early MRI may help identify a group of very preterm born infants with an increased risk of later cognitive impairment. Such information may provide an important key to improve follow-up strategies and to allow better risk stratification for future clinical trials which enroll very preterm born infants.

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Qualitative Brain MRI at Term and Cognitive Outcomes at 9 Years After Very Preterm Birth

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METHODOLOGY

$P_{ET}CO_2$ measured by a new lightweight mainstream capnometer with very low dead space volume offers accurate and reliable noninvasive estimation of $PaCO_2$

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Objective: Although capnometers are widely used in adult and pediatric intensive care units, they are not widely used in neonatal intensive care units due to issues such as the weight of sensors, dead space, and leakage from tracheal intubation tubes. These authors developed a light and low dead space airway adaptor of end-tidal carbon dioxide pressure ($P_{ET}CO_2$) and evaluated the correlations between $P_{ET}CO_2$ and partial CO_2 pressure ($PaCO_2$) in rabbits while changing tidal volume and leakage volume.

Methods: Firstly, Japanese rabbits weighing 2 kg were divided into three tidal volumes (6 mL/kg, 10 mL/kg, or 15 mL/kg), and $P_{ET}CO_2$ and $PaCO_2$ were measured. Secondly, the respiratory apparatus was set to a tidal volume/body weight ratio of 10 mL/kg, leakage rates were divided into seven groups, and $P_{ET}CO_2$ and $PaCO_2$ were measured.

Results: $P_{ET}CO_2$ and $PaCO_2$ were significantly correlated ($r^2 = 0.9099$, $P < 0.0001$) when there was no leakage in the tracheal intubation tubes. No significant differences were observed between $PaCO_2$ and $P_{ET}CO_2$ ($P_{a\text{-}ET}CO_2$) in the three tidal volume/body weight groups or for groups in which leakage rate was <60%, but significant deviations in $P_{a\text{-}ET}CO_2$ were noted in groups with leakage rate 60%.

Conclusion: There was a strong correlation between $P_{ET}CO_2$ and $PaCO_2$ when tidal volume/body weight ratio was 6–15 mL/kg with leakage rate <60%. Lightweight mainstream capnometer with a low amount of dead space airway adaptor might be useful in very low birth weight infants with small tidal volume.

Keywords: capnography, mainstream, neonate

Introduction

Advancements in the treatment of neonatal respiratory failure, including exogenous surfactant,[1,2] inhaled nitric oxide,[3,4] and a growing repertoire of assisted ventilation strategies,[5] have decreased morbidity and mortality rates. Patient monitoring has played a critical role in the safe and effective application of these advanced therapies.

Maintaining partial carbon dioxide pressure ($PaCO_2$) within the desired range by frequent arterial sampling can increase the need for multiple transfusions in the neonatal intensive care unit,[6] so methods for continuous noninvasive monitoring of CO_2 levels would prove extremely useful. Both hypocarbia and hypercarbia are detrimental to extremely low birth weight infants and have been implicated as causative factors in periventricular leukomalacia, intraventricular hemorrhage, and chronic lung disease.[7–9] Critical event analyses have documented hypoxemia secondary to depressed

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respiratory activity as a principal risk factor for near misses and death.[10,11]

Capnography, which displays the level and waveform of CO_2 in exhaled air, provides information on cell metabolism, blood perfusion, and alveolar ventilation[12] with noninvasive indirect methods. Use of end-tidal CO_2 pressure ($P_{ET}CO_2$) for monitoring and as a tool for verifying endotracheal tube position is a common practice in the operating room and in adult and pediatric intensive care units.[12] However, so far capnometers are not used widely in neonates due to issues such as the weight of sensors and water droplets within circuits, dead space, and leakage from tracheal intubation tubes.

These authors developed a new light (6 g) and low dead space (1 mL) airway adapter (YG-213T; Nihon-Kohden Corporation, Tokyo, Japan) for mainstream capnography (Cap-ONE TG-970P; Nihon-Kohden). The objective of this study was to evaluate the correlations between $P_{ET}CO_2$ and $PaCO_2$ in rabbits while changing tidal volume (TV) and leakage volume with this device.

Material and methods

Ten adult Japanese rabbits weighing 2 kg were anesthetized by intramuscular injection of ketamine at 10 mg/kg and xylazine at 5 mg/kg, and a 24 G catheter (JELCO, Smiths Medical Italia Srl, Mirano, Italy) was placed in the ear vein for continuous intravenous infusion of anesthetic agents. Rabbits were placed in a supine position throughout the experiment. After tracheotomy, an endotracheal tube (internal diameter, 2.5 mm; Mallinkrodt, Inc, St Louis, MO) was inserted. A 24 G catheter was placed in the internal carotid artery to allow collection of samples for blood gas analysis and to monitor arterial blood pressure and heart rate using biomedical research system (LEG-1000; Nihon-Kohden). Anesthesia was provided by continuous intravenous infusion of ketamine (5 mg/kg/hour) and paralysis was maintained by continuous administration of pancuronium (0.1 mg/kg/hour). Animals were administered a mixture of 0.45% sterile saline and 10% glucose solution at 3 mL/kg/hour during the experiment. Body temperature was continuously monitored and maintained at 38.5°C–39.5°C using a heating pad.

Rabbits were ventilated mechanically with a time-cycled pressure-limited ventilator (Humming II; Metran Co, Ltd, Saitama, Japan) adjusted to: fraction of inhaled oxygen at 0.6; inspiratory time at 0.6 seconds; and positive end-expiratory pressure at 5 cmH_2O. Peak inspiratory pressure was settled at the TV maintained at TV/body weight (BW) ratios of 6 mL/kg, 7 mL/kg, 10 mL/kg, and 15 mL/kg, as measured by pneumotachography (ARFEL-VR; Aivision Corporation, Tokyo, Japan). Respiratory rate was adjusted to a level at which normocapnia was maintained (41.6 ± 3.8 mmHg). In the investigation of the effects on TV/BW, TV/BW was divided into three groups (6 mL/kg, 10 mL/kg, and 15 mL/kg), and $P_{ET}CO_2$ and $PaCO_2$ was measured.

$P_{ET}CO_2$ was monitored in intubated rabbits by mainstream capnography (Cap-One TG-970P; Nihon-Kohden). Mainstream $P_{ET}CO_2$ was measured via a capnograph connected to the proximal end of the endotracheal tube. Before each blood sampling, an adequate reading of $P_{ET}CO_2$ and a reliable waveform on the mainstream capnograph (continuous steady waveform of expired CO_2 throughout the ventilatory cycle) was taken. Data was continuously recorded on a laptop computer using the software programmed by LabVIEW (National Instruments Corporation, Austin, TX) for each rabbit. $PaCO_2$ was measured from samples withdrawn intermittently from intraarterial lines indwelling in the internal carotid artery (ABL700; Radiometer Medical ApS, Bronshoj, Denmark). The amount of leakage was adjusted by fixation of the tracheal intubation tubes. The leakage ratio was calculated using the following equation:

$$\text{Leakage ratio} = (\text{inspiratory TV} - \text{expiratory TV}) / \text{inspiratory TV } 100 \times 100$$

The end-tidal volume was measured by mainstream capnography (CO2SMO 8100, Fukuda Denshi, Tokyo, Japan) and also leakage rate was calculated with CO2SMO 8100. The respiratory apparatus was set to a TV/BW ratio of 10 mL/kg, then leakage rates were divided into seven groups (0%–10%, 10%–30%, 30%–50%, 50%–60%, 60%–70%, 70%–80%, and 80%–100%), and $P_{ET}CO_2$ and $PaCO_2$ were measured.

