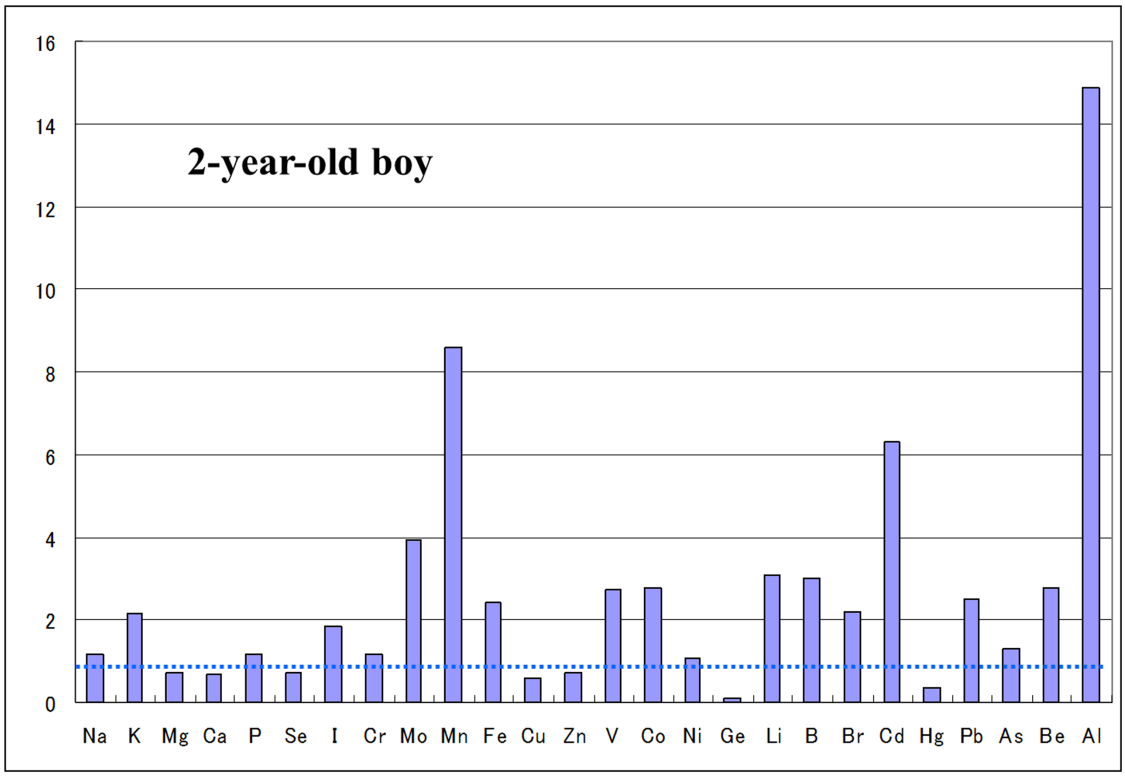

Figure 5 | Metallome profile of an autistic child with high aluminium and manganese burdens. Another typical metallome profile of 2-year-old boy diagnosed with autism is shown, exhibiting simultaneous high burdens with aluminium (55.9 ppm), manganese (926 ppb) and cadmium (44 ppb). Each bar represents the relative concentration of the respective trace element in his scalp hair specimen. The dotted horizontal line at 1.0 represents the reference control level of each trace element.

disorders and that there is a critical term "infantile window" in neurodevelopment and for its therapy. Therefore, it is possible that autistic infants may respond to nutritional approach supplementing deficient nutrients and detoxifying accumulated toxic metals on the basis of the evidence. This evidence-based nutritional approach may yield a new pathway into treatment and prevention of infantile patients with autistic disorders including the suspects. Well-controlled intervention studies for this novel nutritional therapy are desired to establish the epigenetic roles of infantile mineral deficiencies and/or toxic metal burdens in the pathogenesis of neurodevelopment disorders such as autism spectrum disorders and ADHD.

In conclusion, this preliminary study demonstrates that many of infantile patients diagnosed with autism have been suffered from marginal to severe zinc- and magnesium-deficiency and/or high toxic metal burdens of aluminium, cadmium, lead and so on, suggesting that these mineral disorders may epigenetically play principal roles as environmental factors in the pathogenesis of autism spectrum disorders.

Methods

Samples and trace element analysis. On the basis of informed consent, scalp hair samples from 1,967 (male: 1,553; female: 414) autistic Japanese subjects aged 0–15 years were collected in the period from June 2005 to September 2007, although 0 year-old subject was only one (11 month-old female). These subjects were comprised of the children diagnosed with autistic spectrum disorders by their physicians. Hair sampling was recommended to cut as close to the scalp of the occipital area as possible.

Hair sample of 75 mg was weighed into 50 ml plastic tube, and washed with acetone and then with 0.01% Triton solution, as recommended by the Hair Analysis Standardization Board[54]. The washed hair sample was mixed with 10 ml 6.25% tetra methyl ammonium hydroxide (TMAH, Tama Chemical, Kawasaki, Japan) and 50 μl 0.1% gold solution (SPEX Certi Prep, Metuchen, NJ, USA), and then dissolved at 75 centigrade with shaking for 2 hours. After cooling the solution to room temperature, internal standard (scandium, gallium and indium) solution was added, and after adjusting its volume gravimetric, the obtained solution was used for multi-mineral analysis. The trace element concentrations were determined with inductively coupled plasma mass spectrometry (ICP-MS; 7500 ce, Agilent Technologies, Santa Clara, CA, USA) as reported previously[19,20] and expressed as ng g^{-1} hair (ppb) or μg g^{-1} hair (ppm). Human hair certified reference material (NIES CRM No. 13) was used to check for the accuracy of analysis. The inter-daily variation of zinc, magnesium, calcium, aluminium, cadmium, lead, mercury and arsenic determination was 2.2, 9.6, 6.3, 8.2, 6.9, 11.1, 9.4 and 6.9%. The control geometric mean value and reference range for each trace element was obtained from the data for 436 male healthy subjects aged 21–40 year-old, as previously reported[19,20,25].

This study has been approved by review of the ethical committee of La Belle Vie Research Laboratory. All of the data obtained are held securely in such a form as to ensure anonymity.

Statistical analysis. Because each trace element concentration in scalp hair was almost log-normally distributed, the mineral concentration was converted to logarithm, and the geometric rather than arithmetic mean is used as representative of its hair concentration. The relation between age and zinc/magnesium concentration of the subjects was examined by Pearson's correlation coefficient test.

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Author contributions

H.Y. performed this study, analysed the data and wrote the manuscript with the help of M.K., Y.Y. and T.T.

Additional information

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Masahiro Kobayashi did not contribute directly to this study and, at the request of all the authors, has been removed from the author list in both the PDF and HTML versions of the Article. The Author Contributions section should read "H.Y. performed this study, analysed the data and wrote the manuscript with the help of Y.Y. and T.T."