

unidentified signaling pathways and also that hydrogen may functionally interact with other gaseous signaling molecule, NO, carbon monoxide (CO) and hydrogen sulfide (H₂S). In an attempt to corroborate our hypotheses, we focused on inflammation because of the following reasons. First, it has been reported that hydrogen alleviates several inflammatory diseases [10,11], the mechanism of which, however, remains unknown. Second, in the inflammatory processes, LPS/IFN γ -induced activation of signal transduction leads to expression of iNOS, resulting in up-regulation of NO.

In the present study, we demonstrated that hydrogen suppresses LPS/IFN γ -induced NO release from macrophage cells (Fig. 1A), which was due to inhibition of LPS/IFN γ -induced expression of iNOS (Fig. 1B and C). We also revealed that LPS/IFN γ -induced phosphorylation of p38, JNK and I κ B α was specifically suppressed by hydrogen (Fig. 2A and B). These results suggest that hydrogen may reduce binding to the iNOS promoter of several transcription factors such as AP1 and NF κ B via inhibition of signal transduction.

Since our results showed that LPS/IFN γ -induced activation of p38 and JNK is inhibited by hydrogen, we investigated the effects of hydrogen on phosphorylation of ASK1, which is an upstream signaling molecule of both kinases [13]. Indeed, ASK1 phosphorylation was inhibited by hydrogen (Fig. 2C). We also asked if hydrogen directly inhibits phosphorylation of ASK1 *in vitro*, but found that ASK1 phosphorylation is not changed by the presence of hydrogen (Supplementary Fig. 1). On the other hand, ROS-dependent activation of the TRAF6–ASK1–p38 pathway is selectively required for TLR4-mediated innate immunity [15]. In addition, ASK1 is an important effector of NOX in the redox signaling [16]. We thus examined the effects of hydrogen on LPS/IFN γ -induced activation of NOX and subsequent production of ROS, but neither of them was affected by hydrogen treatment (Fig. 3).

In addition to ASK1, hydrogen strongly inhibited the NF κ B signaling (Fig. 2B) as represented by a marked decrease in LPS/IFN γ -induced expression of COX2 as well as iNOS (Fig. 1B). In TLR4 signaling, LPS induces interaction of TLR4 with TRAF6, an E3 ubiquitin–protein ligase, and promotes TRAF6 auto-ubiquitination, leading to ubiquitination and activation of TAK1 [18]. TAK1 phosphorylates I κ B kinases, which in turn phosphorylate I κ B, resulting in I κ B degradation and NF κ B translocation into the nucleus. TAK1 also activates JNK and p38 MAP kinases by phosphorylating MKK4/7 and MKK3/6, respectively. We therefore investigated if hydrogen could inhibit LPS/IFN γ -induced phosphorylation of TAK1, but TAK1 activation was not affected by treatment with hydrogen (Fig. 2C).

Taken together, we demonstrated that hydrogen inhibits LPS/IFN γ -induced phosphorylation of ASK1 and its downstream signaling molecules (p38 and JNK) as well as I κ B α in macrophage cells. Although we were unable to identify the exact molecule(s) that hydrogen directly modulates, we could narrow down the potential target sites. In TLR4 signaling, after formation of the LPS/CD14/TLR4 ligand receptor complex, MyD88 and IRAK as well as TRAF6 and TAK1 are recruited, and their interaction mediates downstream signaling. Our studies suggest that hydrogen modulates molecular events at the receptor or immediately downstream of it. In type I allergy, although the presence of the feed-forward loop in the Fc ϵ RI-mediated signal transduction prevented us from identifying direct target(s) of hydrogen, we proposed as a plausible mechanism that hydrogen may compromise the initial step of signal transduction, phosphorylation of Lyn kinase [3]. Together with the findings from the present studies, it is tempting to speculate that molecular hydrogen acts at or around the receptors. Nevertheless, the hypothesis remains to be proved by further studies.

In cultured macrophage cells, we showed that hydrogen inhibits TLR4 signaling that plays a critical role in induction of pro-inflammatory genes, providing the molecular bases for the hydrogen effects on inflammatory diseases. Finally, we studied

in vivo effects of hydrogen on inflammation using a mouse model for human rheumatoid arthritis. It was found that oral intake of hydrogen-rich water suppresses inflammation and ameliorates anti-type II collagen antibody-induced arthritis (Fig. 4). Although we and others have demonstrated beneficial effects of hydrogen on inflammatory diseases in animal models, its efficacy in humans needs to be established in clinical trials.

We confirmed that hydrogen is capable of modulating signal transduction and suggested a role for molecular hydrogen as a signal modulator. Since a number of functional interactions among NO, CO and H₂S have been reported [19], it is conceivable that hydrogen may interact with other gas molecules. Indeed, we demonstrated a functional interaction between NO and hydrogen and elucidated the underlying mechanisms. Future studies will provide information about interactions among four gaseous signaling molecules and their physiological significance.

Competing interest statement

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.06.116.

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RESEARCH

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Open-label trial and randomized, double-blind, placebo-controlled, crossover trial of hydrogen-enriched water for mitochondrial and inflammatory myopathies

Mikako Ito^{1†}, Tohru Ibi^{2†}, Ko Sahashi³, Masashi Ichihara⁴, Masafumi Ito⁵ and Kinji Ohno^{1*}

Abstract

Background: Molecular hydrogen has prominent effects on more than 30 animal models especially of oxidative stress-mediated diseases and inflammatory diseases. In addition, hydrogen effects on humans have been reported in diabetes mellitus type 2, hemodialysis, metabolic syndrome, radiotherapy for liver cancer, and brain stem infarction. Hydrogen effects are ascribed to specific radical-scavenging activities that eliminate hydroxyl radical and peroxynitrite, and also to signal-modulating activities, but the detailed molecular mechanisms still remain elusive. Hydrogen is a safe molecule that is largely produced by intestinal bacteria in rodents and humans, and no adverse effects have been documented.

Methods: We performed open-label trial of drinking 1.0 liter per day of hydrogen-enriched water for 12 weeks in five patients with progressive muscular dystrophy (PMD), four patients with polymyositis/dermatomyositis (PM/DM), and five patients with mitochondrial myopathies (MM), and measured 18 serum parameters as well as urinary 8-isoprostane every 4 weeks. We next conducted randomized, double-blind, placebo-controlled, crossover trial of 0.5 liter per day of hydrogen-enriched water or placebo water for 8 weeks in 10 patients with DM and 12 patients with MM, and measured 18 serum parameters every 4 weeks.

Results: In the open-label trial, no objective improvement or worsening of clinical symptoms was observed. We, however, observed significant effects in lactate-to-pyruvate ratios in PMD and MM, fasting blood glucose in PMD, serum matrix metalloproteinase-3 (MMP3) in PM/DM, and serum triglycerides in PM/DM. In the double-blind trial, no objective clinical effects were observed, but a significant improvement was detected in lactate in MM. Lactate-to-pyruvate ratios in MM and MMP3 in DM also exhibited favorable responses but without statistical significance. No adverse effect was observed in either trial except for hypoglycemic episodes in an insulin-treated MELAS patient, which subsided by reducing the insulin dose.

Conclusions: Hydrogen-enriched water improves mitochondrial dysfunction in MM and inflammatory processes in PM/DM. Less prominent effects with the double-blind trial compared to the open-label trial were likely due to a lower amount of administered hydrogen and a shorter observation period, which implies a threshold effect or a dose-response effect of hydrogen.

* Correspondence: ohnok@med.nagoya-u.ac.jp

† Contributed equally

¹Division of Neurogenetics, Center for Neurological Diseases and Cancer, Nagoya University Graduate School of Medicine, 65 Tsurumai, Showa-ku, Nagoya 466-8550, Japan

Full list of author information is available at the end of the article

Background

Ohsawa and colleagues first reported an effect of hydrogen gas on cerebral infarction in June 2007 [1]. Effects of hydrogen administered in the forms of inhaled gas, drinking water, instillation, and intraperitoneal injection have been reported for 31, 4, and 5 diseases in animal models, cells, and humans, respectively [2]. Hydrogen exhibits prominent effects especially on oxidative stress-mediated diseases and inflammatory diseases in rodents. Hydrogen scavenges hydroxyl radicals and less efficiently peroxynitrite [1]. The radical-scavenging activities, however, are unlikely to be an exclusive mechanism, because the amount of radical oxygen species generated in rodents and humans is much more than the amount of hydrogen molecules taken up by the body. Indeed, the amount of hydrogen taken up by drinking hydrogen-enriched water (HEW) is 100 or more times less than that by inhaling 2% hydrogen gas, but drinking HEW exhibits beneficial effects as good as or even better than inhaling 2% hydrogen gas in rodents [2-4], which suggests the lack of a simple dose-response effect. Our previous study on type 1 allergy also indicates that hydrogen suppresses type 1 allergy by acting as a gaseous signal modulator not as a radical scavenger [5].

Effects of hydrogen in humans have been examined in five studies. First, a randomized, double-blind, placebo-controlled crossover study of 900 ml/day of HEW for 8 weeks in 30 patients with diabetes mellitus type 2 demonstrated significant decreases of electronegative charge-modified LDL cholesterol, small dense LDL, and urinary 8-isoprostanes [6]. Second, an open-label trial of electrolyzed hydrogen-enriched hemodialysis solution in 9 patients for 4 months [7] and 21 patients for 6 months [8] showed significant decreases of systolic blood pressure before and after dialysis, as well as of plasma monocyte chemoattractant protein 1 and myeloperoxidase. Third, an open-label trial of 1.5-2.0 liters per day of HEW for 8 weeks in 20 subjects with metabolic syndrome exhibited a 39% increase of urinary superoxide dismutase (SOD), a 43% decrease of urinary thiobarbituric acid reactive substances (TBARS), an 8% increase of high density lipoprotein (HDL)-cholesterol, and a 13% decrease of total cholesterol/HDL-cholesterol ratio [9]. Fourth, a randomized placebo-controlled study of 1.5-2.2 liters/day of HEW for 6 weeks in 49 patients receiving radiotherapy for malignant liver tumors showed marked improvements of QOL scores [10]. As the study was not blinded, subjective QOL scores tended to be overestimated by a placebo effect, but objective markers for oxidative stress were also significantly decreased. Fifth, drip infusion of hydrogen-enriched saline in combination with Edaravone, a clinically approved radical scavenger for cerebral infarction, for 7 days in 8 patients with brain stem infarction was

compared to 24 such patients receiving Edaravone alone [11]. Although the study was not randomized and not blinded, MRI markers of patients infused with hydrogen showed significant improvements and accelerated normalization.