$PaCO_2$ was measured with the additional dead space caused by attachment of a CO_2 sensor and additional dead space in the setting of TV/BW ratios of 6 mL/kg, 7 mL/kg, and 10 mL/kg.

Statistical analyses were conducted using SPSS Statistics version 17.0 software (SPSS, Inc, Chicago, IL). To determine whether $P_{ET}CO_2$ values were representative of $PaCO_2$, the relationship between $P_{ET}CO_2$ and $PaCO_2$ was analyzed by simple linear regression. Bland–Altman plots, representing a visual assessment of agreement between two methods of measurement, were created to assess the measurement of $P_{ET}CO_2$. Bland–Altman plots demonstrate "good agreement" not only when differences between methods are consistent across all measurements but also when the differences are small. In a situation in which difference between the two measurements is expected to change based on a third

variable, Bland–Altman plots lose importance. The precision of $P_{ET}CO_2$ measurements and agreement between $P_{ET}CO_2$ and $PaCO_2$ values were assessed by bias ($PaCO_2 - P_{ET}CO_2$), standard deviation, and 95% confidence interval (CI) for the bias. One-factor analysis of variance and the Mann–Whitney U test were used to compare differences between subgroups of TV/BW and leakage ratio. Values of $P < 0.05$ were considered significant. The present study was conducted with the approval of the Institutional Animal Care and Use Committee of Nagano Children's Hospital, Nagano, Japan.

Results

A total of 43 measurements of $P_{ET}CO_2$ and $PaCO_2$ were analyzed. Under conditions of no leakages in the tracheal intubation tubes, $P_{ET}CO_2$ and $PaCO_2$ were significantly correlated ($r^2 = 0.9099$, $P < 0.0001$; Figure 1) and the Bland–Altman plot test showed a mean difference (bias) for $P_{ET}CO_2$ of −0.876 mmHg (Figure 2). The 95% CI for this bias was −1.80–0.04 mmHg. The 95% CI of limits of agreement was −6.74–4.99 mmHg.

In the comparison of differences between $PaCO_2$ and $P_{ET}CO_2$, when setting the TV/BW to 6 mL/kg, 10 mL/kg or 15 mL/kg, no significant differences were observed between the three groups (Figure 3).

Figure 4 shows the effects of additional dead space on $PaCO_2$ under TV conditions of 12 mL, 14 mL, or 20 mL. Under TV conditions of 12 mL and 14 mL (6 mL/kg and 7 mL/kg, respectively), $PaCO_2$ was significantly increased by a dead space increase of only 1 mL, but under TV conditions of 20 mL (10 mL/kg), a dead space increase of 1 mL did not significantly increase $PaCO_2$. $PaCO_2$ thus seemed to be increased by dead space increases representing 7% of TV.

Figure 5 shows the effects of leakage rate on $PaCO_2/P_{ET}CO_2$ at a TV/BW of 10 mL/kg. For all leakage rates <60%, no significant differences were seen between $PaCO_2$ and $P_{ET}CO_2$ compared to the group with a leakage rate of 0%–10%. However, groups with leakage rate 60% showed significant deviations between $PaCO_2$ and $P_{ET}CO_2$.

Regarding the expiratory plateau phase, a relatively constant PCO_2 was maintained when alveolar mixed gas was expired, as shown by the capnogram for 10 mL/kg TV/BW and leakage ratio <60% (Figure 6).

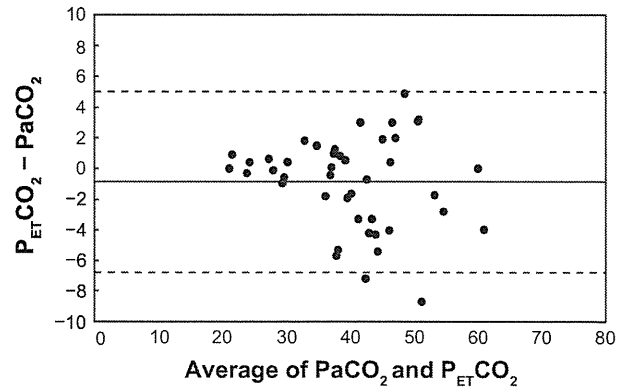

Figure 2 In the Bland–Altman plot test, the mean difference (bias) and standard deviation of differences for end-tidal carbon dioxide pressure ($P_{ET}CO_2$) was −0.876 ± 2.99 mmHg. The 95% confidence interval for bias was −6.74 to 4.99 mmHg.
Abbreviation: $PaCO_2$, partial CO_2 pressure.

Discussion

It was found that $P_{ET}CO_2$ measured by mainstream capnography cap-ONE was an accurate and reliable noninvasive method for estimating $PaCO_2$, showing a good correlation with $PaCO_2$ ($r^2 = 0.9099$, $P < 0.0001$), and there is no bias in the measurement (95% CI: −1.80–0.04 mmHg).

$P_{ET}CO_2$ can be feasibly measured by mainstream or sidestream capnography. Measuring $P_{ET}CO_2$ reportedly underestimates alveolar CO_2 due to the relatively low TVs and rapid respiratory rates of newborns.[13,14] The CO_2 waveform (capnogram) has been characterized for adult patients with normal and abnormal pulmonary function.[15,16] In newborns,

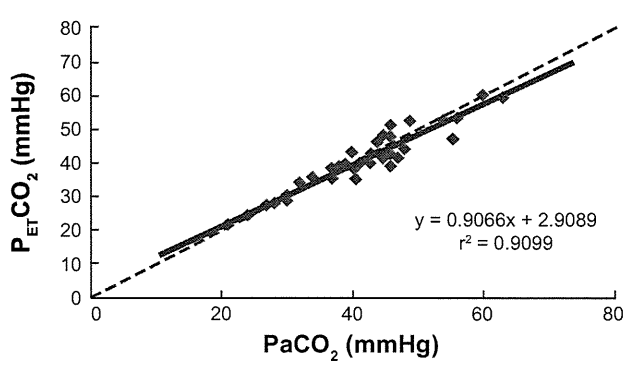

Figure 1 End-tidal carbon dioxide pressure ($P_{ET}CO_2$) and partial CO_2 pressure ($PaCO_2$) were significantly correlated ($r^2 = 0.9099$, $P < 0.0001$).
Note: Dotted line is the line of identity.

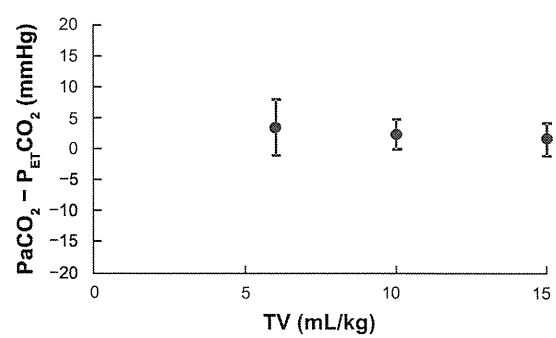

Figure 3 In the comparison of differences between end-tidal carbon dioxide pressure ($P_{ET}CO_2$) and partial CO_2 pressure ($PaCO_2$), no significant differences were observed between the three groups with tidal volume (TV)/body weight ratio of 6 mL/kg, 10 mL/kg, and 15 mL/kg.

