Figure 4. Cooperation of MLL-AF10 with activated K-ras induced acute monoblastic leukemia. (A) Kaplan-Meier survival analysis of mice receiving transplants of human HSCs transfected with EV (n = 8), K-ras ^{G12V} (n = 12), MLL-AF10 (n = 6), or MLL-AF10 plus K-ras^{G12V} (n = 6) vectors. (B) GFP and Venus expression in peripheral blood cells at the indicated weeks after transplantation with human HSCs co-transfected with the MLL-AF10 and K-ras^{G12V} genes. (C) May-Giemsa staining of the peripheral blood of mice engrafted with human HSCs co-transfected with the MLL-AF10 and K-ras^{G12V} genes. Morphologic leukemia cells were found in the peripheral blood of these mice 50 days after transplantation. (D) Splenomegaly in the MLL-AF10/K-ras^{G12V} mice. Spleens from mice engrafted with EV-transduced HSCs (left) and MLL-AF10/K-ras^{G12V} co-transduced HSCs (right) are shown. The graph shows the mean ± SD of the spleen weights from mice receiving transplants of EV-transduced HSCs (n = 6) or of MLL-AF10/K-ras^{G12V} co-transduced HSCs (n = 6). ** represents $p<0.01$.
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transplantation (Figure 4D), and the average weight of their spleens was 5 times greater than in the other groups (Figure 4D). By 10 weeks after transplantation, 100% of the mice that had received MLL-AF10/K-ras^{G12V} co-transduced HSCs were dead, while all the mice in the other 3 groups survived and remained healthy 25 weeks after transplantation (Figure 4A).

We next investigated by FACS the composition of the blood cells in the BM, spleen, and liver of the MLL-AF10/K-ras^{G12V}-expressing HSC-treated mice. Most of the hematopoietic cells in these tissues were GFP$^+$ Venus$^+$ double positive (Figure 5A). The double-positive cells were confirmed to express both the MLL-AF10 and K-ras^{G12V} genes by RT-PCR with specific primers for the exogenously transduced genes (Figure 5B). Interestingly, no cells expressing MLL-AF10 alone (GFP$^+$ Venus$^-$) or K-ras^{G12V} alone (GFP$^-$Venus$^+$) were observed in any mice in the co-transduced group, even though MLL-AF10 alone could effectively promote multilineage hematopoiesis (Figures 1 and 2). More importantly, in the group receiving K-ras^{G12V} alone-transduced HSCs, there were no Venus$^+$ K-ras^{G12}-expressing blood cells in any tissues examined, even in the BM (data not shown). These results indicate that the co-expression of MLL-AF10 and K-ras^{G12V} (GFP$^+$Venus$^+$) was necessary for the *in vivo* induction of leukemia from human HSCs.

Additional FACS data showed that the GFP$^+$Venus$^+$ human CD45$^+$ blood cells in the all recipient mice transfused with the co-transfected HSCs had a uniform surface marker profile, CD33$^+$CD11b$^+$HLA-DR$^+$CD14$^+$CD15$^+$ (Figure 5C), which was fully compatible with the FAB M5 phenotype. This finding suggested that the abnormal blood cells observed in the mice receiving co-transduced HSCs were leukemia cells. We next examined the cellular clonality of the abnormal blood cells by Southern blotting. As shown in Figure 5D, an EGFP probe that recognizes both EGFP and Venus (because the nucleotide sequences between EGFP and Venus are 98% identical) revealed one or two bands in the DNA of the abnormal leukocytes from individual mice in the co-transduced group, indicating the monoclonality of the abnormal leukocytes.

Taken together, we have successfully established a human leukemia model in which acute monoblastic leukemia cells are derived from human normal HSCs *in vivo*.