Being prompted by the prominent effects of hydrogen on inflammatory diseases and oxidative stress-mediated diseases especially in rodents, we performed an open-label trial of drinking 1.0 liter per day of HEW for 12 weeks in 14 patients with muscle diseases, and identified improvement in four parameters: (i) a decrease of the lactate-to-pyruvate ratio in mitochondrial myopathies (MM) and progressive muscular dystrophy (PMD); (ii) a decrease of serum matrix metalloproteinase-3 (MMP3) in polymyositis/dermatomyositis (PM/DM), (iii) a decrease of fasting glucose in PMD, and (iv) a decrease of serum triglycerides in PM/DM. We then conducted a randomized, double-blind, placebo-controlled, crossover trial of 0.5 liter per day of HEW for 8 weeks in 12 MM and 10 DM cases. We observed that HEW significantly improved serum lactate in MM. In both studies, some patients reported subjective improvement of fatigability, diarrhea, and myalgia, but others reported floating sensation and worsening of diarrhea. We observed no objective improvement or worsening of clinical symptoms during each study. Our studies imply that HEW improves clinical parameters in MM and PM/DM, but 0.5 liter/day for 8 weeks is likely to be insufficient to demonstrate statistically significant effects.

Patients and methods

Patients

For the open-label trial, we recruited 5 patients with PMD, 4 patients with PM/DM, and 5 patients with MM. The PMD patients comprised 1 male with Miyoshi myopathy and 4 females with limb girdle muscular dystrophy type 2B with an average age and SD of 50.4 ± 15.9 years (range 25 - 66). The PM/DM patients comprised 2 males and 2 females with an average age of 53.8 ± 24.8 years (range 29 - 83). All the PM/DM cases were taking 5 - 10 mg of prednisolone per day and were well controlled. The MM patients comprised 4 cases with MELAS (2 males and 2 females with an average age of 45.8 ± 12.3 years, range 37 - 64) and a 54-year-old female with chronic progressive external ophthalmoplegia (CPEO).

For the randomized, double-blind, placebo-controlled, crossover trial, we recruited 12 patients with MM and 10 patients with DM. The MM patients comprised 5 cases with MELAS (2 males and 3 females with an average age of 44.6 ± 17.6 years, range 20 - 65), as well as 7 cases with CPEO (3 males and 4 females with an average age of 49.1 ± 11.1 years, range 29 - 61). The DM patients comprised 3 males and 7 females with an

average age of 49.6 ± 13.7 years (range 32 - 66). All the DM patients were well controlled with 5 - 10 mg prednisolone per day. Three MM and three DM patients participated in both trials. Both trials were approved by the Ethical Review Board of the Aichi Medical University. Informed consent was obtained from each patient.

Protocols

We purchased 500 ml HEW or placebo water in aluminum pouch from Blue Mercury Inc. (Tokyo, Japan). We measured hydrogen concentrations using an H₂-N hydrogen needle sensor attached to a PA2000 2-Channel Picoammeter (Unisense Science, Aarhus, Denmark). The hydrogen concentrations were ~0.5 ppm (~31% saturation). We also confirmed that hydrogen in placebo water was undetectable with our system. For each trial, we instructed patients to evacuate the air from the pouch and to close a plastic cap tightly every time after they drink water to keep the hydrogen concentration as high as possible.

For the open-label trial, patients took 1.0 liter per day of HEW in five to ten divided doses for 12 weeks. We measured 18 serum and one urinary parameters and recorded clinical symptoms at 0, 4, 8, 12, 16 weeks.

For the double-blind trial, patients took 0.5 liter per day of HEW or placebo water in two to five divided doses for 8 weeks. Between the 8-week trials with HEW and placebo, we placed a 4-week washout period. We measured 18 serum parameters and recorded clinical symptoms at 0, 4, 8, 12, 16, 20, 24 weeks. In the double-blind trial, we did not measure urinary 8-isoprostane levels.

The data were statistically analyzed using one-way repeated measures ANOVA for the open-label trial and two-way repeated measures ANOVA for the double-blind trial, both followed by the Bonferroni's multiple comparison test using Prism version 4.0c (Graphpad Software, San Diego, CA).

Results

Open-label trial

Fourteen patients with PMD, PM/DM, and MM participated in the study and no patient dropped out of the study. Patients took 1.0 liter of HEW for 12 weeks and we measured 18 serum and one urinary parameters every 4 weeks (Table 1). We observed no objective improvement or worsening of clinical symptoms during the study. All the patients reported increased micturition frequency. Two MELAS patients reported improvement of fatigability, and another MELAS patient complained mild occasional floating sensation. We estimated statistical significance using one-way repeated measures ANOVA analysis and detected five parameters (Figure 1). Serum lactate-to-pyruvate (L/P) ratios of MM patients were high before the study, and were

decreased during the study (Figure 1A). Serum L/P ratios and fasting glucose levels of PMD patients were elevated after the study, but the values were still within normal ranges (Figures 1B and 1C). Serum MMP3 levels of DM patients were decreased down to 72.9% of those before HEW, which were again increased after the study (Figure 1D). Serum triglyceride levels of DM patients were elevated after the study (Figure 1E).

Randomized, double-blind, placebo-controlled, crossover trial

Twelve MM and ten DM patients participated in the study and no patient dropped out of the study. Patients took 0.5 liter of HEW or placebo water for 8 weeks and we measured 18 serum parameters every 4 weeks (Table 2). An MM patient reported increased micturition frequency on HEW. A DM patient reported subjective improvement of fatigability and diarrhea on HEW, but an MM patient rather complained increased diarrhea at first on HEW. Another DM patient reported an improvement of myalgia on HEW. A MELAS patient had hypoglycemic episodes only on HEW, but the episodes subsided after the insulin dose was decreased. We observed no objective improvement or worsening of clinical symptoms during the study. Two-way repeated measures ANOVA analysis revealed that only serum lactate levels were significantly decreased in MM by HEW (Figure 2A). Temporal profiles of serum L/P ratios in MM (Figure 2B) and of serum MMP3 levels in DM (Figure 2C) also demonstrated favorable responses to HEW but without statistical significance.

Discussion

We performed open-label and double-blind studies of HEW on myopathic patients. In the open-label study, we observed statistical significance of hydrogen effects in four parameters: L/P ratios in MM and PMD; fasting glucose in PMD; MMP3 in PM/DM; and triglycerides in PM/DM (Figure 1). In the double-blind study, serum lactate levels were significantly improved in MM. L/P ratios in MM and MMP3 in DM were also improved but without statistical significance (Figure 2). Small numbers of participants in both the open-label and double-blind studies might have failed to disclose statistically significant effects of HEW.

In MM, the mitochondrial electron transfer system (mETS) is compromised by mutations in mitochondrial DNA [12]. This results in a decreased influx of NADH into mETS and elevates NADH levels in the cytoplasm, which facilitates conversion of pyruvate to lactate by lactate dehydrogenase. Thus, lactate and L/P ratio are useful surrogate markers to estimate functions of mETS, and are usually abnormally elevated in MM [12]. Defective mETS also causes leakage of electrons from

Table 1 Open-label trial of HEW in 14 myopathic patients

| | Progressive muscular dystrophy (PMD) | | | Polymyositis (PM)/Dermatomyositis (DM) | | | Mitochondrial myopathies (MM) | | |
|----------------------------------|--------------------------------------|---------------|---------------|--|---------------|---------------|-------------------------------|---------------|---------------|
| | Before | 12 weeks | After | Before | 12 weeks | After | Before | 12 weeks | After |
| CK (U/L) | 3067 ± 1492 | 3419 ± 1610 | 3107 ± 2382 | 124 ± 31 | 180 ± 97 | 140 ± 86 | 187 ± 75 | 124 ± 47 | 156 ± 40 |
| HbA1c (%) | 5.25 ± 0.44 | 5.14 ± 0.31 | 5.16 ± 0.42 | 6.68 ± 1.61 | 6.70 ± 1.53 | 6.90 ± 2.03 | 7.40 ± 1.70 | 7.32 ± 1.48 | 7.38 ± 1.74 |
| Fasting glucose (mmol/L) | 5.52 ± 0.16** | 5.51 ± 0.08** | 5.82 ± 0.11** | 7.66 ± 0.11 | 7.29 ± 1.57 | 7.69 ± 1.85 | 8.94 ± 3.24 | 9.31 ± 4.18 | 8.96 ± 3.19 |
| Lactate (mmol/L) | 0.95 ± 0.34 | 1.15 ± 0.40 | 1.35 ± 0.40 | 1.42 ± 0.18 | 1.66 ± 0.32 | 1.30 ± 0.27 | 1.84 ± 0.50 | 1.87 ± 0.78 | 1.73 ± 0.65 |
| L/P ratio | 12.1 ± 0.7* | 10.7 ± 1.3* | 13.6 ± 2.2* | 13.1 ± 0.9 | 15.0 ± 3.2 | 12.7 ± 1.0 | 20.7 ± 2.9* | 14.9 ± 3.5* | 20.3 ± 3.1* |
| Creatinine (μmol/L) | 34.8 ± 3.1 | 34.5 ± 6.9 | 34.7 ± 8.8 | 58.6 ± 13.7 | 56.3 ± 10.7 | 56.6 ± 14.1 | 48.8 ± 9.0 | 47.7 ± 9.7 | 48.6 ± 8.8 |
| BUN (mmol/L) | 4.74 ± 1.16 | 4.20 ± 0.60 | 4.21 ± 1.05 | 4.33 ± 0.71 | 4.11 ± 0.48 | 4.68 ± 0.74 | 5.28 ± 1.69 | 5.89 ± 1.09 | 5.00 ± 1.58 |
| Uric acid (μmol/dL) | 295 ± 46 | 315 ± 61 | 300 ± 30 | 319 ± 44 | 331 ± 71 | 329 ± 40 | 208 ± 50 | 220 ± 60 | 217 ± 45 |
| Urinary 8-isoprostane (ng/mg Cr) | 303 ± 155 | 392 ± 173 | n.d. | 222 ± 88 | 237 ± 86 | n.d. | 274 ± 117 | 261 ± 59 | n.d. |
| T-chol (mmol/L) | 5.42 ± 0.99 | 5.74 ± 1.02 | 5.70 ± 0.81 | 5.28 ± 0.31 | 5.55 ± 0.93 | 5.97 ± 1.35 | 4.52 ± 0.75 | 4.53 ± 0.34 | 4.42 ± 0.77 |
| LDL-chol (mmol/L) | 3.30 ± 1.05 | 2.86 ± 1.46 | 2.99 ± 1.15 | 2.66 ± 0.28 | 3.21 ± 0.99 | 3.13 ± 1.02 | 2.44 ± 0.43 | 2.27 ± 0.44 | 2.22 ± 0.45 |
| HDL-chol (mmol/L) | 1.62 ± 0.21 | 1.49 ± 0.19 | 1.62 ± 0.18 | 2.01 ± 0.73 | 1.99 ± 0.76 | 2.02 ± 0.67 | 1.14 ± 0.79 | 1.03 ± 0.72 | 1.04 ± 0.69 |
| Triglycerides (mmol/L) | 1.31 ± 0.46 | 3.62 ± 4.83 | 3.07 ± 3.67 | 2.17 ± 0.63* | 2.01 ± 1.21* | 3.09 ± 1.22* | 0.78 ± 0.34 | 0.89 ± 0.45 | 0.73 ± 0.37 |
| WBC (10 ⁹ /L) | 5.30 ± 1.32 | 5.40 ± 1.12 | 4.80 ± 0.38 | 10.78 ± 2.08 | 8.35 ± 3.41 | 10.10 ± 0.17 | 5.12 ± 1.27 | 7.27 ± 2.29 | 5.93 ± 1.35 |
| RBC (10 ¹² /L) | 4.17 ± 0.64 | 3.79 ± 0.42 | 3.87 ± 0.80 | 4.01 ± 0.62 | 4.34 ± 0.36 | 4.49 ± 0.45 | 4.33 ± 0.43 | 4.31 ± 0.78 | 4.35 ± 0.59 |
| Platelets (10 ⁹ /L) | 262 ± 42 | 260 ± 33 | 270 ± 20 | 337 ± 123 | 265 ± 82 | 270 ± 65 | 215 ± 25 | 207 ± 28 | 217 ± 25 |
| Hematocrit | 0.375 ± 0.040 | 0.374 ± 0.038 | 0.407 ± 0.055 | 0.337 ± 0.069 | 0.376 ± 0.041 | 0.395 ± 0.045 | 0.381 ± 0.036 | 0.370 ± 0.047 | 0.378 ± 0.038 |
| MMP3 (ng/ml) | n.d. | n.d. | n.d. | 307.8 ± 59.1* | 224.3 ± 53.4* | 283.3 ± 77.6* | n.d. | n.d. | n.d. |
| IgG (mg/dl) | n.d. | n.d. | n.d. | 1343 ± 470 | 1396 ± 550 | 1429 ± 581 | n.d. | n.d. | n.d. |