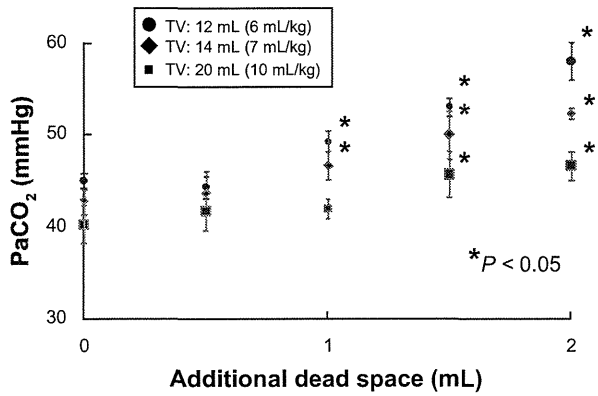

Figure 4 Effects of additional dead space on partial carbon dioxide pressure ($PaCO_2$) under conditions of 12 mL or 20 mL of tidal volume (TV). Under conditions of 12 mL of TV (6 mL/kg), $PaCO_2$ was significantly increased by a dead space increase of only 1 mL. Under conditions of 20 mL of TV (10 mL/kg), even a dead space increase of 1 mL did not significantly increase $PaCO_2$. $PaCO_2$ appears to be affected by a dead space increase of >7% of TV.

CO_2 waveform slurring (a spurious decrease in the slope of the ascending phase of the capnogram) occurs due to dilution of exhaled gas when small, rapid breath packets are measured in a relatively large sample cell.[17]

Condensed water and patient secretions may also impede both mainstream and sidestream technologies. Furthermore, relative inaccuracy is seen in conditions of ventilation-perfusion mismatch.[18,19]

Transcutaneous CO_2 monitoring is a noninvasive technique for measuring CO_2 levels. However, transcutaneous CO_2 monitoring is not tolerated in very low birth weight infants because of their fragile skin, and the technique is affected by acidosis and hypoxia.[20,21]

Mainstream capnography has been found to be more accurate,[22–24] but the sensor position used for mainstream capnography is connected inline between the proximal

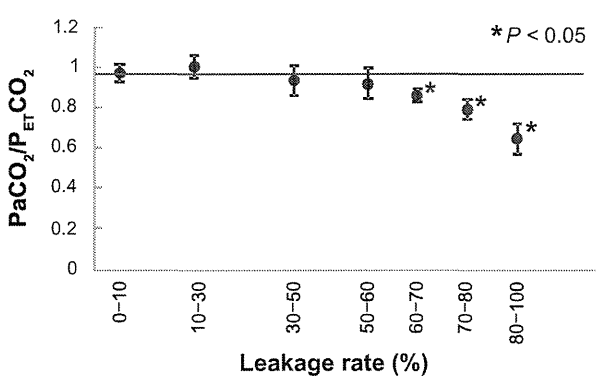

Figure 5 Effects of leakage rate on end-tidal carbon dioxide pressure ($P_{ET}CO_2$) and partial CO_2 pressure ($PaCO_2$). When leakage rate was <60%, no significant differences were seen between $PaCO_2$ and $P_{ET}CO_2$ compared to the group in which the leakage rate was 0%–10%. However, groups with leakage rate 60% showed significant deviations between $PaCO_2$ and $P_{ET}CO_2$.

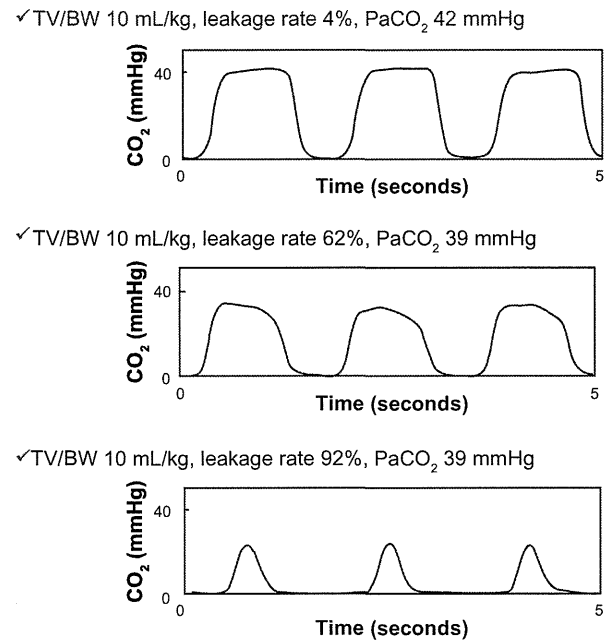

Figure 6 Regarding the expiratory plateau phase, when alveolar mixed gas was expired, a relatively constant partial CO_2 pressure ($PaCO_2$) was maintained, as shown by the capnogram for the >6 mL/kg tidal volume/body weight (TV/BW) group.

endotracheal tube and ventilator circuit. As a result, dead space is increased, the sensor competes for TV, and sensor weight may kink the endotracheal tube. When a flow sensor is connected to the endotracheal tube, use of mainstream capnography is even more cumbersome.[25] Given these limitations, most neonatal intensive care units do not routinely perform capnography to assess and manage ventilatory status. These problems might be reduced in measurement of $P_{ET}CO_2$ if the sensor is lightweight and the dead space is small.

In the comparison of differences between $PaCO_2$ and $P_{ET}CO_2$, when setting the TV/BW to 6 mL/kg, 10 mL/kg, or 15 mL/kg, no significant differences were observed between the three groups even at the 6 mL/kg setting. Conversely, Sakamoto et al reported a lower correlation between $PaCO_2$ and $P_{ET}CO_2$ with ≤6 mL/kg compared to >6 mL/kg in porcine neonates.[26] They measured $P_{ET}CO_2$ by sidestream capnography, so $P_{ET}CO_2$ might have been underestimated.

Figure 4 shows the effects of additional dead space on $PaCO_2$. Under conditions of 6 mL/kg of TV, $PaCO_2$ was significantly increased by a dead space increase of only 1 mL, representing >7% of TV. Figueras et al[27] reported that the introduction of the pneumotachometer, which has a dead space of 1.6 mL for 10 minutes, led to a transcutaneous PCO_2 increase of 5.40 ± 2.66 mmHg, from 39.76 ± 8.69 mmHg to 45.17 ± 9.22 mmHg ($P = 0.0000$) in newborn infants. However, they did not measure $PaCO_2$ directly, nor they did change the amount of dead space when measuring transcutaneous PCO_2.

With respect to lung injury, too high volumes/pressures and/or too low positive end expiratory pressure promotes degradation of the lungs, so-called ventilator-induced lung injury, and is associated with high mortality.[28] Such injuries are known to result in local alterations in lung compliance and pulmonary edema secondary to capillary leakage and are important contributing factors to the pathogenesis of chronic lung disease in neonates.[29,30]

To the best of the authors' knowledge, no studies have described the effects of endotracheal tube leakage on $P_{ET}CO_2$. Leakage is more of a problem in children than in adults because of the use of uncuffed endotracheal tubes. Capnography offers direct monitoring of the inhaled and exhaled concentrations or partial pressure of CO_2. The amount of endotracheal tube leakage around an uncuffed endotracheal tube is thus larger, and significant differences between $PaCO_2$ and $P_{ET}CO_2$ might arise.

No significant differences were found between $PaCO_2$ and $P_{ET}CO_2$ when the leakage rate was <60%, similar to the group in which leakage rate was 0%–10%. However, in groups with a leakage rate 60%, significant deviations were seen between $PaCO_2$ and $P_{ET}CO_2$.