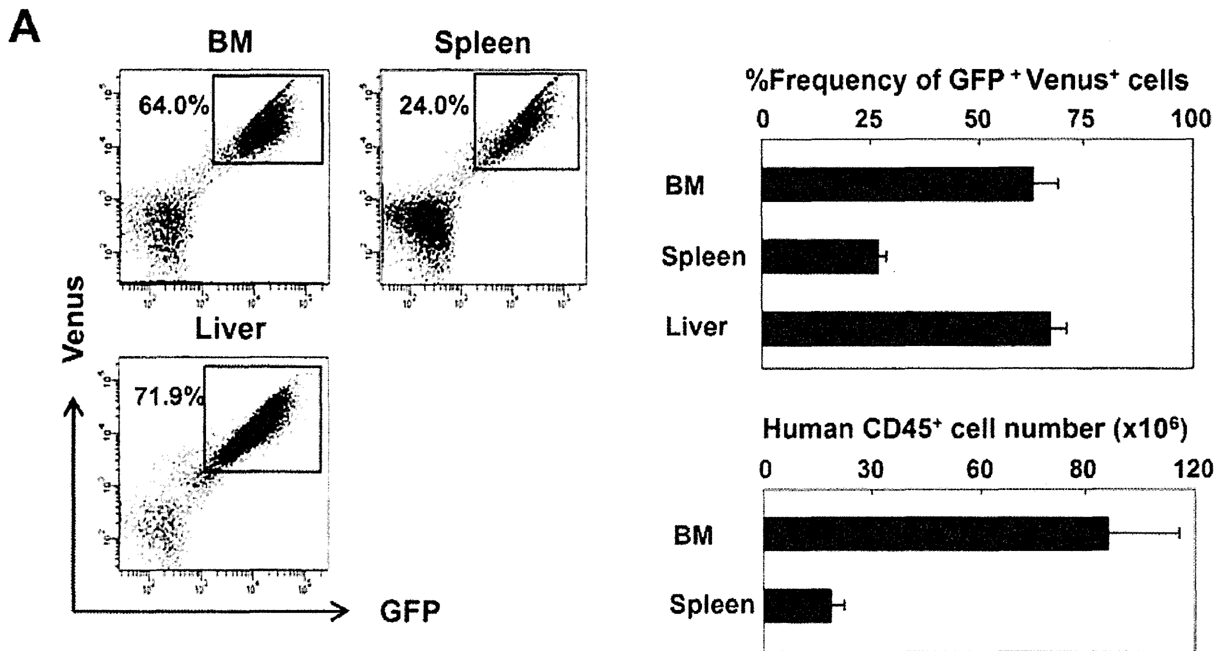
Pathological Phenotypes of the Leukemia

Pathological examination of our human leukemia model showed that the spleens were extensively infiltrated with human hematopoietic cells, and the architecture of the red pulp and the white pulp was disrupted (Figures 6A and 6B). The tibia bones looked very pale (data not shown), and the BM was occupied by uniform blood cells, which expressed human CD45 (Figures 6A and 6B). The periportal regions of the liver were also massively infiltrated with human hematopoietic cells (Figure 6A and 6B). Hepatosplenomegaly is a common symptom in patients with acute monoblastic leukemia with rearranged MLL genes, and leukemia cell infiltration into the intrahepatic periportal regions is also a common pathological manifestation of human MLL-rearranged monoblastic leukemia. Therefore, our model may be a good representation of human MLL-rearranged monoblastic leukemia.