Values represent mean ± SD. n.d., not determined. *p < 0.05 and **p < 0.005 by one-way repeated measures ANOVA.

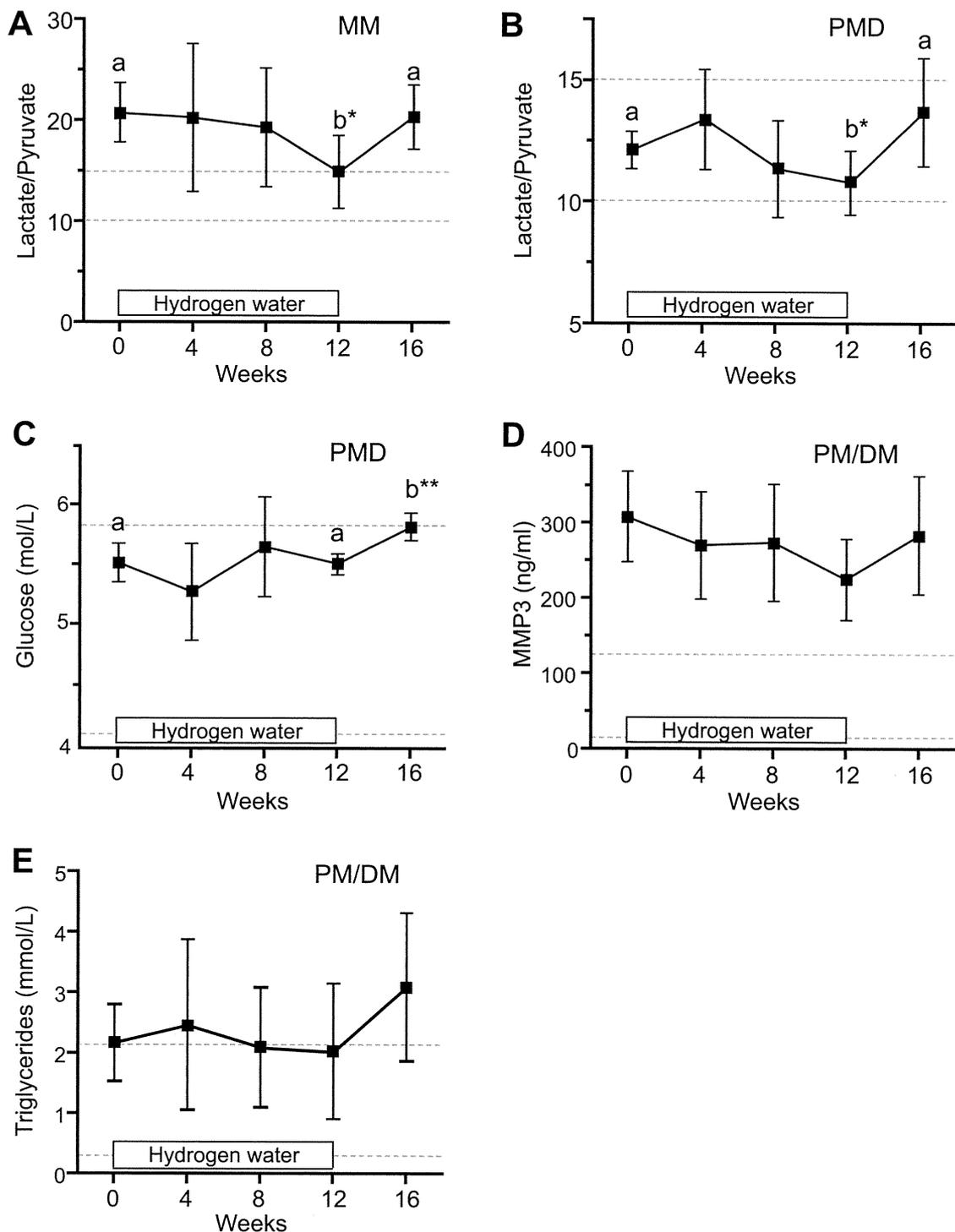


Figure 1 Temporal profiles of four parameters that demonstrate statistical significance by one-way repeated measures ANOVA in the open-label trial. Ratios of serum lactate/pyruvate (L/P) in 5 mitochondrial myopathies (MM) patients (A) and 4 progressive muscular dystrophy (PMD) patients (B). Note abnormally high L/P ratios in MM patients. (C) Fasting glucose in 4 PMD patients. (D) Serum MMP3 in 5 Polymyositis (PM)/Dermatomyositis (DM) patients. (E) Serum triglycerides in 4 PMD patients. Twelve weeks on HEW are indicated by a box in each panel. Means and SD are plotted. Statistically different values by the Bonferroni's multiple comparison test are indicated by 'a' and 'b' with * $p < 0.05$ and ** $p < 0.01$. Bonferroni's test reveal no statistical difference between any two values in (D) and (E). Broken lines show a normal range of each parameter.

Table 2 Randomized, double-blind, placebo-controlled, crossover trial of HEW in 10 DM and 12 MM patients

| | Dermatomyositis (DM) | | | | Mitochondrial myopathies (MM) | | | |
|--------------------------------|----------------------|---------------|---------------|---------------|-------------------------------|---------------|---------------|---------------|
| | Hydrogen water | | Placebo water | | Hydrogen water | | Placebo water | |
| | 0 week | 8 weeks | 0 week | 8 weeks | 0 week | 8 weeks | 0 week | 8 weeks |
| CK (U/L) | 88.7 ± 24.3 | 106.1 ± 88.2 | 93.5 ± 45.0 | 99.5 ± 86.7 | 165 ± 86.6 | 120 ± 55.5 | 142.0 ± 69.4 | 221 ± 235 |
| HbA1c (%) | 6.23 ± 1.28 | 6.34 ± 1.55 | 6.27 ± 1.44 | 6.16 ± 1.28 | 6.09 ± 0.94 | 6.12 ± 1.05 | 6.06 ± 1.22 | 6.06 ± 1.02 |
| Fasting glucose (mmol/L) | 8.27 ± 3.62 | 7.81 ± 2.91 | 6.70 ± 2.11 | 6.48 ± 1.90 | 6.05 ± 1.43 | 5.67 ± 1.99 | 6.02 ± 1.46 | 6.11 ± 1.69 |
| Lactate (mmol/L) | 1.93 ± 0.78 | 1.81 ± 0.87 | 1.80 ± 0.89 | 1.65 ± 0.77 | 1.76 ± 0.67* | 1.61 ± 0.48* | 1.49 ± 0.49* | 1.70 ± 0.57* |
| L/P ratio | 13.1 ± 6.0 | 11.5 ± 2.6 | 12.1 ± 2.88 | 15.2 ± 8.3 | 18.7 ± 8.8 | 17.9 ± 7.7 | 7.12 ± 13.4 | 17.7 ± 8.6 |
| Creatinine (μmol/L) | 59.1 ± 15.6 | 59.1 ± 13.6 | 58.3 ± 15.0 | 59.0 ± 19.7 | 53.6 ± 18.3 | 52.0 ± 17.4 | 54.8 ± 20.3 | 57.5 ± 22.3 |
| BUN (mmol/L) | 5.36 ± 1.48 | 4.82 ± 1.57 | 5.78 ± 1.71 | 4.86 ± 1.56 | 6.08 ± 2.09 | 5.39 ± 1.54 | 6.24 ± 1.46 | 6.34 ± 2.76 |
| Uric Acid (μmol/dL) | 303 ± 88 | 320 ± 68 | 313 ± 75 | 321 ± 83 | 413 ± 316 | 375 ± 229 | 447 ± 387 | 408 ± 284 |
| T-chol (mmol/L) | 5.23 ± 0.78 | 5.15 ± 0.85 | 5.21 ± 0.61 | 4.95 ± 0.92 | 4.61 ± 0.78 | 4.80 ± 0.57 | 4.69 ± 0.78 | 4.68 ± 0.71 |
| LDL-chol (mmol/L) | 3.06 ± 0.82 | 2.93 ± 0.75 | 2.97 ± 0.80 | 3.03 ± 0.84 | 2.69 ± 0.68 | 2.82 ± 0.58 | 2.73 ± 0.67 | 2.79 ± 0.60 |
| HDL-chol (mmol/L) | 1.59 ± 0.48 | 1.56 ± 0.36 | 1.53 ± 0.47 | 1.43 ± 0.48 | 1.47 ± 0.04 | 1.55 ± 0.34 | 1.52 ± 0.34 | 1.45 ± 0.35 |
| Triglycerides (mmol/L) | 1.54 ± 0.65 | 1.83 ± 0.76 | 1.86 ± 0.80 | 1.85 ± 0.48 | 1.16 ± 0.58 | 0.92 ± 0.35 | 0.97 ± 0.36 | 1.02 ± 0.49 |
| WBC (10 ⁹ /L) | 11.7 ± 6.4 | 10.9 ± 3.1 | 9.66 ± 2.68 | 11.2 ± 4.7 | 5.5 ± 0.1 | 5.7 ± 1.8 | 5.81 ± 1.78 | 5.8 ± 1.9 |
| RBC (10 ¹² /L) | 4.28 ± 0.05 | 4.32 ± 0.35 | 4.37 ± 0.44 | 4.35 ± 0.44 | 4.15 ± 0.04 | 4.17 ± 0.61 | 4.26 ± 0.67 | 4.15 ± 0.56 |
| Platelets (10 ⁹ /L) | 271 ± 10 | 302 ± 69 | 306 ± 54 | 312 ± 79 | 203 ± 40 | 214 ± 44 | 216 ± 48 | 225 ± 50 |
| Hematocrit (%) | 0.380 ± 0.004 | 0.384 ± 0.040 | 0.392 ± 0.052 | 0.391 ± 0.047 | 0.373 ± 0.004 | 0.378 ± 0.060 | 0.386 ± 0.063 | 0.376 ± 0.058 |
| MMP3 (ng/ml) | 245 ± 122 | 232 ± 84.1 | 217 ± 93.5 | 221 ± 112 | n.d. | n.d. | n.d. | n.d. |
| IgG (mg/dl) | 1211 ± 357 | 1244 ± 305 | 1202 ± 340 | 1282 ± 353 | n.d. | n.d. | n.d. | n.d. |

Values represent mean ± SD. n.d., not determined. *p < 0.05 by two-way repeated measures ANOVA.

mitochondrial inner membranes and increases production of reactive oxygen species (ROS), which further damages mETS [13,14]. Reduction of the L/P ratios in the open-label and double-blind studies suggests that hydrogen alleviates mETS dysfunction in MM either by scavenging ROS or by yet unidentified signaling mechanisms.