Also, regarding the expiratory plateau phase, a relatively constant PCO_2 was maintained, as shown by the capnogram for 10 mL/kg TV/BW and leakage ratio <60% (Figure 6).

This analysis of rabbits weighing 2 kg revealed good correlation and agreement between $PaCO_2$ and $P_{ET}CO_2$. However, only deep anesthetized rabbits weighing 2 kg were used. In addition, $P_{ET}CO_2$ was measured in rabbits using the setting of limited mechanical ventilation. Further studies using different conditions are warranted to further elucidate this area.

Conclusion

A strong correlation was obtained and there was no bias between $P_{ET}CO_2$ and $PaCO_2$ when TV/BW was 6–15 mL/kg and the leakage rate was <60%. Lightweight and low amounts of dead space capnometer may be used in very low birth weight infants with small TV.

Disclosure

The authors report no conflicts of interest in this work.

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Oral manifestations of patients with hypophosphatasia

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Abstract Hypophosphatasia is a rare inherited disorder characterized by defective bone mineralization and deficiency of tissue non-specific alkaline phosphatase (TNSALP) activity. The disease is caused by mutations in the liver/bone/kidney alkaline phosphatase gene (*ALPL*) encoding TNSALP. As for dental manifestations, premature loss of deciduous teeth due to disturbed cementum formation is well known. However, few reports of multiple cases have been presented. The oral manifestations of patients diagnosed with hypophosphatasia were analyzed by collecting clinical records of cases from a nationwide survey of pediatric dentistry clinics affiliated with 29 university dental hospitals in Japan. We inquired regarding the number of cases and clinical findings of diagnosed patients. We obtained information for 9 children diagnosed with hypophosphatasia from our university and 10 from 6 other universities. The main oral manifestation was early exfoliation of deciduous teeth, which was found in 15 of the 19 cases. Early exfoliation of mandibular deciduous anterior teeth was recognized in 14, whereas there were no cases of early exfoliation of a permanent tooth. The main oral finding of hypophosphatasia was early exfoliation of deciduous teeth, predominantly in the mandibular anterior region of children aged 1 to 4 years old.

Key words
Cementum,
Early exfoliation,
Hypophosphatasia,
Mandibular anterior teeth,
Primary teeth

Introduction

Hypophosphatasia is a rare inherited disorder related to deficiency of tissue non-specific alkaline phosphatase (TNSALP) activity and characterized by defective bone mineralization[1]. The frequency of severe forms of hypophosphatasia has been estimated to be 1 per 100,000 newborns, while mild forms of the disease are considered to be more common[2–4]. Hypophosphatasia is inherited as an autosomal recessive trait, though autosomal dominant inheritance has been reported in some milder cases[5]. The disease is caused by mutations in the liver/bone/kidney alkaline phosphatase gene (*ALPL*) encoding TNSALP[6], with more than 200 mutations in the *ALPL* gene reported[5]. In Japanese patients, the F301L and T1559del mutation types are commonly found in the TNSALP gene[7], of which the former is reported to be associated with relatively mild forms and the latter type with the lethal form[8].

Six clinical forms of hypophosphatasia, perinatal lethal, perinatal benign, infantile, childhood, adult, and odonto-hypophosphatasia, are currently recognized, which are classified based on the age at diagnosis, and the severity of associated signs and symptoms[3,4]. The severity of the disease is generally correlated with the onset period, except for the odonto type[7]. Symptoms in patients with hypophosphatasia range from stillbirth without mineralized bone to isolated premature loss of primary teeth[3,4].

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Table 1 Summary of patients diagnosed with hypophosphatasia

Case	Gender	Phenotype	First and last examination	Early exfoliated or extracted teeth	Genetic analysis
1[a]	F	Perinatal (benign)	2Y7M–14Y3M	A | A	F301L
2[a]	F	Childhood	7Y0M–18Y0M	None	Unknown
3[b]	M	Odonto	3Y0M–8Y9M	A	A23V/E174G
4[b]	M	Childhood	3Y0M–8Y9M	CBA | AB	A23V/E174G
5	M	Perinatal (benign)	8Y8M–12Y4M	None	F301L
6	M	Childhood	2Y2M–3Y9M	C C / CBA | ABC	Unknown
7	M	Odonto	2Y2M–3Y7M	BA | AB	Unknown
8	F	Odonto	4Y5M	A	Unknown
9	F	Perinatal (benign)	1Y9M–4Y4M	C | BA	Unknown
10	F	Odonto	2Y8M–15Y6M	A / CBA | AB	Unknown
11	M	Childhood	3Y10M–4Y8M	B / A | A C	Unknown
12	M	Odonto	5Y2M–23Y11M	None	Exon 10 point mutation
13	F	Odonto[c]	4Y0M–8Y10M	BA | A C / BA | A	E218V/1559delT
14	M	Childhood	1Y5M–7Y	BA / C A | C	Unknown
15	F	Childhood	1Y7M–10Y2M	A / CBA | ABC	Unknown
16	M	Childhood	3Y5M–11Y	CBA | ABC / A | A	Unknown
17	M	Infantile	7Y8M–12Y8M	None	p327L/1559delT
18	M	Childhood	3Y2M–7Y3M	BA | A	Unknown
19	F	Childhood	2Y7M–	AB / BA | AB	Unknown

[a]: Siblings, [b]: Fraternal twins, [c]: Odonto type, possibly childhood type, general information is lacking.

Most common oral manifestations in patients with hypophosphatasia are known to be premature loss of primary teeth due to impaired formation of the cementum, especially in childhood cases and odonto-hypophosphatasia type, with the latter not associated with abnormalities of the skeletal system[3,4]. Histopathological examinations of spontaneously exfoliated teeth have shown the lack of both cellular and acellular cementum[8,9]. In general, hypophosphatasia in childhood is often recognized by pediatric dentists when the patient visits a dentist for early and spontaneous exfoliation of a primary tooth[9].

In the present study, the clinical records of 19 hypophosphatasia cases from 7 pediatric dental clinics of 29 university dental hospitals were examined and the oral manifestations presented are summarized.