Discussion

A detailed understanding of leukemogenesis requires the development of experimental murine models that can accurately mimic this process. Some studies have sought to recapitulate the harboring of MLL fusion genes in human AML using mouse HSCs [6]. However, the leukemia occurring in mice often does not faithfully recapitulate human leukemia, in part because of the biological differences between human and mouse HSCs [8,27]. In two improved models, the MLL-ENL or MLL-AF9 gene was recently shown to be capable of initiating human leukemogenesis in NK-cell-depleted NOD/SCID mice [18] or human cytokine transgenic NOD/SCID mice [19]. However, overexpression of the MLL-AF4 fusion gene in human CD34$^+$ cells is not sufficient to initiate leukemia [20]. Similar to the latter report, we found that MLL-AF10 was insufficient to induce leukemogenesis at least by 25 weeks after transplantation in the present model. In our experiments, the infectious efficiency of the MLL-AF10 vector in human HSCs was low (<2.0%). Although the efficiency of MLL-ENL and MLL-AF9 in the former report was unclear [18], differences in the infectious efficiency of the vectors used among these studies might have been responsible for the different leukemogenic effects. In another model with MLL-AF9 [19], human SCF, GM-CSF, and IL-3 were transgenically expressed in the recipient NOD/SCID mice. These cytokines may provide the MLL-AF9-transdused HSCs with additional signals for cell growth and survival, which might work as an oncogenic promoter. Comparing our model with this model, ectopic expression of K-ras^{G12V} in our model may in part compensate the cytokine (SCF, GM-CSF, and IL-3) signals in the MLL-AF9 model since stimulation with SCF and cytokines directly activates the ras signaling pathway in leukemogenesis [28]. However, it is also likely that each MLL-rearranged gene (MLL-ENL, MLL-AF4, MLL-AF9, and MLL-AF10) has a different leukemogenic efficacy, because the different partners of MLL may confer different biological functions to the produced MLL-fusion molecule [2].

Accumulating evidence points to a multistep pathogenesis for leukemia development and progression [29,30]. In the multi-genetic step models of leukemogenesis, particularly in the two-hit model of leukemia, the initial genetic hit often leads to abnormal cell differentiation (Type II mutation), while subsequent mutations may activate specific signaling pathways that are involved in cell growth, such as the ras/MAP kinase pathway (Type I mutation) [3,29,31]. Indeed, MLL-rearranged AML in neonatal bloodspots was shown to be of prenatal origin, supporting the idea that MLL rearrangement is one of the earliest events (first hit) in leukemogenesis [32]. In this line, the effect of MLL-AF10 as the first hit is thought to be differentiation arrest or the promotion of a specific hematopoietic lineage, because two previous studies demonstrated that MLL-AF10 expression in mouse HSCs results in skewed myeloid hematopoiesis [21,22]. However, in human HSCs, MLL-AF10 did not give rise to skewed hematopoiesis, but



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24.0%
71.9%
Venus
GFP
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Human CD45⁺ cell number (x10⁶)
0
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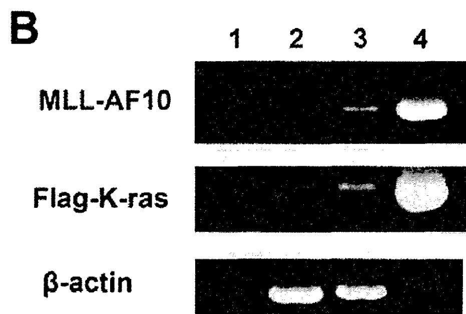
Spleen



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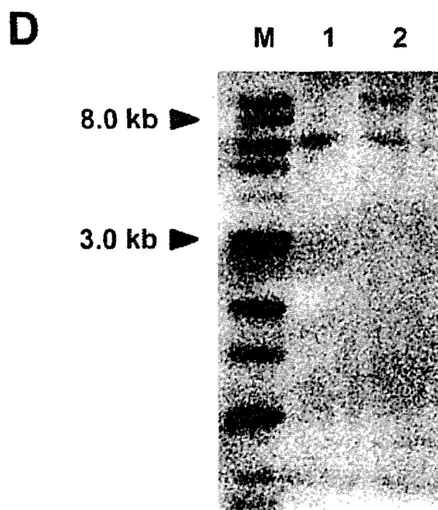

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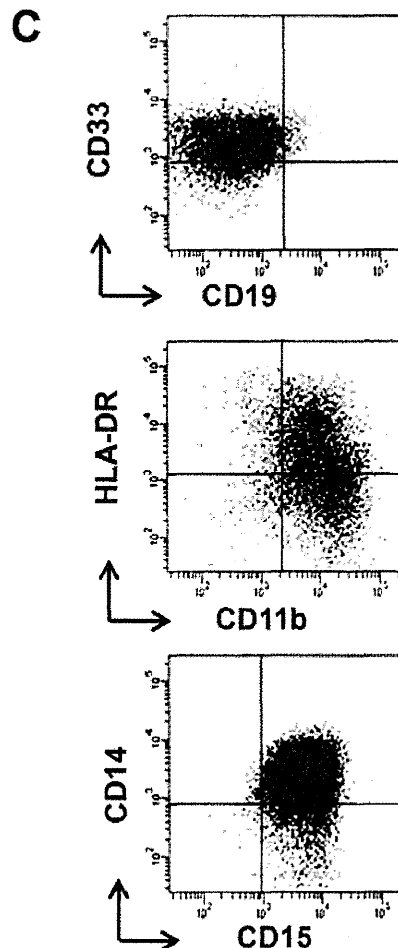