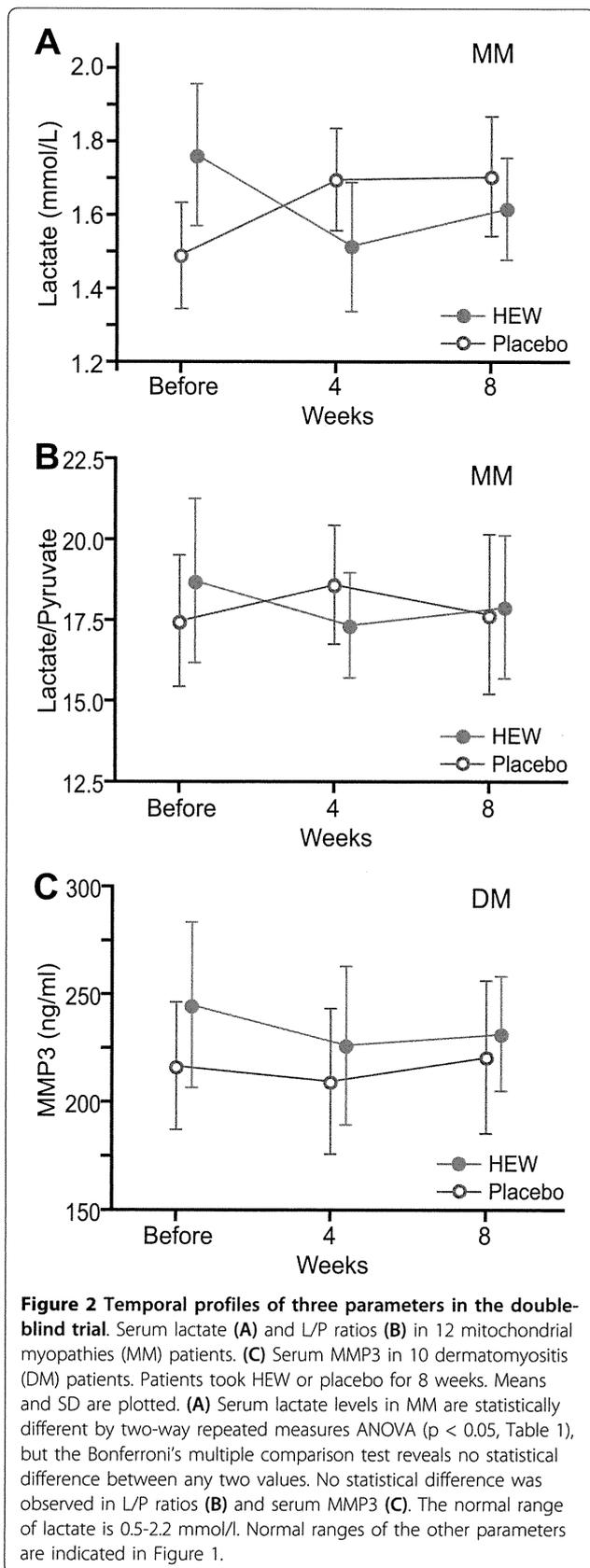
MMP3 belongs to a family of calcium-dependent zinc proteinases induced by cytokines and secreted by inflammatory cells. MMPs enhance T-cell migration and adhesion, and also degrade the extracellular matrix proteins [15]. MMP3 is increased in a fraction of DM patients [16]. MMP3 may facilitate lymphocyte adhesion and enhance T-cell-mediated cytotoxicity by degrading extracellular matrix proteins in DM. Hydrogen improved serum MMP3 levels in the open-label and double-blind studies, which is expected to ameliorate pathogenic inflammatory processes that culminates in muscle fiber destruction.

We observed less prominent effects with the double-blind study compared to the open-label study. The lack of statistically significance in the double-blind study is possibly due to a lower amount of HEW (1.0 vs. 0.5 liter per day) and to a shorter observation period (12 vs. 8 weeks). In the open-label study, drinking 1.0 liter of HEW was not readily accommodated by most myopathic patients. Hydrogen does not show simple dose-

response relationship in rodents [2-4], and *ad libitum* administration of even 5%-saturated HEW markedly attenuates development of Parkinson's disease in mice [17]. We thus reduced the amount of hydrogen to 0.5 liter in the double-blind trial, and also shortened the observation period to minimize the burden on the participants. This, however, might have masked effects of HEW. Indeed, when we compare studies of diabetes mellitus type 2 [6], the current open-label trial, and metabolic syndrome [9], the participants took 0.9, 1.0, and 1.5-2.0 liters of HEW, respectively. Ratios of total cholesterol/HDL-cholesterol are available at 8 weeks in all the studies, and are changed to 103.8%, 98.6%, and 95.8%, respectively, of those before hydrogen administration, which is in accordance with a dose-response effect of HEW. Additionally, among the two previous studies [6,9] and the current open-label and double-blind studies, the most prominent effects are observed with 1.5-2.0 liters of HEW. As drinking a large amount of HEW is not easily accommodated by most patients especially in winter, a threshold effect and/or a dose-response effect should be further elaborated for each pathological state.

Conclusions

HEW is effective for mitochondrial dysfunction in MM and inflammatory processes in DM. Hydrogen may have



a threshold effect or a dose-response effect and 1.0 liter or more per day of HEW is likely to be required to exert beneficial effects.

Abbreviations

HEW: hydrogen-enriched water; PMD: progressive muscular dystrophy; PM: polymyositis; DM: dermatomyositis; MM: mitochondrial myopathies; CPEO: chronic progressive external ophthalmoplegia; MELAS: mitochondrial myopathy with lactic acidosis and stroke-like episodes; MMP3: matrix metalloproteinase-3.

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Author details

¹Division of Neurogenetics, Center for Neurological Diseases and Cancer, Nagoya University Graduate School of Medicine, 65 Tsurumai, Showa-ku, Nagoya 466-8550, Japan. ²Faculty of Pathophysiology and Therapeutics, Aichi Medical University College of Nursing, 21 Karimata Yazako, Nagakute-cho, Aichi-gun, Aichi 480-1195, Japan. ³Department of Neurology, Aichi Medical University School of Medicine, 21 Karimata Yazako, Nagakute-cho, Aichi-gun, Aichi 480-1195, Japan. ⁴Department of Biomedical Sciences, College of Life and Health Sciences, Chubu University, 1200 Matsumoto, Kasugai, Aichi 487-8501, Japan. ⁵Department of Longevity and Aging Research, Gifu International Institute of Biotechnology, 1-1 Nakafudogaoka, Kakamigahara, Gifu 504-0838, Japan.

Authors' contributions

TI and KS examined patients and acquired data. MI¹ and TI organized data and performed statistical analysis. MI¹ and KO wrote the paper. MI⁴, MI⁵, and KO conceived the study. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Hyperuricemia cosegregating with osteogenesis imperfecta is associated with a mutation in *GPATCH8*

Hiroshi Kaneko · Hiroshi Kitoh · Tohru Matsuura ·
Akio Masuda · Mikako Ito · Monica Mottes ·
Frank Rauch · Naoki Ishiguro · Kinji Ohno

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Abstract Autosomal dominant osteogenesis imperfecta (OI) is caused by mutations in *COL1A1* or *COL1A2*. We identified a dominant missense mutation, c.3235G>A in *COL1A1* exon 45 predicting p.G1079S, in a Japanese family with mild OI. As mutations in exon 45 exhibit mild to lethal phenotypes, we tested if disruption of an exonic splicing *cis*-element determines the clinical phenotype, but detected no such mutations. In the Japanese family, juvenile-onset hyperuricemia cosegregated with OI, but not in the previously reported Italian and Canadian families with c.3235G>A. After confirming lack of a founder haplotype in three families, we analyzed *PRPSAP1* and *PRPSAP2* as candidate genes for hyperuricemia on chr 17 where *COL1A1* is located, but found no mutation. We next resequenced the whole exomes of two siblings in the Japanese family and identified variable numbers of previously

reported hyperuricemia-associated SNPs in *ABCG2* and *SLC22A12*. The same SNPs, however, were also detected in normouricemic individuals in three families. We then identified two missense SNVs in *ZPBP2* and *GPATCH8* on chromosome 17 that cosegregated with hyperuricemia in the Japanese family. *ZPBP2* p.T69I was at the non-conserved region and was predicted to be benign by *in silico* analysis, whereas *GPATCH8* p.A979P was at a highly conserved region and was predicted to be deleterious, which made p.A979P a conceivable candidate for juvenile-onset hyperuricemia. *GPATCH8* is only 5.8 Mbp distant from *COL1A1* and encodes a protein harboring an RNA-processing domain and a zinc finger domain, but the molecular functions have not been elucidated to date.