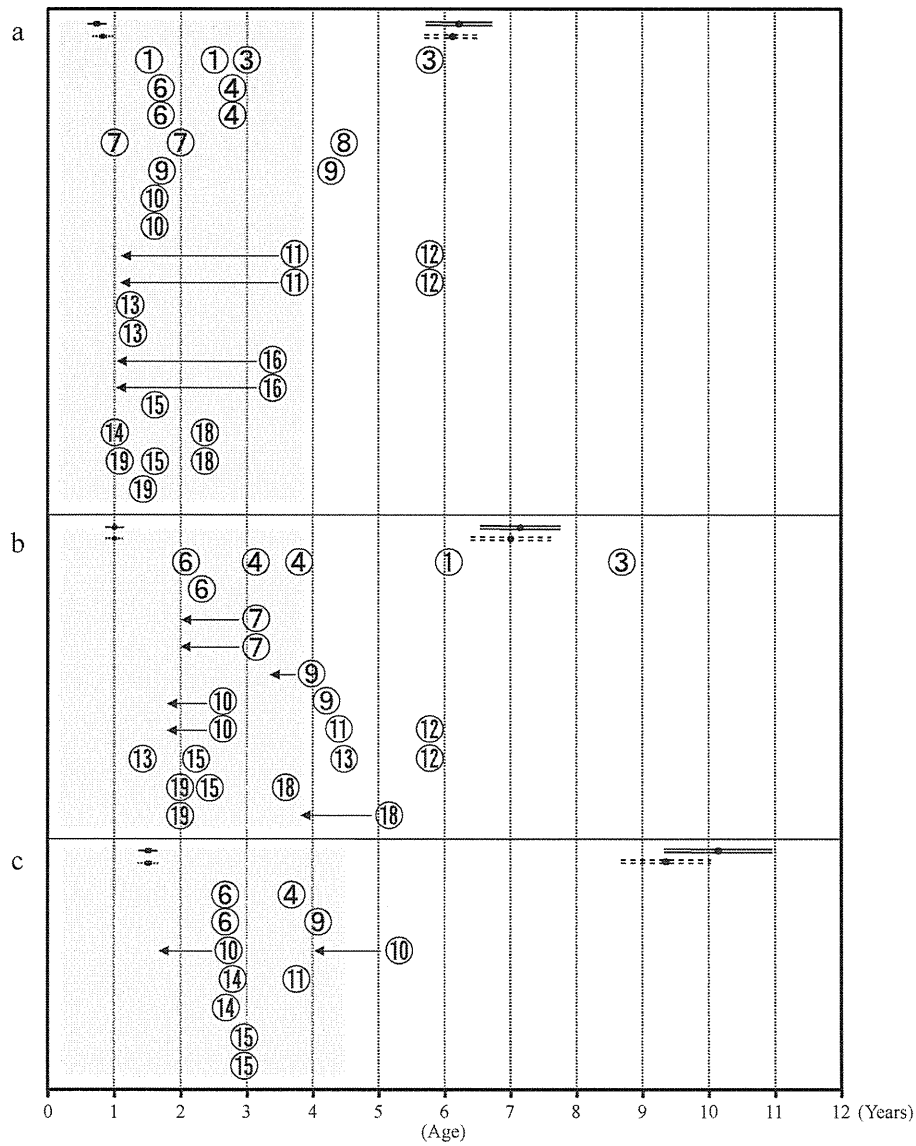

Fig. 1 Time of spontaneous exfoliation of right and left mandibular primary incisors and canines

Numbers in circles indicate case numbers. Arrows indicate estimated periods of exfoliation. Bold line indicates the mean time (center dot) of emergence of mandibular primary incisors and canines in boys, with an SD of 1. The double bold line indicate the same for the permanent successors and the center dot indicates the mean. Bold dotted and double-dotted lines indicate results for girl patients. Shadow indicates the term within 3 years after eruption of the primary tooth.
a: Central incisors, b: Lateral incisors, c: Canines.

Subjects and Methods

Collection of subjects with hypophosphatasia

There were 9 children diagnosed with hypophosphatasia at our clinic, with the clinical oral features of 4 of those previously reported (Cases 1 to 4 in Table 1)[10]. In order to collect information from additional cases, we contacted clinics of pediatric dentistry at 28 other university dental hospitals in Japan and inquired regarding the existence of patients diagnosed with hypophosphatasia. In reply, we received information for 10 children from 6 universities, thus the total number of cases analyzed was 19 (11 males, 8 females). There were no cases reported from the other 22 university dental hospitals contacted.

Clinical analyses

The patient information was summarized in regard to gender, phenotype, chronological age at the

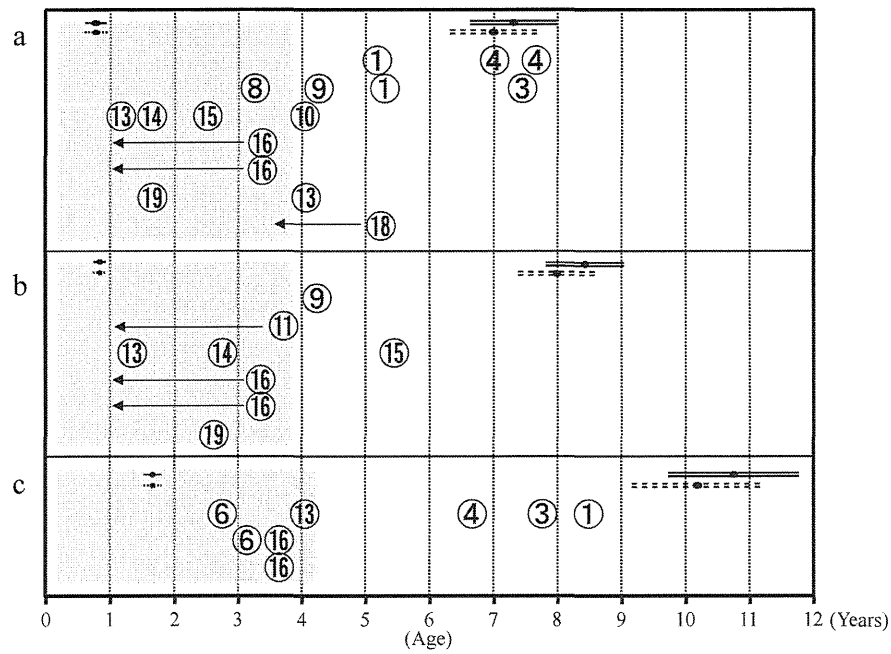

Fig. 2 Time of spontaneous exfoliation of right and left maxillary primary incisors and canines

Numbers in circles indicate case numbers. Arrows indicate estimated periods of exfoliation. Bold line indicates the mean time (center dot) of emergence of mandibular primary incisors and canines in boys, with an SD of 1. The double bold line indicate the same for the permanent successors and the center dot indicates the mean. Bold dotted and double-dotted lines indicate results for girl patients. Shadow indicates the term within 3 years after eruption of the primary tooth.
a: Central incisors, b: Lateral incisors, c: Canines.

time of the first and last examinations, location of spontaneous early exfoliated teeth, and genotypes. An early exfoliated or extracted tooth was defined as a primary tooth exfoliated or extracted because of periodontal disease within 3 years after eruption of the primary tooth, which was determined from the mean eruption time of primary teeth in Japanese children[11].

Results

Phenotypes

The most frequent phenotype seen in the present study was the childhood type (9 cases), followed by odonto (n=6), perinatal benign (n=3), and infantile (n=1) types. In addition, general information was not available for 1 of the patients (Case 13), who was classified as childhood type.

Oral manifestations

Early exfoliated or extracted primary teeth were found in 15 cases (Table 1). Most of those were identified in the anterior region, while there were no cases of early exfoliated or extracted permanent teeth.

The time of early exfoliation of primary anterior teeth is illustrated in Figs. 1 and 2, with findings for the right and left mandibular primary incisors and canines shown in Fig. 1. Early exfoliation of the mandibular primary central incisors before 4 years of age was seen in 74% (14/19 cases) of all cases and comprised 66% (25/38 teeth; 2 teeth per case) of all exfoliated teeth. In addition, the rate of early exfoliation of mandibular primary lateral incisors was 47% of all cases and comprised 40% of the total number of mandibular primary central incisors. On the other hand, early exfoliation of the mandibular primary canines before 4.5 years of age was seen in 37% of all cases and comprised 26% of all exfoliated teeth. Figure 2 shows the period of time required for spontaneous exfoliation of the right and left maxillary primary incisors and canines. Early exfoliation of maxillary primary anterior teeth was seen in 47% of all cases and comprised 18% of all exfoliated teeth. In addition, the abnormalities of the numbers and the shape of teeth were not observed in all cases.