C
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CD11b
CD14
CD15

Figure 5. Immunophenotype and clonality of the MLL-AF10/K-ras-induced leukemia. (A) Frequencies of GFP^+/$Venus^+$ cells or human $CD45^+$ cells in the BM, spleen, and liver at 8 weeks after transplantation with human HSCs co-transfected with the MLL-AF10 and K-ras^{G12V} genes were examined by flowcytometric analysis. The flowcytometry data shown are representative of 6 to 8 mice per group in one representative experiment of two (left). The average of %frequencies of the GFP^+ and $Venus^+$ cells in whole cells in the indicated organs is shown with the standard deviation (right, upper; n = 6). The absolute cell number of human $CD45^+$ cells in the indicated organs is shown with the standard deviation (right, lower; n = 6). (B) Representative RT-PCR results confirming the stable, long-term expression of the MLL-AF10 and Flag-K-ras^{G12V} transcripts in human hematopoietic cells in the BM of mice 8 weeks after transplantation. (C) Lineage distribution of the GFP^+ and $Venus^+$ cells in the BM of a mouse engrafted with HSCs expressing MLL-AF10 and activated K-ras. (D) Southern blot analysis of DNA prepared from the human blood cells in the spleen of mice receiving transplants of MLL-AF10/K-ras^{G12V} co-transduced HSCs. Independent leukemia samples derived from two mice (lane 1; mouse 1 and lane 2; mouse 2) were examined. DNA was digested with Bgl II and probed with an EGFP probe. M: marker.
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enhanced the growth (or survival) of hematopoietic cells (Figures 1B and 2). Therefore, the biological impact of MLL-AF10 as the first hit remains controversial.

The active mutation of ras genes is a known additional hit in MLL-rearranged leukemia [27,29,33], and was so in our model. The hematopoietic cells undergoing the first hit must survive until the additional hit occurs during leukemogenesis. Since no HSCs transdused with K-ras^{G12V} alone could be found in the BM of the humanized mice even 25 weeks after transplantation (data not shown), it is unlikely that the ras mutation was the first hit in our model. Based on the present results, MLL-AF10 as the first hit might alter the self-renewal regulation of HSCs because of its promotion effect on multi-lineage hematopoiesis (Figures 1B and 2), and the HSCs (or leukemic precursor cells) might then receive the additional hit (K-ras^{G12V}), leading to leukemia. A recent report similarly demonstrated that co-transduction of BMI1 and Bcr-Abl oncogenes in HSCs induced leukemia, in which ectopic expression of BMI1 probably functioned as the additional hit, in NOD/SCID mice [34]. Although further examination will be required to test the additional hit hypothesis in leukemogenesis, our leukemia model may be a useful and unique experimental system with which to examine the multi-hit model of leukemogenesis.

Previous studies in which primary human leukemia cells were transplanted into immunodeficient mice provided important information about leukemia biology, in particular, about the biological significance of leukemic stem cells. Ishikawa et al recently demonstrated using NSG mice that the $CD34^+CD38^-$ cell population of the donor human leukemia most easily expands *in vivo* and survives by associating with the osteoblast-like stroma cells [9,10]. These findings shed light on how the leukemic stem cells are maintained *in vivo*. However, evaluating the transplantability or tumorigenic potential of each subpopulation from primary leukemia into immunodeficient mice might be insufficient to define leukemic stem cells. Intriguingly, a recent report