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H. Kaneko · T. Matsuura · A. Masuda · M. Ito · K. Ohno (✉)
Division of Neurogenetics, Center for Neurological Diseases
and Cancer, Nagoya University Graduate School of Medicine,
65 Tsurumai, Showa-ku, Nagoya 466-8550, Japan
e-mail: ohnok@med.nagoya-u.ac.jp

H. Kaneko · H. Kitoh · N. Ishiguro
Department of Orthopaedic Surgery,
Nagoya University Graduate School of Medicine,
Nagoya, Japan

M. Mottes
Department of Life and Reproduction Sciences,
University of Verona, Verona, Italy

F. Rauch
Genetics Unit, Shriners Hospital for Children and McGill
University, Montreal, QC, Canada

Introduction

Osteogenesis imperfecta (OI) is a heritable connective tissue disorder characterized by bone fragility and low bone mass. Clinical severities are widely variable ranging from intrauterine fractures and perinatal lethality to very mild forms without fractures. Patients also exhibit associated features including blue sclera, dentinogenesis imperfecta, hyperlaxity of ligaments and skin, and hearing loss (Rauch and Glorieux 2004). The widely used classification initially described by Silience et al. (1979) distinguishes types I, II, III and IV (MIM# 166200, 166210, 259420, and 166220, respectively) on the basis of clinical and radiographic findings. Recently, five additional types of V, VI, VII, VIII and IX (MIM# 610967, 610968, 610682, 610915, and 259440, respectively) have been reported (Cabral et al. 2007; Glorieux et al. 2000, 2002; van Dijk et al. 2009; Ward et al. 2002). OI type I is the mildest form characterized by fractures with little or no limb deformity and

normal or mildly short stature, whereas type II is a perinatal lethal form, mostly due to respiratory failure resulting from multiple rib fractures. Type III is characterized by progressive deformities and fractures that are often present at birth. Severities of types IV, V, VI and VII are between those of types I and III. Type VIII and IX carry features of both types II and III.

Type I collagen is the most abundant bone protein. Most patients (>90%) with OI types I–IV have dominant or recessive mutation(s) in either of two genes, *COL1A1* (MIM# 120150) on chromosome (chr) 17q21.31–q22 and *COL1A2* (MIM# 120160) on chr 7q22.1 that encode the $\alpha 1$ and $\alpha 2$ chains of type I procollagen, respectively (Rauch and Glorieux 2004). A genetic cause of type V remains undetermined to date. Types VI to IX are caused by recessive mutations. Type VI is caused by mutations in *FKBP10* (MIM# 607063) encoding FK506-binding protein 65 (FKBP65) that is a chaperone in type I procollagen folding (Alanay et al. 2010). Type VII is caused by mutations in *CRTAP* (MIM# 605497) encoding cartilage-associated protein (CRTAP) (Morello et al. 2006). Type VIII is caused by mutations in *LEPRE1* (MIM# 610339) encoding prolyl 3-hydroxylase 1 (P3H1) (Cabral et al. 2007). Type IX is caused by mutations in *PP1B* (MIM# 123841) encoding cyclophilin B (CYPB) (van Dijk et al. 2009). *CRTAP*, *P3H1* and *CYPB* form an intracellular collagen-modifying complex that 3-hydroxylates proline at position 986 in the $\alpha 1$ chain of type I collagen, which is essential for correct folding and stability of the collagen triple helix. Mutations in *CRTAP* and *LEPRE1* are also identified in severe OI phenotypes including type II (Baldrige et al. 2008; Morello et al. 2006). Recently, recessive mutations in *SERPINH1* (MIM# 600943) encoding a chaperone-like protein for collagens, heat shock protein 47 and in *SP7/Osterix* (MIM# 606633) encoding an osteoblast-specific transcription factor have been identified in patients with types III and IV, respectively (Christiansen et al. 2010; Lapunzina et al. 2010).

Two copies of the $\alpha 1$ chain and one copy of the $\alpha 2$ chain form a core triple helix comprising 338 uninterrupted Gly-X-Y triplet repeats, where X is often proline and Y is often hydroxyproline. Gly repeats at every third position are essential for the stability of collagen because larger amino acids cannot be accommodated in the tightly packed core without disruption of the triple helix (Bodian et al. 2008). The most common mutations (>80%) affect one of the repeated Gly residues in the triple helix. More than 800 mutations in *COL1A1* and *COL1A2* are currently deposited in the human type I collagen mutation database (<http://www.le.ac.uk/genetics/collagen/>) (Dalglish 1997; Marini et al. 2007). Clinical phenotypes may be determined by the chain in which the Gly substitution occurs, the position of the mutation within the chain and/or the nature

of the mutant amino acids (Bodian et al. 2008; Marini et al. 2007; Rauch and Glorieux 2004), but we still cannot predict a clinical phenotype of a given mutation. On the other hand, mutations that create a premature stop codon within *COL1A1* mostly exhibit a milder OI type I. This is because a truncation mutation is unlikely to have a dominant negative effect, but the abundance of type I collagen chain is half of the normal (Marini et al. 2007; Rauch and Glorieux 2004).

Pre-mRNA splicing is regulated by intronic and exonic splicing *cis*-elements. Both constitutively and alternatively spliced exons harbor exonic splicing enhancers (ESEs) and silencers (ESSs). Splicing *trans*-factors are expressed in a developmental stage-specific and tissue-specific manner, and their expressions tightly regulate alternative splicing of an exon carrying ESEs/ESSs. A mutation in the coding region is predicted to exert its pathogenicity by compromising a functional amino acid, but nonsense, missense and even silent mutations potentially disrupt an ESE/ESS, thereby resulting in aberrant splicing (Cartegni et al. 2002, 2003). Indeed, more than 16–20% of exonic mutations are predicted to disrupt an ESE (Gorlov et al. 2003).

Exome resequencing is a powerful and efficient method to identify a novel gene associated with a rare monogenic disorder, especially when the number of unrelated patients or the number of family members of a patient are too small to apply linkage studies. Filtering against existing SNP database and the exomes of unaffected individuals can remove common variants to identify a causal gene. Ng et al. (2009) sequenced exomes of 12 humans, including four unrelated individuals with autosomal dominant Freeman-Sheldon syndrome (MIM# 193700) and eight Hap-Map individuals. They successfully identified mutations in *MYH3* (MIM# 160720) in all the affected individuals. Ng et al. (2010b) also sequenced exomes of four patients in three families with autosomal recessive Miller syndrome (MIM# 263750) and de novo identified compound heterozygous mutations in *DHODH* (MIM# 126064). Ng et al. (2010a) additionally sequenced exomes of ten unrelated patients with autosomal dominant Kabuki syndrome (MIM# 147920) and de novo identified nonsense or frameshift mutations in *MLL2* (MIM# 602113). Similarly, Lalonde et al. (2010) sequenced two unrelated fetuses with autosomal recessive Fowler syndrome (MIM# 225790) and de novo identified compound heterozygous mutations in *FLVCR2* (MIM# 610865).

In a Japanese family with OI type I, hyperuricemia co-segregated with OI. To our knowledge, association of hyperuricemia with OI has been reported in two families, in which two of three OI patients had gouty arthritis and hyperuricemia at young ages (Allen et al. 1955). Underexcretion of urate is causally associated with mutations in *UMOD* (MIM# 191845) encoding uromodulin (Hart et al.

2002) as well as with SNPs in three genes: *SLC2A9* (MIM# 606142) encoding glucose transporter 9 (Doring et al. 2008; Vitart et al. 2008), *ABCG2* (MIM# 603756) encoding ATP-binding cassette subfamily G member 2, a urate transporter (Dehghan et al. 2008; Kolz et al. 2009; Stark et al. 2009; Woodward et al. 2009), and *SLC22A12* (MIM# 607096) encoding URAT1, a renal urate-anion exchanger (Graessler et al. 2006; Tabara et al. 2010). On the other hand, overproduction of urate is caused by mutations in *PRPS1* (MIM# 311850) encoding PRPP synthetase I (Roessler et al. 1993) and *HPRT1* (MIM# 308000) encoding hypoxanthine guanine phosphoribosyltransferase I (Gibbs and Caskey 1987).

We here identified a dominant missense mutation, c.3235G>A in *COL1A1* exon 45 predicting p.G1079S, in a Japanese family with OI type I and hyperuricemia, and analyzed the molecular bases of two clinical features. First, we tested if mild to lethal phenotypes of mutations in exon 45 of *COL1A1* were accounted for by preservation or disruption of an ESE/ESS element, and found that ESE/ESS elements were not involved in disease severities. Second, we traced a cause of the hyperuricemia by exome resequencing of two siblings and found that a missense mutation in *GPATCH8* encoding the G patch domain-containing protein 8 close to *COL1A1* cosegregated with hyperuricemia.

Patients and methods

Samples and ethical considerations

We obtained blood from each family member and isolated genomic DNA (gDNA) from 2 ml of peripheral blood using the QIAamp DNA Blood Midi Kit (Qiagen) according to the manufacturer's instructions. We also obtained skin biopsy of an affected individual (II-3) of a Japanese family (F1) and cultured non-transformed fibroblasts for splicing and sequencing analysis. Informed consent was obtained from all family members. The studies have been approved by the Institutional Review Boards of the Nagoya University, the University of Verona and the McGill University. Clinical features and mutational analyses have been previously reported in the Italian and Canadian families (Mottes et al. 1992; Roschger et al. 2008).

Microsatellite analysis of *COL1A1* and *COL1A2* to identify a mutation in the Japanese family

We genotyped all family members for three microsatellite markers flanking *COL1A1* (D17S1293, –16 Mbp; D17S1319, –14 kbp; and D17S788, 2 Mbp). As no annotated microsatellite markers were available close to *COL1A2*, we posted three new microsatellite markers to

DDBJ (AB499843, –17 kbp; AB499844, 29 kbp; and AB499845, 123 kbp) and analyzed them in the family. We fluorescently labeled the 5' end of each forward primer with 5FAM (Sigma-Aldrich), and amplified microsatellite markers with the HotStarTaq Plus Master Mix (Qiagen) using gDNA and primers indicated in Supplementary Table 1.

We mixed 1.5 μ l of 20-times diluted PCR product with 0.5 μ l of GeneScan-500 ROX Size Standard (Applied Biosystems) and 24.5 μ l of formamide, and incubated the mixture at 95°C for 3 min. The mixture was run by capillary electrophoresis on an ABI PRISM 310 Genetic Analyzer and was analyzed with the GeneScan and GeneMapper software (Applied Biosystems).

Sequence analysis of *COL1A1*

After the microsatellite analysis suggested that *COL1A1* was more likely to be a causative gene, we amplified all exons and flanking intronic regions of ~40 bp, as well as 5' and 3' UTRs of *COL1A1* from gDNA of II-3 by PCR. We performed the dye terminator cycle sequencing reaction with the GenomeLab DTCS Quick Start Kit (Beckman Coulter) and ran on the CEQ 8000 Genetic Analysis System (Beckman Coulter) according to the manufacturer's instructions. We compared the chromatograms with the GenBank reference sequences of *COL1A1* gDNA using the Mutation Surveyor software version 2.61 (SoftGenetics). We numbered *COL1A1* mutations with the translation initiator methionine as amino acid +1, and the A of the ATG codon as nucleotide +1 according to the Human Genome Variation Society (<http://www.hgvs.org/mutnomen/recs.html>). We numbered exons according to the human type I collagen mutation database (<http://www.le.ac.uk/genetics/collagen/>), in which *COL1A1* exon 33 is named *COL1A1* exon 33–34 to match the exonic annotations of *COL1A2*.