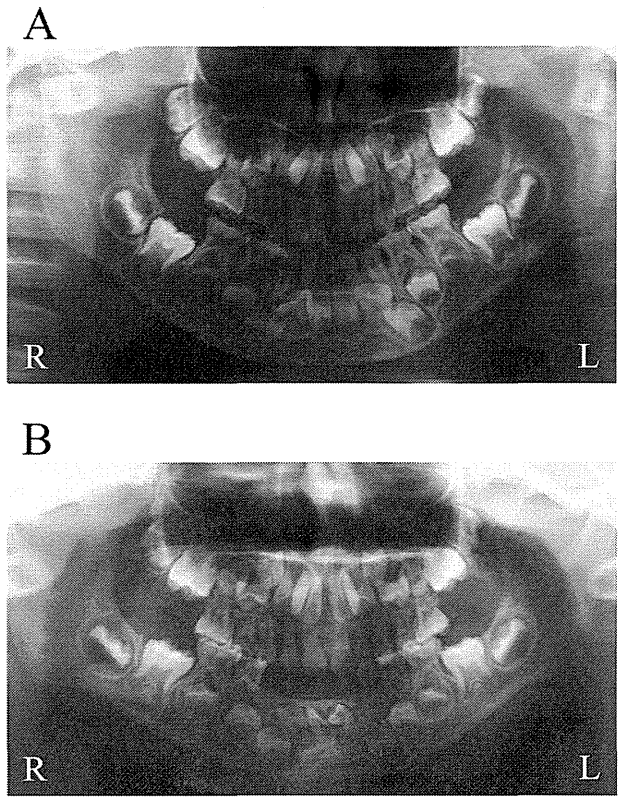

Fig. 3 Orthopantomograph of fraternal twins obtained at 6 years 3 months of age
(A) Case 3, (B) Case 4.

Genetic findings

Information from results of genetic analyses was available for 7 cases and is summarized in Table 1. The F301L mutation type was associated with 2 perinatal lethal cases (Cases 1 and 5). In those, 2 early exfoliated teeth were noted in Case 1, whereas no teeth were spontaneously exfoliated in Case 5. In addition, an A23V/E174G type mutation was found in fraternal twins (Cases 3 and 4) (Fig. 3), in whom a mandibular central incisor (Case 3) and 5 primary mandibular anterior teeth (Case 4) were exfoliated. Furthermore, an E218V/1559delT type mutation was identified in Case 13, which showed 7 early exfoliated teeth, while a p327L/1559delT type mutation was identified in case 17 without early exfoliated teeth.

Discussion

The frequency of severe forms of hypophosphatasia in the general population is estimated to be 1 per 100,000 individuals[2,6], whereas that of moderate forms is expected to be much higher due to the number of patients with dominant forms of the disease[2,3,12]. In the present study, we analyzed 19 cases of hypophosphatasia from information collected at pediatric dentistry clinics of 29 university hospitals throughout Japan including ours. However, 9 of the cases were treated at our medical hospital by pediatricians engaged in management of child patients with hypophosphatasia. We consider it important to collaborate with medical doctors when treating children with hypophosphatasia, since oral manifestations can be identified in most of these patients. Patients in this study referred to our clinic were treated as soon as they were diagnosed with hypophosphatasia by a pediatrician. In addition, a few of the patients were suspected to have hypophosphatasia based on findings in our dental clinic and referred to a pediatrician.

It is generally known that one of the common findings of this disease is early spontaneous exfoliation of primary teeth and the present analysis revealed that exfoliation of a primary incisor was identified by 4 years of age at a frequency of 74%. Since exfoliation of a primary incisor is generally observed at around 6 years of age, early exfoliation occurring at an age younger than 4 years may be an indicator of hypophosphatasia for general dentists as well as medical doctors.

A feature of premature loss of primary teeth is considered to be derived from disturbed cementum formation[13–25]. The present findings showed that early exfoliated teeth generally occur in the area of the primary central incisors, especially the mandibular primary central incisors, which are the first to erupt into the oral cavity among primary dentition. The primary incisors are small in size and have a single root as a morphological feature. In addition, a short time after their eruption, the mandibular central incisors receive high levels of occlusal force from peripheral soft tissues. Although incisors with intact cementum can accept such pressure, those with impaired cementum, such as that seen in hypophosphatasia patients, cannot accept high pressure levels, possibly resulting in irreversible impairment of periodontal tissues and induction of bone loss.

We found no cases of early exfoliated permanent teeth in this study, though a few case reports have described such affected permanent teeth[26–29]. In those cases, in addition to the primary incisors and canines, the primary molars were also exfoliated

before the permanent teeth began to erupt. Hence, a few primary teeth remain in the oral cavity when the permanent incisors and/or first molars erupt into the oral cavity. As with the mandibular central incisors, these permanent teeth in patients with hypophosphatasia also receive high levels of pressure, which may induce early exfoliation. To prevent early exfoliation of permanent teeth in hypophosphatasia patients, a denture should be applied to reduce pressure before the permanent teeth begin to erupt.

To our knowledge, no effective approaches for early exfoliation of primary teeth in cases with hypophosphatasia have been presented. Thus, it is important to pay special attention to changes in periodontal conditions to discern the onset of periodontitis as early as possible. It is generally considered that periodontitis in children is a quite rare clinical finding, though cases of gingivitis are commonly found[30]. When conditions similar to periodontitis are found in these patients, it is important to pay special attention to prevent the development of lesions. Since impaired cementum tissue may produce favorable sites for colonization of periodontitis-related bacteria[10], maintenance of oral hygiene is most vital. In the present study, we noted predominant periods for early spontaneous exfoliation of primary teeth, which might be also important for monitoring the periodontal conditions of patients with hypophosphatasia.

When cases with early exfoliation of primary teeth are encountered, application of a partial denture is recommend to disperse occlusal pressure and resolve esthetic problems. However, it is possible that the wire clasps of a partial denture may impose a severe burden on the remaining primary teeth. Therefore, it is important to confirm the mobility of teeth with clasps during periodic examinations, otherwise a non-clasp denture may be more suitable.

Presently, there is no radical treatment recommended for cases of hypophosphatasia. On the other hand, several possible treatments have been presented, including enzyme replacement therapy using the serum of a patient with Paget's disease[31], administration of parotoid hormone[32,33], transplantation of bone fragments and cultured osteoblasts[34], and allogenic mesenchymal stem cell transplantation[35]. Furthermore, animal experiments using *TNALP* knockout mice, a model of infantile hypophosphatasia[36], showed that enzyme replacement therapy with a deca-aspartate-tagged enzyme was successful[37]. That therapy also prevented hypomineralization of alveolar bone, dentin, and cementum[38]. Clinical trials with this modified enzyme are now in progress, though the therapy requires repeated administrations of large amounts of enzymes for long-term correction[5]. Gene therapy by means of a single injection may prove to be a better treatment. In previous studies, *TNALP* knockout mice were treated with lentivirus gene therapy[39] and adeno-associated virus serotype 8 mediated gene therapy[40]. Accumulation of such experimental findings may result in novel approaches for patients with hypophosphatasia in the near future.

Four of the present cases with different types of disease did not show early exfoliation of primary teeth. However, the present results did not reveal information regarding the relationships between early exfoliation and subtypes. The two most common types of mutations for *ALPL* (F301L and T1559delT) are reported to be associated with relatively mild and lethal forms, respectively, of hypophosphatasia in Japanese patients[7]. That study also noted that the genotype-phenotype relationship is consistent with the enzymatic activities of mutant ALP proteins. Furthermore, patients with F301L type were found to retain some residual activities of ALP, whereas those with the T1559del type had a complete loss of those activities. In addition, they showed that F301L is associated with relatively mild forms of hypophosphatasia, whereas T1559 is associated with lethal forms. In the present study, the F301L mutation was detected in Cases 1 and 5 with the perinatal benign type, though their dental phenotypes were different. In addition, Cases 3 and 4, fraternal twins, had the same mutation, while their dental phenotypes were completely different. It was previously reported that the relationships between phenotypes and genotypes are not fully understood[5], and additional studies are required.