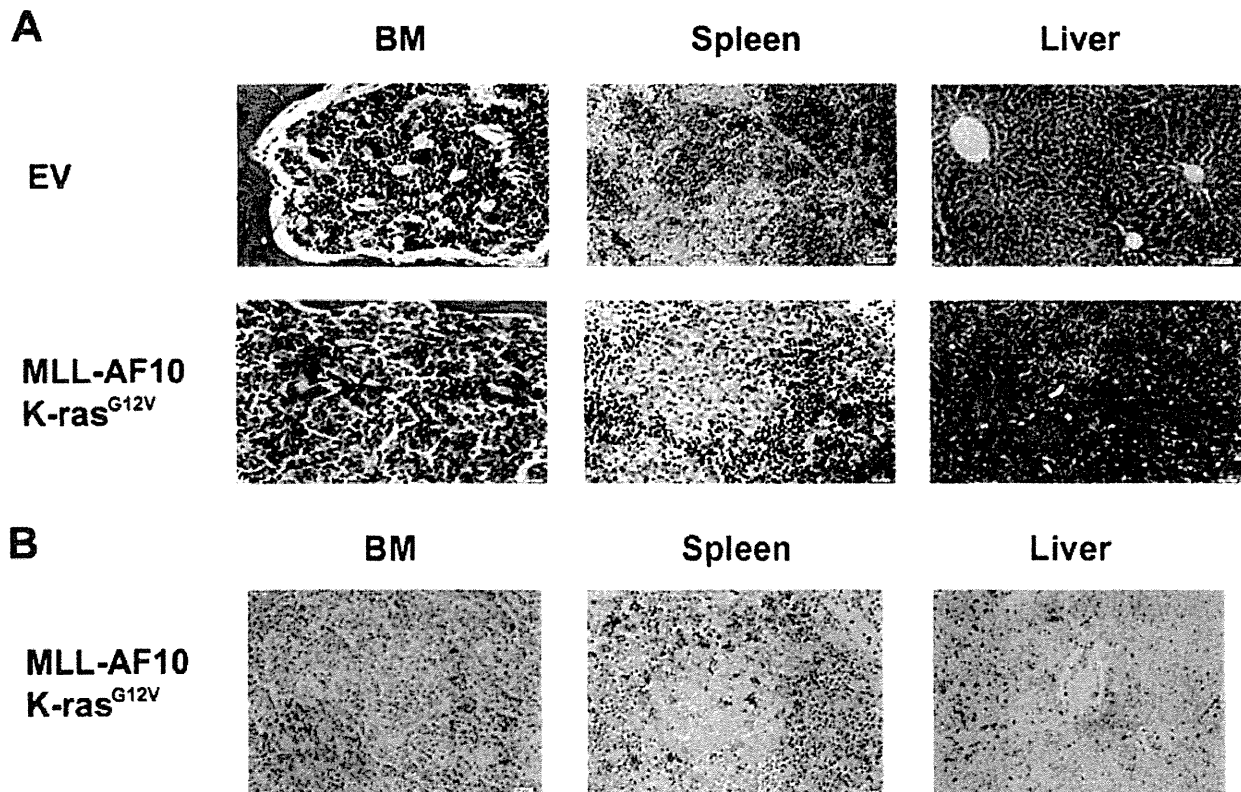

Figure 6. Pathological phenotypes of the leukemia. (A) Hematoxylin and eosin staining showing the infiltration of leukemic cells in the indicated organs of mice engrafted with HSCs expressing the MLL-AF10 and K-ras^{G12V} genes compared to control mice. (B) Immunostaining by a human CD45 mAb in the BM, spleen, and liver in mice engrafted with HSCs expressing the MLL-AF10 and K-ras^{G12V} genes.
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demonstrated using primary human melanoma cells that most of the cells (putative non-stem cells) that were not transplantable in NOD/SCID mice, which have NK cells, could easily survive and expand, like stem cells, in NSG mice, which lack NK cells [35]. This indicates the possibility that the high transplantability of the putative stem cell population as defined in the NOD/SCID mice reflected simply their resistibility to NK-cell-killing. Therefore, the observations in a certain transplantation model using immunodeficient mice may not necessarily reflect the physiological pathogenesis of human leukemic stem cells.