Allele-specific primer (ASP)-PCR to trace the *COL1A1* mutation

We traced the mutation in family members using ASP-PCR. The wild-type ASP was 5'-TCCCGCCGGTCCTGTAGG-3', and the mutant ASP was 5'-TCCCGCCGGTCTGTAAAG-3', where the mutated nucleotide is underlined, and an artificially introduced mismatch is shown in bold. The reverse primer was 5'-GCCACGGTGACCCTTTATGC-3'.

Prediction of effects of mutations on pre-mRNA splicing

Five missense mutations in exon 45 of *COL1A1* cause mild to lethal OI phenotypes (Fig. 2a) (Constantinou et al. 1989;

Hartikka et al. 2004; Lund et al. 1997; Marini et al. 2007; Mottes et al. 1992; Roschger et al. 2008). We predicted the effects on pre-mRNA splicing of 18 sequence variants with or without each mutation in the presence or absence of each of two SNPs (rs1800215 and rs1800217) in exon 45 of *COLIA1* (Fig. 2b) using five Web-based programs: ESEfinder 3.0 (<http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home>) (Cartegni et al. 2003), ESR-search (<http://ast.bioinfo.tau.ac.il/>) (Goren et al. 2006), FAS-ESS (<http://genes.mit.edu/fas-ess/>) (Wang et al. 2004), PESXs (<http://cubweb.biology.columbia.edu/pesx/>) (Zhang and Chasin 2004; Zhang et al. 2005), and RESCUE-ESE (<http://genes.mit.edu/burgelab/rescue-ese/>) (Fairbrother et al. 2002). We also predicted the effects of the mutations on splice site strength of the 18 sequence variants using two Web-based programs: the NetGene2 Server (<http://www.cbs.dtu.dk/services/NetGene2/>) (Brunak et al. 1991; Hebsgaard et al. 1996) and the Splice Site Prediction by Neural Network (http://www.fruitfly.org/seq_tools/splice.html) (Reese et al. 1997).

Splicing analysis of fibroblasts of the OI patient (II-3)

We first examined splicing of *COLIA1* exon 45 in the patient's fibroblasts. We extracted total RNA from cultured fibroblasts of II-3 using Trizol reagent (Invitrogen) and synthesized cDNA using the oligo(dT)_{12–18} primer (Invitrogen) and the ReverTra Ace reverse transcriptase (Toyobo). We examined skipping of *COLIA1* exon 45 using 5'-GGTCCCCTGGACGAGAC-3' on exon 43 and 5'-TCCAGAGGGACCTTGTTAC-3' on exon 47. We also sequenced RT-PCR products as described above to scrutinize splicing consequences. As skipping of exon 45 results in an in-frame deletion of 54 nucleotides, we did not downregulate the nonsense-mediated mRNA decay (NMD) before harvesting cells.

COLIA1 minigene constructs

We amplified exons 44–46 and the intervening introns of *COLIA1* (Fig. 2b) by PCR. The PCR primers introduced a *Hind*III site and the Kozak consensus sequence of 5'-CCACCATG-3' to the 5' end, as well as a TAA stop codon and a *Bam*HI site at the 3' end of the PCR product, so that the minigene transcript is tolerant to NMD (Ohno et al. 2003). We inserted the PCR product into the pcDNA3.1(+) mammalian expression vector (Invitrogen) and confirmed the lack of PCR artifacts by sequencing the entire insert. We next constructed 17 variant minigenes using the QuikChange site-directed mutagenesis kit (Stratagene) (Fig. 2b). We again confirmed presence of the introduced

mutations and absence of artifacts by sequencing the entire inserts.

Splicing assays of *COLIA1* exon 45

We transfected 500 ng of a minigene construct into 50% confluent HEK293 cells in a 12-well plate using the FuGENE 6 transfection reagent (Roche Applied Science) according to the manufacturer's instructions. After 48 h, we extracted total RNA from HEK293 cells and synthesized cDNA as described above. To prevent amplification of the endogenous *COLIA1*, we used generic primers of 5'-TTAATACGACTCACTATAGGGAGACC-3' and 5'-T TAAGGAGGGCCAGGGG-3' located on pcDNA3.1. Untransfected cells were used as a negative control.

Founder analysis of three families with *COLIA1* c.3235G>A

To examine if the *COLIA1* c.3235G>A mutation arose from a common founder in the Japanese, Italian (Mottes et al. 1992) and Canadian (Roschger et al. 2008) families, we genotyped three microsatellite markers flanking *COLIA1* described above and sequenced an intragenic SNP (rs2075554) of *COLIA1* in the Italian and Canadian families (Fig. 1).

Sequence analysis of *PRPSAP1* and *PRPSAP2*

To seek for a responsible gene for hyperuricemia, we sequenced the entire coding regions of *PRPSAP1* and *PRPSAP2* on chr 17 using cDNA synthesized from cultured fibroblasts of II-3.

Resequencing of exome-enriched DNA

As we found no mutation in *PRPSAP1* and *PRPSAP2*, we enriched exonic regions of genomic DNA of II-2, II-3 and VIII-2 using the SureSelect human all exon kit v1 (Agilent Technologies) that covers 1.22% (34 Mbp) of the human genome. We sequenced 50 base pairs of each tag in a single direction using a quarter of a cell of the SOLiD 3 Plus system (Life Technologies) for each sample.

For II-2, II-3 and VIII-2, we obtained 79.1, 68.6 and 109.9 × 10⁶ tags of 50-bp SOLiD reads and mapped 2.18 (69.6%), 1.87 (68.5%) and 3.37 (76.5%) Gbp to the human genome hg19/GRCh37, which yielded a mean coverage of 64.1, 55.1 and 99.0, respectively. Among the mapped tags, 70.1, 72.0 and 73.1% were located on the SureSelect exome probes. Among the 34-Mbp regions where the exome probes were designed, 3.4, 3.6 and 3.5% of nucleotides

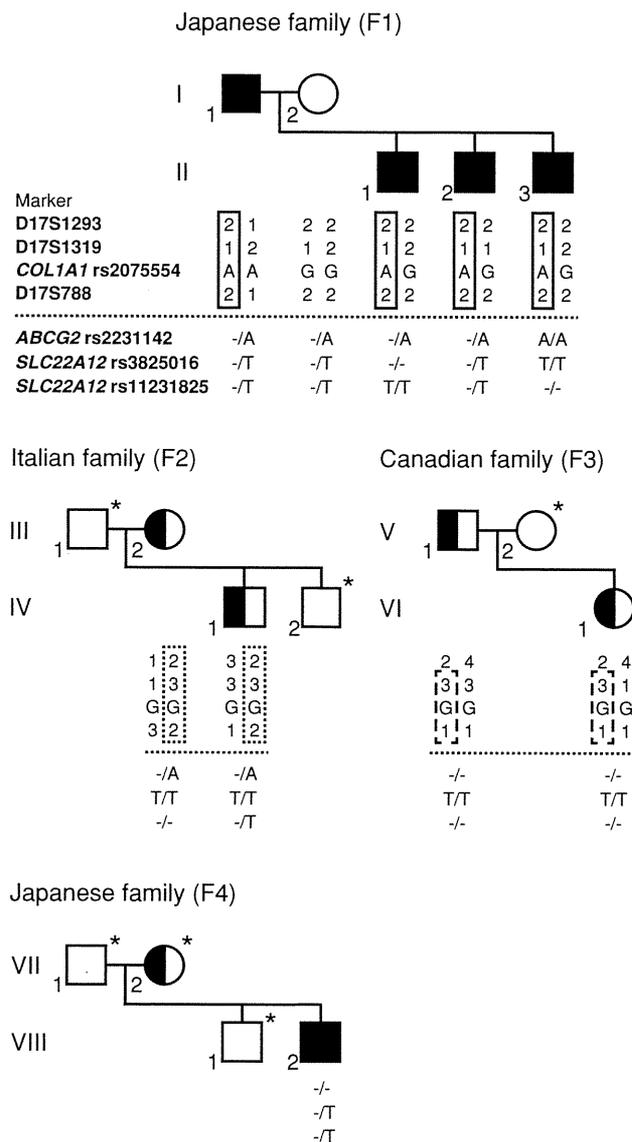


Fig. 1 Pedigrees, haplotypes and genotypes of the Japanese (F1 and F4), Italian (F2) and Canadian (F3) families. Patients in F1, F2 and F3 have c.3235G>A predicting p.G1079S in COL1A1. A patient in F4 has c.577G>T predicting p.G193C in COL1A2. Closed symbols indicate patients with OI and with hyperuricemia. Half-shaded symbols represent OI without hyperuricemia. Asterisks indicate that DNA sample is not available for our studies. Clinical subtypes are all type I except for the Canadian father (V-1), who exhibits type IV. In F1, hyperuricemia cosegregates with OI. Genotypes of three microsatellite markers (D17S1293, -16 Mbp; D17S1319, -14 kbp; and D17S788, 2 Mbp) flanking COL1A1 and an SNP (rs2075554) in intron 11 of COL1A1 are indicated for all the available members in F1, F2, and F3. F1, F2 and F3 carry their unique haplotypes (shown by solid, dotted and broken boxes, respectively). The D17S1293 genotype in F3 is not informative and is not boxed. Genotypes of hyperuricemia-associated SNPs (rs2231142, rs3825016 and rs11231825) are indicated at the bottom of each pedigree tree

were not sequenced at all. Search for single nucleotide variants (SNVs) and indels with Bioscope 1.2.1 (Life Technologies) detected 52,436, 56,941 and 60,303

SNVs/indels. SNVs and indels were compared to dbSNP Build 132.

Analysis of variants in KRBA2, ZPBP2 and GPATCH8

To trace if variants in KRBA2, ZPBP2 and GPATCH8 cosegregated with hyperuricemia, we analyzed all family members in the Japanese family (F1) by capillary sequencing.

We traced two variants, ZPBP2 c.206C>T and GPATCH8 c.2935G>C, in the Italian and Canadian families and 100 normal human genomes using ASP-PCR. The forward primers of ZPBP2 and GPATCH8 were 5'-CGT GTCTTCAGCACAAAATGG-3' and 5'-AGAAGCCGTA GCACCACTCC-3', respectively. The reverse primers were 5'-GGCCCAATCCATAAGTACAT-3' and 5'-CCCA TGATCTCTTCCTGGAG-3', respectively. The mutated nucleotide is underlined, and an artificially introduced mismatch is shown in bold.

To search for the identified variants in ZPBP2 and GPATCH8 in normal controls, we mapped 50 Tibetan exome reads (SRA accession number SRP002446) (Yi et al. 2010) to a 200-bp region spanning c.206C>T in exon 3 of ZPBP2 and a 200-bp region spanning c.2935G>C in exon 8 of GPATCH8 with the bowtie alignment tool version 0.12.7 (Langmead et al. 2009) using default parameters.