Child onset hypophosphatasia is often recognized first by pediatric dentists, who are generally consulted for premature spontaneous exfoliation of fully rooted primary teeth[9]. In the present study, most of the cases of child and odonto types treated at our clinic were suspected to be hypophosphatasia based on dental findings. Pediatric dentists should refer patients suspected to have hypophosphatasia based on premature exfoliation of a primary anterior tooth to a pediatrician as early as possible for additional examinations.

Conclusion

The main oral finding of hypophosphatasia was early exfoliation of deciduous teeth, predominantly in the mandibular anterior region, at the age of 1 to 4 years old. Pediatric dentists should investigate the possibility of hypophosphatasia when an uncommon case of early exfoliation of a primary tooth is encountered in a young patient.

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ORIGINAL RESEARCH

Skeletal Analysis of the Long Bone Abnormality (*lbab/lbab*) Mouse, A Novel Chondrodysplastic C-Type Natriuretic Peptide Mutant

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Abstract Long bone abnormality (*lbab/lbab*) is a strain of dwarf mice. Recent studies revealed that the phenotype is caused by a spontaneous mutation in the *Nppc* gene, which encodes mouse C-type natriuretic peptide (CNP). In this study, we analyzed the chondrodysplastic skeletal phenotype of *lbab/lbab* mice. At birth, *lbab/lbab* mice are only slightly shorter than their wild-type littermates. Nevertheless, *lbab/lbab* mice do not undergo a growth spurt, and their final body and bone lengths are only ~60% of those of wild-type mice. Histological analysis revealed that the growth plate in *lbab/lbab* mice, especially the hypertrophic chondrocyte layer, was significantly thinner than in wild-type mice. Overexpression of CNP in the cartilage of *lbab/lbab* mice restored their thinned growth plate, followed by the complete rescue of their impaired endochondral bone growth. Furthermore, the bone volume in *lbab/lbab* mouse was severely decreased and was recovered by CNP overexpression. On the other hand, the thickness of the growth plate of *lbab/+* mice was not different from that of wild-type mice; accordingly, impaired endochondral bone growth was not observed in *lbab/+* mice. In organ culture experiments, tibial explants from fetal *lbab/lbab* mice were significantly shorter than those from *lbab/+* mice and elongated by addition of 10^{-7} M CNP to the same extent as *lbab/+* tibiae treated with the same dose of CNP. These results demonstrate that *lbab/lbab* is a novel mouse model of chondrodysplasia caused by insufficient CNP action on endochondral ossification.

Keywords C-type natriuretic peptide · Long bone abnormality (*lbab*) · Chondrodysplasia · Endochondral bone growth · Organ culture

C-type natriuretic peptide (CNP) is a member of the natriuretic peptide family and exerts its biological actions through the accumulation of intracellular cyclic GMP via a subtype of membranous guanylyl cyclase receptor, guanylyl cyclase-B (GC-B) [1, 2]. We previously demonstrated that the CNP/GC-B system is a potent stimulator of endochondral bone growth: transgenic mice with targeted overexpression of CNP in cartilage under the control of type II collagen promoter [3] or those with elevated plasma CNP concentrations under the control of human serum amyloid P component promoter [4] exhibit a prominent skeletal overgrowth phenotype. On the other hand, the physiological importance of the CNP/GC-B system on endochondral bone growth has been revealed by the phenotypes of hypomorphs. We generated complete CNP or GC-B null mice and demonstrated that they exhibit an impaired bone growth phenotype [5, 6]. We have also reported that in two lines of spontaneous mutant mice, *cn/cn* and *slw/slw*, disproportionate dwarfism is caused by loss-of-function mutations in the murine GC-B gene [7, 8].

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The skeletal phenotypes of these mutant mice resemble those of GC-B knockout mice. Furthermore, recent studies have elucidated that loss-of-function mutations in the human GC-B gene are the causes of acromesomelic dysplasia type Maroteaux (AMDM), one form of skeletal dysplasia with a disproportionate short stature phenotype [9]. The impaired skeletal growth phenotype observed in patients suffering from AMDM is similar to the skeletal phenotype of *cn/cn*, *slw/slw*, and GC-B knockout mice.

The long bone abnormality (*lbab/lbab*) mouse was first identified in The Jackson Laboratory (Bar Harbor, ME) as a spontaneous autosomal recessive mutant characterized by impaired growth of the long bones [10]. Recent studies have elucidated that the impaired growth of *lbab/lbab* mice is caused by a hypomorphic mutation in the CNP gene; Jiao et al. [11] found that its impaired growth phenotype is associated with a single point mutation in the mouse CNP gene, and we showed that this phenotype is completely recovered by CNP overexpression [12]. Yoder et al. [13] characterized the mutant CNP in *lbab/lbab* mice and demonstrated that it is less biologically active than authentic CNP; in whole-cell cGMP elevation and membrane guanylyl cyclase assays, 30-fold to greater than 100-fold more mutant CNP is required to activate GC-B compared to authentic CNP. We also confirmed that the mutant CNP in *lbab/lbab* mice retains only about 10% activity to induce cyclic GMP production through GC-B compared to authentic CNP in an in vitro transfection assay using COS-7 cells [12]. Collectively, *lbab/lbab* is a novel chondrodysplastic mouse model with insufficient CNP action on endochondral bone growth. Nevertheless, the skeletal phenotypes of *lbab/lbab* mice have only been partially described in short reports, including our own brief communication [11–13], and have not yet been fully studied. In this study, we performed further analyses of the skeletal phenotypes of *lbab/lbab* mice.

Materials and Methods

Mice

Heterozygous (*lbab/+*) mice (C57BL/6 J background) were obtained from The Jackson Laboratory, and the strain was maintained by sib mating of heterozygotes. Transgenic mice with targeted overexpression of CNP in the growth plate chondrocytes under the control of the mouse pro-α_1(II) (*Col2a1*) promoter (CNP-Tg) were created as reported previously [3]. To perform genetic rescue of *lbab/lbab* mice, CNP-Tg mice were mated with *lbab/+* mice, and F_1 offspring heterozygous for the transgene and for the *lbab* allele were mated with those with only the *lbab* allele to generate *lbab/lbab* mice with the transgene expression (*lbab/lbab*·CNP-Tg/+ mice) [12]. Genotypes for the CNP transgene and the *lbab* allele were determined by PCR analysis using mouse genomic DNAs extracted from tails. Because there was no tendency of gender differences in the growth of each genotype (data not shown), we used only female mice in our experiments. Animal care and all experiments were conducted in accordance with the Guidelines for Animal Experiments of Kyoto University and were approved by the Animal Research Committee, Graduate School of Medicine, Kyoto University.

Skeletal Analysis

For 10 weeks after birth, body lengths of female mice were measured weekly. Body length was measured as the length from the nose to the anus (nasoanal length) or that from the nose to the tip of the tail (nose–tail length). Body weights were also measured weekly. Skeletal analysis was performed as previously described [14]. Briefly, mice were subjected to soft X-ray analysis (30 kVp, 5 mA for 1 min; Softron type SRO-M5; Softron, Tokyo, Japan), and lengths of the bones were measured on the X-ray films. CT scanning of the humerus was performed using a ScanXmate-L090 Scanner (Comscantechno, Yokohama, Japan). Three-dimensional microstructural image data were reconstructed and structural indices calculated using TRI/3D-BON software (RATOC System Engineering, Tokyo, Japan).