In this context, the leukemia that arose *in vivo* from human HSCs as demonstrated here may better reflect the physiological biology of human leukemic stem cells. Furthermore, the primary leukemia cells from different patients with leukemia showing even the same phenotypic markers should be different. Thus, the *in vivo* results from mice receiving transplants of different donor cells may be difficult to interpret, even if the leukemia cells from individual donors show the same phenotype. In contrast, the M5 leukemia established here was uniform in terms of cell morphology, symptoms (including selective tissue infiltration), and even survival prognosis. Importantly, the high reproducibility by which the same gene combination (MLL-AF10 and K-ras^{G12V}) induced the same leukemia in individual mice may represent a considerable advantage of these mice for the *in vivo* modeling of human leukemia over previous models.

To examine whether the leukemia is transplantable in a second recipient, we transplanted the leukemic cells from the spleen and BM to healthy adult NOG mice at least 20 times. Unexpected, we, however, could not find any GFP$^+$ cells in any second recipients even 10 months after transplantation (data not shown). Since GFP$^+$Venus$^+$ leukemic cells in the BM of the first hosts contained less than 0.1% CD34$^+$CD38$^-$ putative AML stem cells (data not shown), leukemic stem cells might have lost their self-renewal ability and been run out in the first host. To understand the unexpected phenomenon, Further investigation will be required.

Over the last decade, the outcome in pediatric AML has improved significantly, with up to 60% of children suffering from MLL-rearranged AML currently surviving [3]. However, improving the outcome of pediatric AML using current treatment protocols is hampered by treatment-related deaths and long-term side effects. In addition, most patients with MLL-rearranged ALL still indicate a poor prognosis. Therefore, to improve the outcome in pediatric MLL-rearranged leukemia, the development of leukemia-specific targeting drugs is an important strategy [36]. We hope that this newly established model using humanized mice will contribute to future studies aimed at revealing the molecular mechanisms for MLL-rearranged gene related leukemogenesis and developing new therapies against MLL-related malignancies.

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Author Contributions

Conceived and designed the experiments: NI KM SK ST KS. Performed the experiments: KM MS YW TT. Analyzed the data: TU YS. Contributed reagents/materials/analysis tools: YA SK MM. Wrote the paper: NI KM.

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Two Novel Mutations in the Lactase Gene in a Japanese Infant with Congenital Lactase Deficiency

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Intestinal lactase is required for the hydrolysis of lactose that is the most essential carbohydrate in milk and the primary diet source of newborn. Congenital lactase deficiency [CLD (MIM 223000)] is a severe gastrointestinal disorder and is characterized by watery diarrhea due to an extremely low or the lack of lactase activity in the intestinal wall from birth. CLD is a rare disease and occurs more frequently in Finland. Recent studies have shown that mutations in the coding region of the lactase (*LCT*) gene underlie CLD in patients from Finland and other European countries. Here, we report two novel mutations in the *LCT* gene in a Japanese female infant with clinical features consistent with those of CLD. She suffered from severe watery diarrhea from the age of 2 days on breast milk/lactose containing cow's milk formula. With the lactose-free hydrolyzed cow's milk formula, diarrhea was stopped, and she has now developed well on a lactose-free diet. She shows a lactose-intolerance pattern on the lactose challenge test. Sequence analysis revealed the two mutations in her *LCT* gene: c.4419C>G (p.Y1473X) in exon 10 transmitted from her mother and c.5387delA (p.D1796fs) in exon 16 transmitted from her father. Both mutations cause premature truncation of lactase polypeptide and are supposed to be responsible for CLD. To our knowledge, this is the first report on mutations in the *LCT* gene in Japan. We suggest that an increased awareness is required regarding CLD.