We analyzed amino acid conservations of ZPBP2 and GPATCH8 using the evolutionary annotation database, Evola, at the H-Inv DB (<http://www.h-invitational.jp/evola/>). We also predicted functional effects of amino acid substitutions using two Web-based programs: PolyPhen-2 (<http://genetics.bwh.harvard.edu/pph2/>) (Adzhubei et al. 2010) and SIFT (<http://sift.jcvi.org/>) (Kumar et al. 2009).

Results

Hyperuricemia cosegregated with OI type I in the Japanese family (F1)

In a Japanese family (F1), a father (age 56 years) and his three sons (ages 29, 26 and 23 years) had OI type I with blue sclera, dentinogenesis imperfecta and joint laxity (Fig. 1). Two sons (II-1 and II-3) had histories of more than ten fractures before age 13 years, but the father (I-1) and another son (II-2) experienced no bone fracture. One son (II-1) had hearing loss from age 10 years likely due to fractures or deformities of small bones in the middle ear and had hip joint deformities due to repeated femoral fractures. Interestingly, all the affected members had hyperuricemia of ~8 mg/dl that was diagnosed at ages 15–30 years. One son (II-3) had a gout attack, and the other two (I-1 and II-1) had urinary stones. Hyperuricemia is currently well controlled by medication in all the members.

Heteroallelic c.3235G>A mutation in *COL1A1* in the Japanese family (F1)

Genotypes of three microsatellite markers flanking *COL1A1* cosegregated with the OI phenotype in F1 (Fig. 1), whereas genotypes of three markers flanking *COL1A2* did not (data not shown). We thus sequenced the entire exons and the flanking noncoding regions and identified a heteroallelic c.3235G>A mutation in exon 45 and a heteroallelic G/A SNP (rs2075554) in intron 11 of *COL1A1*. The c.3235G>A mutation predicts p.G1079S. We genotyped c.3235G>A in family members by ASP-PCR, and found that all affected members were heterozygous for c.3235G>A (Fig. 1).

Phenotypic variability of osteogenesis imperfecta is not accounted for by disruption of splicing *cis*-elements

In addition to c.3235G>A, four more mutations and two SNPs have been reported in *COL1A1* exon 45 with variable phenotypes ranging from mild type I to perinatal lethal type II (Fig. 2a) (Constantinou et al. 1989; Hartikka et al. 2004; Lund et al. 1997; Marini et al. 2007; Mottes et al. 1992; Roschger et al. 2008). We thus hypothesized that disruption or de novo generation of a splicing *cis*-element determines the clinical phenotype. The five mutations and two SNPs in *COL1A1* exon 45 were predicted to affect 16 putative splicing *cis*-elements by ESEfinder, ESRsearch and PESXs (Table 1). FAS-ESS and RESCUE-ESE predicted no splicing *cis*-elements. All the five mutations with or without two SNPs in *COL1A1* exon 45 variably but slightly weaken acceptor and/or donor splice site strengths according to the NetGene2 (Table 2). The splice site prediction by neural network also predicted that c.3235G>A generates a weak cryptic splice acceptor site in *COL1A1* exon 45 (Table 2). We first examined cultured fibroblasts of II-3 by RT-PCR and found that the *COL1A1* c.3235G>A mutation did not induce aberrant splicing of *COL1A1* (data not shown). NMD was unlikely to have masked aberrant splicing, because we observed heterozygous peaks at c.3235G>A in sequencing the RT-PCR product. We next constructed 18 *COL1A1* minigenes with or without each of the five mutations in the presence or absence of each of the two SNPs (Fig. 2b). RT-PCR analysis of transfected HEK293 cells showed that all minigene constructs gave rise to a single fragment of 336 bp, indicating that splicing was not affected in any mutations or SNPs (Fig. 2c).

Japanese (F1), Italian (F2) and Canadian (F3) families with *COL1A1* c.3235G>A share no founder haplotype

We previously reported *COL1A1* c.3235G>A in the Italian and Canadian families (Marini et al. 2007; Roschger et al.

2008). Although we have not measured serum urate concentrations in these families, gout or urinary stone has not been documented in either family, which suggests that hyperuricemia is not simply due to c.3235G>A.

To pursue if a gene responsible for hyperuricemia is on the same chr as *COL1A1*, we looked for a founder haplotype for c.3235G>A in three families by genotyping three microsatellite markers flanking *COL1A1* (D17S1293, –16 Mbp; D17S1319, –14 kbp; and D17S788, 2 Mbp) and an SNP (rs2075554) in intron 11 of *COL1A1*. The analysis revealed that each family carried a unique haplotype and shared no founder haplotype (Fig. 1). Thus, the mutation is likely to have occurred independently in three ethnic groups. Alternatively, c.3235G>A is an ancient founder mutation, and subsequent multiple recombinations and divergence of microsatellite repeats have obscured a founder effect. In either case, lack of a found haplotype supports the notion that a gene responsible for hyperuricemia is potentially but not necessarily linked to *COL1A1*.

Hyperuricemia is not caused by mutations in *PRPSAP1* or *PRPSAP2*

We thus first looked into candidate genes for hyperuricemia on chr 17 where *COL1A1* (17q21.31–q22) is located. Two candidate genes for hyperuricemia are on chr 17: *PRPSAP1* (MIM# 601249) at 17q24–q25 encoding PAP39 (Ishizuka et al. 1996) and *PRPSAP2* (MIM# 603762) at 17p12–p11.2 encoding PAP41 (Katashima et al. 1998). PAP39 and PAP41 are subunits of phosphoribosylpyrophosphate (PRPP) synthetase that leads to urate production. No mutation has been reported in either gene in any diseases. We sequenced cDNAs of *PRPSAP1* and *PRPSAP2* in II-3, but found no mutation in either gene. As we detected no heterozygous SNPs in *PRPSAP1* and *PRPSAP2*, a mutant allele carrying a premature stop codon might have been missed due to mRNA degradation by NMD.

Resequencing of exomes reveals hyperuricemia-associated SNPs

We next traced a causative gene for hyperuricemia in two siblings (II-2 and II-3) by exome resequencing with the SureSelect human all exon kit v1 (Agilent) and with the SOLiD 3 Plus sequencer (Life Technologies).

We similarly analyzed an unrelated Japanese male (22-year-old) with OI type I and hyperuricemia (VIII-2 in F4 in Fig. 1). His hyperuricemia was by chance detected at age 14 years when he had fractures. His hyperuricemia has been well controlled by medication since then. Exome resequencing of VIII-2 disclosed a novel heteroallelic c.577G>T mutation in *COL1A2* exon 12 predicting

Table 1 Affected putative splicing *cis*-elements in *COL1A1* exon 45 predicted by ESEfinder, ESRsearch and PESXs

| Sequence variation | Normal allele | Variant allele | Predicted effect | Score ^d | | Putative <i>trans</i> -factor |
|---------------------|------------------|-------------------------------|------------------|--------------------|---------|-------------------------------|
| | | | | Normal | Variant | |
| rs1800215 | CCG <u>CCGG</u> | CC <u>ACCGG</u> ^a | Gain | 1.622 | 4.231 | SRp40 |
| rs1800217 | CTGT <u>TGGC</u> | CTGT <u>CGGC</u> ^c | Gain | | n.a. | |
| c.3226G>A | CGCC <u>GG</u> | CGCC <u>AG</u> ^b | Gain | | n.a. | |
| | CCG <u>GCCT</u> | CC <u>AGTCCT</u> ^c | Gain | | n.a. | |
| c.3226G>A+rs1800215 | CCG <u>CCGG</u> | CC <u>ACCAG</u> ^a | Gain | 1.622 | 3.663 | SRp40 |
| c.3226G>T | CGCC <u>GG</u> | CGCC <u>TG</u> ^b | Gain | | n.a. | |
| c.3226G>T+rs1800215 | CCG <u>CCGG</u> | CC <u>ACCTG</u> ^a | Loss | 3.498 | 0.571 | SF2/ASF |
| c.3235G>A | CTGT <u>CGG</u> | CTGT <u>CAG</u> ^a | Loss | 2.492 | 0.713 | SF2/ASF |
| | CTGT <u>CGGC</u> | CTGT <u>CAGC</u> ^c | Loss | | n.a. | |
| | TGTC <u>GGC</u> | TGTC <u>AGC</u> ^a | Gain | 1.058 | 3.613 | SRp40 |
| | GTC <u>GGC</u> | GTC <u>AGC</u> ^b | Gain | | n.a. | |
| | CGGC <u>CTG</u> | CA <u>GCCCTG</u> ^c | Gain | | n.a. | |
| c.3244G>T | <u>GGCGCCCG</u> | <u>TGCGCCCG</u> ^a | Loss | 3.109 | 1.059 | SC35 |
| | <u>GGCGCC</u> | <u>TGCGCC</u> ^a | Gain | 0.721 | 3.531 | SRp55 |
| c.3244G>T+rs1800217 | TGTT <u>TGGC</u> | TGTT <u>CTGC</u> ^a | Gain | -1.326 | 3.367 | SRp40 |
| c.3253G>A | CGT <u>TGC</u> | CGT <u>AGC</u> ^a | Loss | 2.940 | 2.331 | SRp55 |

Variant nucleotides are shown in bold and underlined

n.a. not applicable

^a ESEfinder

^b ESRsearch

^c PESXs

^d Default threshold values employed by ESEfinder are SRp40 = 2.67, SF2/ASF = 1.867, SC35 = 2.383 and SRp55 = 2.676

12.5% regions of the entire genome. We first sought for a mutation responsible for hyperuricemia in 24 genes (see Table 3 and Suppl. Table 2) that are involved in urate metabolisms and excretion, but found none in either patient. We then scrutinized SNPs in ten genes that are known to be associated with hyperuricemia and identified 12 SNPs (Table 3). In this analysis, we excluded SNPs with a minor allelic frequency of 0.01 or less. Among the 12 SNPs, rs2231142 in *ABCG2* as well as rs3825016 and rs11231825 in *SLC22A12* are previously reported and will be addressed in the discussion. We traced the three SNPs in F1, F2 and F3 by capillary sequencing and found that variable dosages of these SNPs were observed in hyperuricemic as well as in normouricemic individuals (Fig. 1).

A missense mutation in *GPATCH8* is likely to lead to hyperuricemia in the Japanese family (F1)

After eliminating SNPs in the dbSNP132 database, only three non-synonymous variants remained shared between II-2 and II-3 on chr 17; c.602A>G in exon 2 of *KRBA2* at 17p13.1, c.206C>T in exon 3 of *ZPBP2* (MIM# 608499) at 17q12 (Fig. 3a) and c.2935G>C in exon 8 of *GPATCH8* at

17q21.31 (Fig. 3b). Capillary sequencing revealed that variants in *ZPBP2* and *GPATCH8* cosegregated with hyperuricemia in F1, but the *KRBA2* variant did not. These two variants were not detected in F2, F3, F4, 100 normal human individuals or exomes of 50 Tibetans (Yi et al. 2010). In addition, exome-capture resequencing of VIII-2 detected no mutations in *ZPBP2* and *GPATCH8*.