Histological Examination

Tibiae were fixed in 10% formalin neutral buffer, decalcified in 10% EDTA, and embedded in paraffin. Sections (5 μm thick) were sliced and stained with alcian blue (pH 2.5) and hematoxylin–eosin. For immunohistochemistry, sections were incubated with rabbit anti-type X collagen antibody (LSL, Tokyo, Japan), goat anti-Indian hedgehog (Ihh) antibody (Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-matrix metalloproteinase 13 (MMP-13) antibody (Thermo Fisher Scientific, Waltham, MA), and mouse anti–proliferating cell nuclear antigen (PCNA) antibody (Dako, Copenhagen, Denmark). Immunostaining was performed using the Histofine Mousestain Kit (Nichirei Biosciences, Tokyo, Japan) according to the manufacturer's instruction. Peroxidase activity was visualized using diaminobenzidine. Sections were counterstained with hematoxylin, dehydrated, and then mounted with malinol (Muto Pure Chemicals, Tokyo, Japan). To confirm antibody specificity, normal rabbit serum (Sigma-Aldrich, St. Louis, MO), normal goat IgG (Santa Cruz Biotechnology), and mouse IgG (Dako) were used as first antibodies for negative controls.

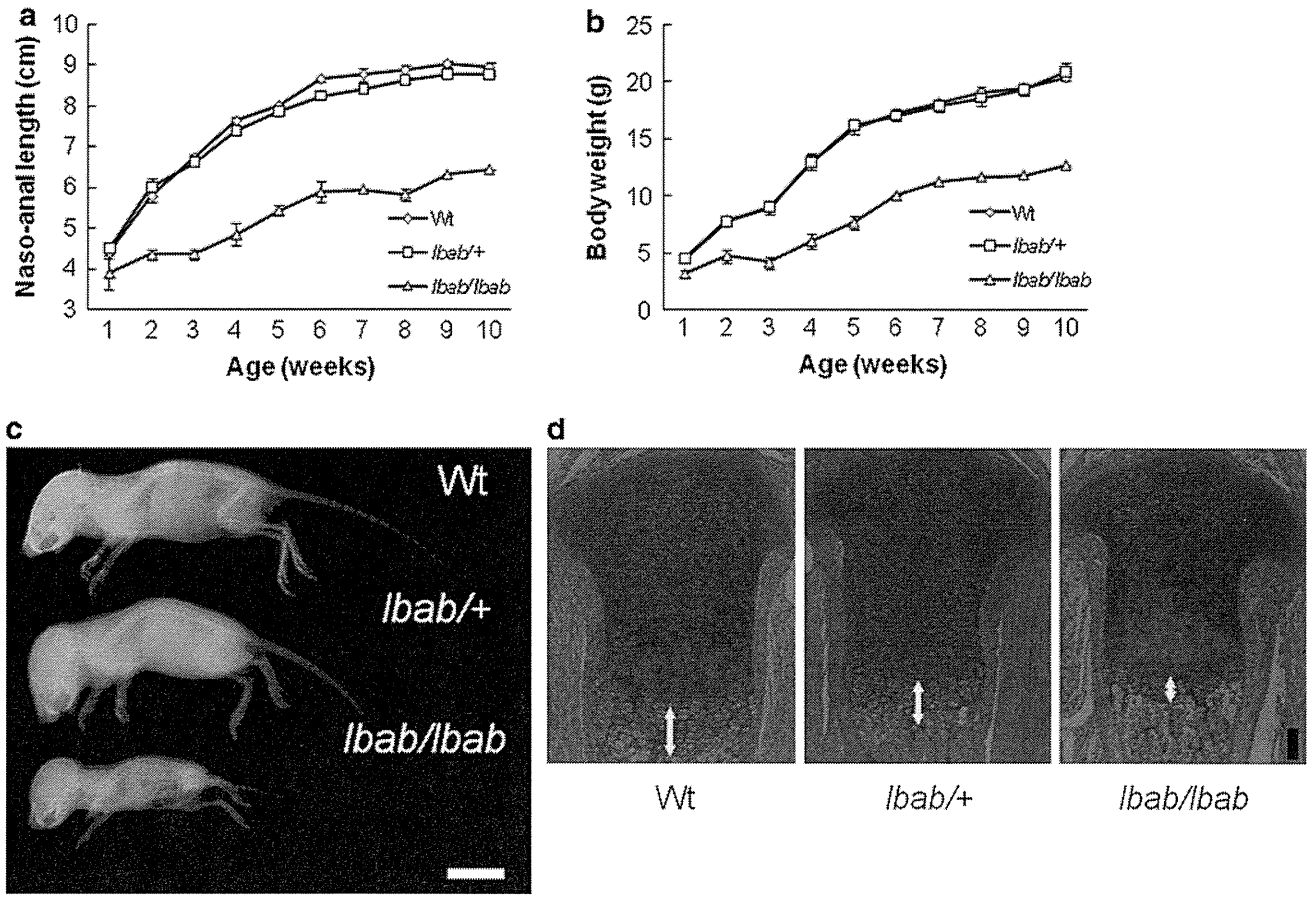

Fig. 1 Growth and skeletal phenotype of *lbab/+* and *lbab/lbab* mice. Nasoanal lengths (**a**) and body weights (**b**) of female wild-type (*Wt, open diamond*), *lbab/+* (*open square*), and *lbab/lbab* (*open triangle*) mice ($n = 2$–8). **c** Whole skeletons of wild-type, *lbab/+* , and *lbab/lbab* mice at 2 weeks of age. *Scale bar* 1 cm. **d** Histological analysis of the tibial growth plates of 3-day-old mice. *Arrows* indicate hypertrophic chondrocyte layers. Alcian blue and hematoxylin–eosin staining. *Scale bar* 100 μm

Organ Culture

Organ culture of fetal mouse tibiae or third metatarsi was performed as described previously [15]. Tibial or metatarsal explants from *lbab/+* mice and their *lbab/lbab* littermates at 16.5 days postcoitus were cultured for 4 days with vehicle or 10^{-7} M CNP (Peptide Institute, Minoh, Japan). Medium was changed every day. Before and after the culture, the maximal longitudinal lengths of tibiae were measured as the total tibial length, the sum of the lengths of proximal and distal cartilaginous primordia (CP), and the length of the osteogenic center (OC), using a linear ocular scale mounted on an inverted microscope. For histological analysis, explants were fixed in 10% formalin neutral buffer and embedded in paraffin. Sections (5 μm thick) were sliced and stained with alcian blue (pH 2.5) and hematoxylin–eosin. Immunohistochemical staining of incorporated bromodeoxyuridine (BrdU) was performed using 5-Bromo-2′-deoxyuridine labeling and detection kit II (Roche Applied Science, Indianapolis, IN) according to the manufacturer's protocol.

Statistical Analysis

Data were expressed as the mean ± SEM. The statistical significance of differences between mean values was assessed using Student's *t*-test.

Results

Analyses of Skeletal Growth of *lbab/lbab* and *lbab/+* Mice

As previously reported, *lbab/lbab* mice developed severe dwarfism characterized by short tails and extremities [11, 12]. At birth, *lbab/lbab* pups were slightly shorter than their wild-type littermates: the nasoanal and nose–tail lengths of *lbab/lbab* mice were 88 and 83% of those of