Keywords: congenital lactase deficiency; cow's milk allergy; *lactase* gene; oral lactose challenge test; watery diarrhea
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Intestinal lactase is required for the hydrolysis of lactose, which is the most essential carbohydrate in milk and the primary diet source of newborn. Congenital lactase deficiency [CLD (MIM 223000)] is a severe gastrointestinal disorder characterized by watery diarrhea due to an extremely low or the lack of activity of lactase in the intestinal wall from birth. Affected infants are suffered from severe watery diarrhea shortly after the first feed with breast milk or lactose-containing formulas (Savilahti et al. 1983). Despite adequate feeding, they are dehydrated and have poor weight gain, because they are unable to hydrolyze lactose that accounts for 40% of energy ingested among infants. This disease is a rare autosomal disorder and occurs more frequently in Finland. Recently, mutations in the coding region of the lactase (*LCT*) gene were revealed to be the underlying cause of CLD and the molecular background is being identified. The *LCT* gene consists of 17 exons encoding 1927 amino acids comprising four homologous domains, I - IV. Domain IV harbors lactase activity. One mutation, c4170T>A (p.Y1390X) in exon 9, is enriched in Finnish population, and 84% of Finnish patients were homozygous for this mutation. Y1390X is located in domain IV, and results in a truncation of lactase (Kuokkanen et al. 2006; Behrendt et al. 2009).

Here, we report a Japanese female infant with clinical features consistent with those of CLD who has two novel mutations in the *LCT* gene in a heterozygous form: c.4419C>G (p.Y1473X) in exon 10 and c.5387delA (p.D1796fs) in exon 16. Both of the mutations are located in the domain IV and supposed to be causative of CLD. To our knowledge, this is the first report on mutations in the *LCT* gene in Japan, and our findings suggest that an increased awareness is required regarding CLD.

Clinical Report

The patient is the first child of healthy nonconsanguineous Japanese parents. She was born at term after an uneventful pregnancy with a birth weight of 3,124 g. When the patient was fed breast milk and lactose-containing formula, she developed watery diarrhea at the age of 2 days. At the age of 4 days, she was admitted to the department of pediatrics because of poor weight gain and dehydration.

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On admission, her weight was 2,722 g, and the serum levels of blood urea nitrogen, creatinine, sodium, and potassium were 29.4 mg/dL, 0.4 mg/dL, 157 mEq/L, and 5.4 mEq/L, respectively. Dehydration resolved after intravenous infusion was initiated, but severe watery diarrhea continued to be observed. The result of a stool culture was negative; in addition, rapid antigen tests for rotavirus and adenovirus were negative. Watery diarrhea promptly disappeared when the oral intake was discontinued at the age of 9 days and did not recur when she was administered a diet with lactose-free hydrolyzed cow's milk formula at the age of 10 days. She showed a remarkable improvement in weight gain, and the intravenous infusion was discontinued at the age of 12 days. She was discharged from the hospital at the age of 17 days, showed good body weight gain, and was free from gastrointestinal symptoms. She was suspected to have CLD or cow's milk protein allergy, and was admitted to our hospital for a lactose challenge test at the age of 4 months. The oral lactose challenge test with 2 g/kg of lactose showed no increase in the blood glucose level within 120 min and was followed by watery diarrhea within a few hours (Fig. 1). The serum level of total immunoglobulin E (IgE) antibody was 3 IU/mL and that of serum-specific IgE antibody to whole cow's milk measured using a chemiluminescent enzyme immunoassay was 0.07 lumicount; class 0 (MAST33; SRL, Tokyo, Japan). Lymphocyte stimulation tests for α-lactalbumin, β-lactoglobulin, α-casein, β-casein, and κ-casein were negative (stimulation indices were 0.7,

Fig. 1. Oral lactose challenge test at the age of 4 months.
After oral administration of 2 g/kg of lactose, no increase was observed in the blood glucose within 120 min and watery diarrhea developed. In contrast, a marked increase was observed in the blood glucose level after drinking 1 g/kg of glucose and no gastrointestinal symptoms were observed.