ZPBP2 c.206C>T and *GPATCH8* c.2935G>C predict amino acid substitutions of p.T69I and p.A979P, respectively. Threonine 69 and the flanking amino acids of *ZPBP2* are not conserved across mammalian species. Additionally, an SNP rs35591738 mutates the N-terminal proline at codon 68, and an SNP rs34272593 causes a frameshift at codon 70 (Fig. 3a). In contrast, alanine 979 and the flanking amino acids of *GPATCH8* are in the serine-rich region and are highly conserved across mammalian species (Fig. 3b). PolyPhen-2 (Adzhubei et al. 2010) predicted that *ZPBP2* p.T69I was benign with a score of 0.025 and *GPATCH8* p.A979P was damaging with a score of 0.988, where 1.0 was the worst score. Similarly, SIFT (Kumar et al. 2009) predicted that *ZPBP2* p.T69I was tolerated with a score of 0.38 and *GPATCH8* p.A979P was damaging with a score of 0.00, where a score < 0.05 was predicted to be deleterious.

Table 2 Splice site strength of *COL1A1* exon 45 predicted by the NetGene2 and the Splice Site Prediction by Neural Network

| Sequence variation | NetGene2 | | Splice Site Prediction by Neural Network | |
|---------------------|------------|-------|--|-------|
| | Confidence | | Score | |
| | Acceptor | Donor | Acceptor | Donor |
| Wild type | 0.97 | 0.93 | 0.98 | 0.95 |
| rs1800215 | – | – | – | – |
| rs1800217 | – | – | – | – |
| c.3226G>A | – | 0.89 | – | – |
| c.3226G>A+rs1800215 | 0.94 | 0.89 | – | – |
| c.3226G>A+rs1800217 | – | 0.89 | – | – |
| c.3226G>T | 0.94 | 0.87 | – | – |
| c.3226G>T+rs1800215 | 0.94 | 0.86 | – | – |
| c.3226G>T+rs1800217 | 0.94 | 0.87 | – | – |
| c.3235G>A | 0.94 | 0.87 | – | – |
| c.3235G>A+rs1800215 | 0.94 | 0.86 | – | – |
| c.3235G>A+rs1800217 | 0.94 | 0.87 | – | – |
| c.3244G>T | 0.94 | 0.86 | – | – |
| c.3244G>T+rs1800215 | 0.94 | 0.86 | – | – |
| c.3244G>T+rs1800217 | 0.94 | 0.86 | – | – |
| c.3253G>A | 0.94 | 0.88 | 0.52 ^a | – |
| c.3253G>A+rs1800215 | 0.94 | 0.87 | 0.52 ^a | – |
| c.3253G>A+rs1800217 | – | 0.89 | – | – |

– symbol represents being identical to the wild-type

^a In addition to the native splice acceptor site of 0.98, a cryptic splice acceptor site ‘AG’ is generated at c.3253_3254

Table 3 Twelve SNPs identified by exome resequencing in five out of ten genes associated with hyperuricemia

| Ch | Gene | Position | Nuc. | Amino acid | AF | dbSNP | II-2 | II-3 | VIII-2 |
|----|------------------------------|-------------|-------|------------|------|------------|------|------|--------|
| 1 | <i>AGL</i> | 100,336,361 | C > T | Syn. | 0.7 | rs2230306 | T/T | –/T | –/T |
| 4 | <i>ABCG2</i> ^b | 89,034,551 | G > A | Syn. | 0.02 | rs35622453 | –/– | –/– | –/A |
| | | 89,052,323 | C > A | Q141K | 0.31 | rs2231142 | –/A | A/A | –/– |
| | | 89,061,114 | G > A | V12M | 0.19 | rs2231137 | –/A | –/– | –/A |
| 4 | <i>SLC2A9</i> ^b | 9,909,923 | C > T | P350L | 0.33 | rs2280205 | –/T | –/T | –/– |
| | | 9,922,130 | G > A | R294H | 0.72 | rs3733591 | –/A | –/– | –/A |
| | | 9,998,440 | G > A | Syn. | 0.54 | rs10939650 | –/A | A/A | –/A |
| | | 10,022,981 | G > A | G25R | 0.43 | rs2276961 | –/A | –/A | –/– |
| | | 10,027,542 | G > A | A17T | 0.06 | rs6820230 | –/– | –/A | –/A |
| 11 | <i>SLC22A12</i> ^b | 64,359,286 | C > T | Syn. | 0.21 | rs3825016 | –/T | T/T | –/T |
| | | 64,360,274 | C > T | Syn. | 0.81 | rs11231825 | –/T | –/– | –/T |
| 12 | <i>PFKM</i> | | n.d. | | | | | | |
| 16 | <i>UMOD</i> ^b | | n.d. | | | | | | |
| 17 | <i>G6PC</i> | | n.d. | | | | | | |
| X | <i>HPRT1</i> ^a | | n.d. | | | | | | |
| X | <i>PRPS1</i> ^a | | n.d. | | | | | | |
| X | <i>MAOA</i> | 43,591,036 | G > T | Syn. | 0.3 | rs6323 | –/– | T/T | T/T |

^a The gene is associated with purine metabolism

^b The gene is associated with renal excretion of urate

– symbol represents in the patients’ genotypes mean being identical to the reference nucleotides

Syn synonymous nucleotide change, *AF* allelic frequency of the changed nucleotide, *n.d.* no SNPs are detected

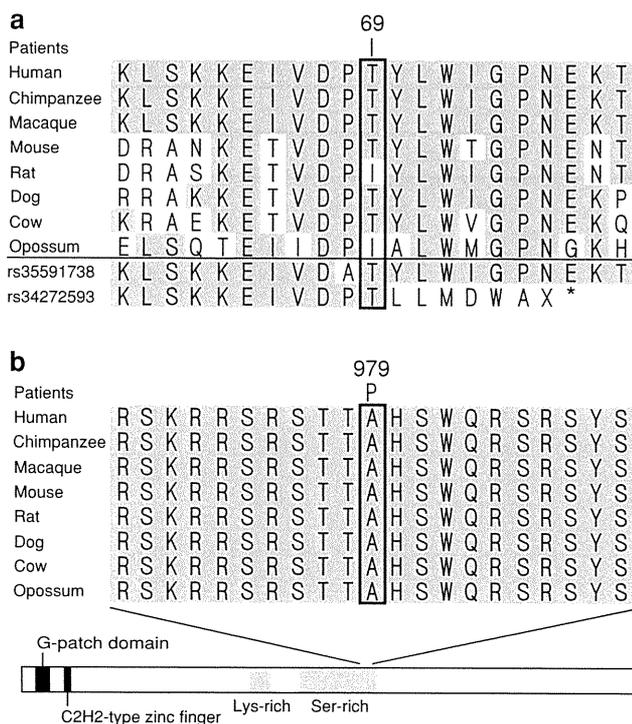


Fig. 3 Conservation of 21 amino acid segments encoded by *ZPBP2* and *GPATCH8* in mammals. Locations of non-synonymous variants identified in the Japanese family (F1) are boxed. Amino acids identical to human are shaded. **a** c.206C>T predicts p.T69I in *ZPBP2*. T69 is not conserved in rat and opossum. An SNP rs35591738 predicts p.P68A, and an SNP rs34272593 induces a frameshift. **b** c.2935G>C predicts p.A979P in *GPATCH8*. The mutation is located at the C-terminal end of the serine-rich region

Discussion

Phenotypic variabilities of OI mutations

We identified a heteroallelic c.3235G>A mutation in *COL1A1* exon 45 in a Japanese family with mild OI type I. The c.3235G>A mutation has been previously reported in six families with OI types I (Hartikka et al. 2004; Mottes et al. 1992; Roschger et al. 2008) and IV (Marini et al. 2007). *COL1A1* exon 45 encodes six of the 338 Gly-X-Y triplet repeats. Four additional mutations have been reported in exon 45 (Constantinou et al. 1989; Lund et al. 1997; Marini et al. 2007), and all substitute Ser or Cys for Gly (Fig. 2a) with mild to lethal phenotypes. Among the five mutations, two mutations introducing Cys result in a lethal type II, whereas three mutations introducing Ser give rise to milder types I, III and IV. This notion, however, cannot be applied to the other exons according to the human type I collagen mutation database (<http://www.le.ac.uk/genetics/collagen/>).

Being prompted by a report that more than 16–20% of exonic mutations disrupt an ESE (Gorlov et al. 2003), we asked if a mutation disrupting an ESE in exon 45 causes

skipping of an inframe exon 45 and exhibits a severe dominant negative phenotype. Three Web-based programs predict that all the five mutations and the two SNPs affect 16 putative exonic splicing *cis*-elements (Table 1). We thus constructed and analyzed 18 minigenes carrying all possible combinations of the five mutations and two SNPs (Fig. 2b), but found that none affected pre-mRNA splicing (Fig. 2c). Our analysis suggests that the currently available algorithms of splicing *trans*-factors cannot efficiently predict splicing *cis*-elements. This is likely because the recognition motifs of splicing *trans*-factors are mostly determined by in vitro SELEX experiments. A recently developed technique, the high throughput sequencing coupled to crosslinking immunoprecipitation method (HITS-CLIP), enables us to extensively determine RNA segments recognized by a specific splicing *trans*-factor in vivo (Licatalosi et al. 2008; Yeo et al. 2009). Accumulation of knowledge with the HITS-CLIP technology will enable us to construct dependable algorithms to efficiently predict splicing *cis*-elements.

In addition to the phenotypic variability among similar mutations in the same exon, the same mutation often exhibits variable phenotypes, although the variability is usually less (Lund et al. 1996). This is also true for our families. In the Japanese family (F1), two sons (II-1 and II-3) experienced many fractures, whereas the father (I-1) and another son (II-2) had no history of fractures. In the Italian family (F2), the son suffered from many fractures, but his affected mother did not (Mottes et al. 1992). In the Canadian family (F3), the father was classified as OI type IV, whereas his daughter as OI type I (Roschger et al. 2008). Variable clinical phenotypes of the c.3235G>A mutation is likely due to differences in environmental factors or to SNPs in disease-modifying genes, but the molecular bases have not been elucidated in any type of OI.

Molecular basis of hyperuricemia

In F1, hyperuricemia cosegregated with OI type I. Although no known genes causing hyperuricemia are on chr 17 where *COL1A1* is located, two candidate genes of *PRPSAP1* and *PRPSAP2* are on chr 17. Capillary sequencing of these genes, however, detected no mutation. We thus employed exome-capture resequencing analysis of two siblings in F1 to search for a responsible gene for hyperuricemia. We first looked into SNPs in the 10 candidate genes that are associated with hyperuricemia, purine metabolism and renal excretion, and found 12 SNPs in 10 genes (Table 3). Among them, three SNPs in *ABCG2* (rs2231142) and *SLC22A12* (rs3825016 and rs11231825) are previously reported markers for hyperuricemia and/or gout.