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ORIGINAL ARTICLE

Adults with germline *CBL* mutation complicated with juvenile myelomonocytic leukemia at infancy

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Juvenile myelomonocytic leukemia (JMML) appears to be a life-threatening disease and showed poor prognosis even after hematopoietic stem cell transplantation (HSCT) because of high relapse rate. On the other hand, recent molecular analysis revealed the heterogeneity of JMML. Here we report that two JMML patients survived >20 years without HSCT and both patients had uniparental disomy of 11q23 where *CBL* is located without the phenomenon found in neither Noonan syndrome nor Noonan syndrome-like disorder. We think that some JMML patients with *CBL* mutation might show the good prognosis in later life after remission of JMML.
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INTRODUCTION

Juvenile myelomonocytic leukemia (JMML) was estimated to show the poor prognosis.[1] Hematopoietic stem cell transplantation (HSCT) was strongly recommended for JMML patients, however, ~30% of patients showed relapse even after HSCT.[2] Recent study revealed that the prognosis might be different from the genetic alterations, *NRAS*, *KRAS*, *PTPN11*, *NF1* and *CBL*.[3]

Perez *et al.*[4] identified a heterozygous germline mutation in the *CBL* gene (Y371H) in three unrelated JMML patients with a Noonan syndrome-like disorder. The mutation occurred *de novo* in two patients and was inherited from an unaffected father in one patient. Leukemic cells of all patients showed somatic loss of heterozygosity (LOH) at chromosome 11q23, including the *CBL* gene. These findings indicate that heterozygous mutation in the *CBL* gene is associated with predisposition for the development of JMML.

We found two JMML patients survived with good health longer than 20 years without HSCT (20-year-old female and 31-year-old male). Molecular analysis revealed that these two patients had the germline *CBL* mutation with different frequency in each organ. Uniparental disomy (UPD) of chromosome 11q23 was also found in the recent peripheral blood, both. Furthermore, there are no typical features of Noonan syndrome nor Noonan syndrome-like disorders in either patients. We will discuss about JMML with the *CBL* mutation.

MATERIALS AND METHODS

We analyzed several genes affecting the JMML leukemogenesis as follows, *NRAS*, *KRAS*, *PTPN11*, *CBL*, *SETBP1* and *JAK3*.[5–9] Written informed consent including picture presentation was obtained from each patient and institutional review board of Okayama University Hospital approved this project. Total DNA was extracted from stored bone marrow mononuclear cells sample, stored or recent peripheral blood mononuclear cells sample, buccal smear cells, nail or hair using QIAamp DNA Mini or Investigator kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. PCR was performed using primer pair as described previously.[5,10–13] PCR product was directly sequenced using ABI 310 or 3130 sequencer (Applied Biosystems, Tokyo, Japan).

We used pyrosequencing to quantify the fraction of mutated alleles in DNA samples from the different somatic tissues. DNA extracted from samples was analyzed using the PyroMarkQ24 Gold Reagents according to the manufacturer's recommendation (QIAGEN). Data analysis was performed using the allelic quantitation software of the PyroMark Q24 system.

Genome-wide analysis for genetic lesions of mutated *CBL* was performed by single-nucleotide polymorphism array analysis. DNA extracted from samples was analyzed using the GeneChip Human Mapping 250K *NspI* array (Affymetrix, Santa Clara, CA, USA). The data thus obtained were processed using CNAG/AsCNAR software.

Patients

Diagnostic criterion of JMML was dependent on WHO 2008 classification.[14]

Case 1. A 6-month-old girl was admitted to our hospital because of abdominal swelling. She did not have the characteristics of Noonan syndrome (Supplementary Figure 1A). She was diagnosed as having JMML. We repeated chemotherapy using low-dose cytarabine ($30\ mg\ m^{-2}$ for 10 days) and 6-mercaptoprine ($40\ mg\ m^{-2}$ for 7 days) for 126 times (~10.5 years). Leukocytosis and hepatosplenomegaly diminished within 1 year after starting chemotherapy. She is now 20-year old and healthy without short stature, hearing loss, optic atrophy, congenital heart defects, malformations of certain blood and lymph vessels, hypertension or cardiomyopathy. Her intelligence

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quotient was borderline (intelligence quotient test was 70–79). On her recent laboratory test, anti-nuclear antigen has been positive, however, she has no symptom of collagen disease.

Case 2. A 9-month-old boy was admitted to our hospital because of hepatosplenomegaly and leukocytosis. He did not have the characteristics of Noonan syndrome (Supplementary Figure 1B). He was diagnosed as having JMML. We continued chemotherapy using cytarabine and 6-mercaptoprine for ~9 years. Hepatosplenomegaly and leukocytosis diminished within 2 years after starting chemotherapy. He is now 31-year old and healthy without short stature, intellectual disability, hearing loss, optic atrophy, congenital heart defects, malformations of certain blood and lymph vessels, hypertension or cardiomyopathy.

RESULTS

The same *CBL* Tyr(Y)371His(H) mutation was found in the recent peripheral blood mononuclear cells in both patients without other gene mutations. Furthermore, the same *CBL* mutation was found in the diagnostic bone marrow mononuclear cells of case 1. Unfortunately, we could not check the diagnostic sample of case 2 because his sample was not available. We identified the same *CBL* Y371H mutation in DNA derived from buccal smear cells, nails of hands and hairs in two patients (Figure 1). The mutated frequency by pyrosequencing was different in each sample (Figure 1). In DNA from buccal smear cells of the both patients' parents, no mutation was detected (Figure 1). We found that both cases are *de novo* mutation.