Fig. 2. Sequence analysis of the *LCT* gene.
a. LCT Ex10 c.4419C>G (p.Y1473X). b. LCT Ex16 c.5387delA (p.D1796fsX)

0.9, 1.1, 1.3, and 1.0, respectively). The oral lactose challenge test was repeated at the age of 5 months, and the patient continued to show a lactose-intolerance pattern (no increase in the blood glucose level).

After experiencing acute gastroenteritis, she passed blood-tinged stools for a few weeks and underwent sigmoid colonoscopy with biopsy. The colonoscopy showed lymphoid nodular hyperplasia with patchy erythema, and histological examination indicated a relatively high number of eosinophils (about 10 per high power field) and a small number of neutrophils in the lamina propria. Although these findings were consistent with those of mild proctocolitis, they were not sufficiently strong to confirm the presence of a food allergy.

To confirm whether lactose intolerance was primary or secondary, we performed sequence analysis of 17 exons of the *LCT* gene after the patient's parents provided written informed consent. The Ethics Committee of the Tohoku University School of Medicine approved the present study. The result showed two novel mutations: c.4419C>G (p.1473X) in exon 10 and c.5387delA (p.D1796fs) in exon 16 (Fig. 2). The p.Y1473X mutation was transmitted from her mother, and the other mutation (p.D1796fs) was transmitted from her father.

The patient is now administered a lactose-free diet, and her psychomotor development was appropriate for her age at the latest examination at the age of 11 months.

Discussion

CLD is one of the rare autosomal disorders commonly occurring in the Finnish population because of a founder effect and genetic drift. A few cases of CLD in patients with different ethnic origins have also been reported. The incidence of CLD was estimated to be 1:60 000 newborns in Finland on the basis of the number of patients who had been diagnosed until 1998 (Järvelä et al. 1998). After the molecular background of CLD was confirmed, the number of patients newly diagnosed with CLD in Finland increased, and the novel *LCT* mutations were reported in the CLD patients with different ethnic origins (Torniainen et al. 2009). In Japan, only few cases of CLD have been reported since Akabane and Arakawa published the first case in 1965 (Akabane 1965; Yabuuchi et al. 1966; Nose et al. 1979). Infants who develop severe watery diarrhea after consuming breast milk/lactose-containing formula are unlikely to be suspected of having CLD because this disease is thought to be very rare.

The nascent lactase polypeptide comprises four homologous domains, I-IV. After posttranslational processing, the mature lactase contains only domains III and IV. Domain IV comprises lactase activity, and domains I-III act as intramolecular chaperone which is critical for the maturation during lactase-folding process (Kuokkanen et al. 2006; Behrendt et al. 2009). To date, nine mutations are known to underlie CLD and there are quite evenly distributed covering both the pro-region and the mature lactase (Kuokkanen et al. 2006; Torniainen et al. 2009). Five of them result in a premature stop codon. One of the missense mutations, G1363S, located in the domain III, leads to defective lactase activity and impaired trafficking of mutant lactase polypeptide to the cell surface at physiological temperature (Behrendt et al. 2009).

In the case of our patient, she was suspected of having CLD or cow's milk protein allergy in the early neonatal period, because her symptoms improved with a change in her diet from breast milk/lactose-containing formula to hydrolyzed cow's milk formula, which is lactose-free. Small bowel biopsy would be useful to distinguish CLD from cow's milk protein allergy (Heyman 2006), but it is an invasive procedure and requires excellent technical skills. Accordingly, since it would need to have been performed in a 4-month old baby, we performed sequence analysis as the diagnostic examination. Sequence analysis revealed that she has one nonsense mutation and one frame-shift mutation in domain IV. These mutations lead to premature truncation of lactase protein being causative of CLD. Sequence analysis would be useful for the diagnosis if a CLD patient also has cow's milk protein allergy.

Our findings suggest that CLD is possibly more common in Japan than it was thought to be. CLD patients may be treated as patients with cow's milk protein allergy, using lactose-free hydrolyzed cow's milk formula. Sequence analysis is useful for diagnosing CLD, which is sometimes difficult to distinguish from cow's milk protein allergy. Pediatricians should have an increased awareness regarding CLD.

Conflict of Interest

The authors have no conflict of interest associated with this article.

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