SNP array data suggested that *CBL* mutations were related to the LOH of chromosome 11q that included the *CBL* gene, both (Figure 2).

DISCUSSION

JMML was estimated to be a life-threatening disease and HSCT was strongly recommended as soon as possible, however, high relapse rate was still observed and resulted in the poor prognosis. Recent study revealed that JMML prognosis might be quite different from the genetic alterations.[5–9]

Our two cases survived for >20 years without HSCT and had the same sporadic germline *CBL* mutation with 11qUPD. Recently, germline *CBL* mutation syndrome was presented.[3,4,15,16] Interestingly, *CBL* mutation in our two patients was found with different frequency in the different organ even after JMML remission. *CBL* mutations are generally associated with LOH of the 11q23 chromosomal region resulting in apparent homozygosity for a *CBL* mutation in JMML,[17] but our cases still showed the LOH of the 11q23 in healthy PB samples, especially case 1. Previous reports suggested that germline heterozygous missense *CBL* mutations were detected in four sporadic and two familial cases (total of seven cases).[4,15] None of the seven individuals with a *CBL* mutation had any hematological or solid tissue malignancy; however, the authors proposed the hypothesis that carriers of a germline *CBL* mutation could be at increased risk for both, analogous to the predisposition to malignancies seen in NF1, another disorder involving the *RAS-MAPK* pathway.[4,15] These studies suggest that germline heterozygous *CBL* mutation carriers are susceptible to malignancy if reduction to homozygosity in somatic tissues occurs owing to acquired UPD. For example, Kato *et al.*[18] reported duplication of *KRAS* due to acquired UPD caused JMML aggressive transformation. However, now our two cases have no malignancy except for JMML at infancy, although they had the germline *CBL* mutation with 11qUPD. The relationship between

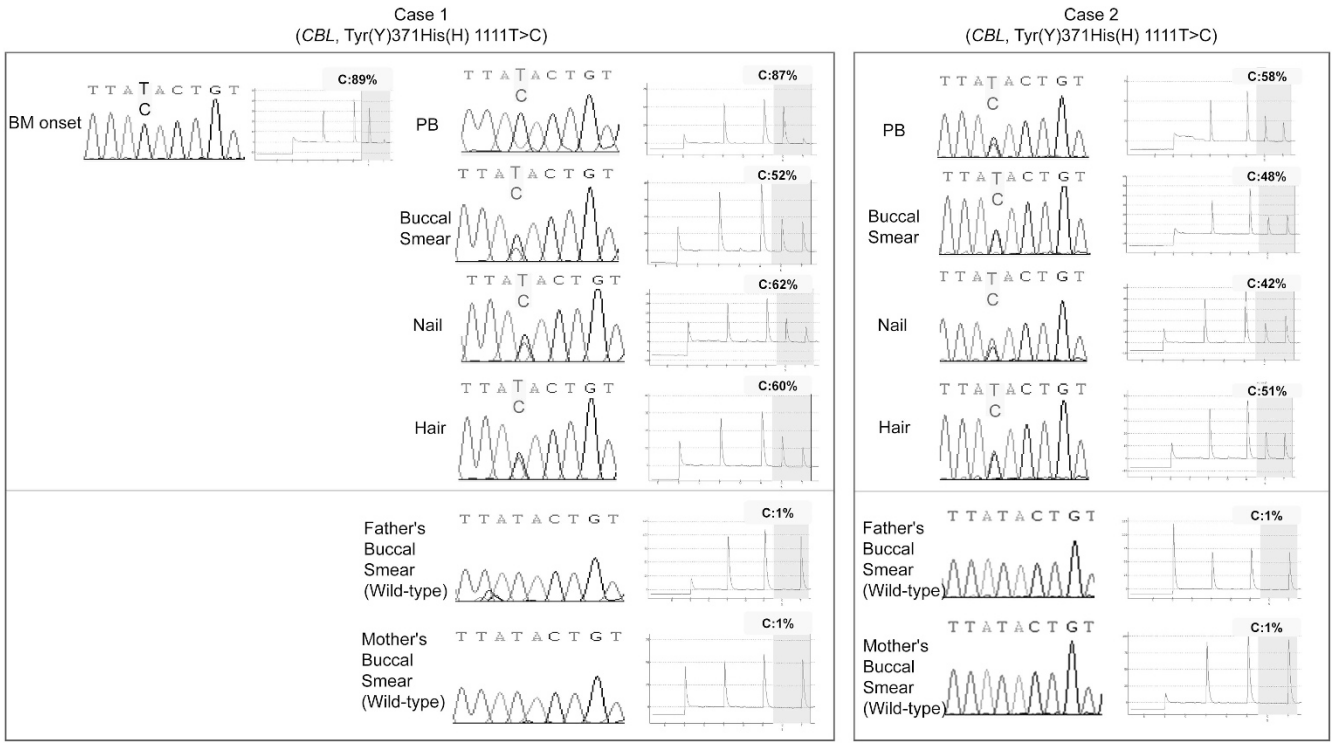

Figure 1 Results of mutation analysis of *CBL* by direct sequencing and pyrosequencing in cases 1 and 2. The same mutation of Y371H, 1111T>C was observed in both patients. The frequencies of mutated allele by pyrosequencing were different from the tissue type as follows, for Case 1, PB-MNCs 87%, buccal smear cells 52%, nails 62%, hair 60%. For Case 2, PB-MNCs 58%, buccal smear cells 48%, nails 42%, hair 51%. Their parents did not have this *CBL* mutation in their buccal smear cells.

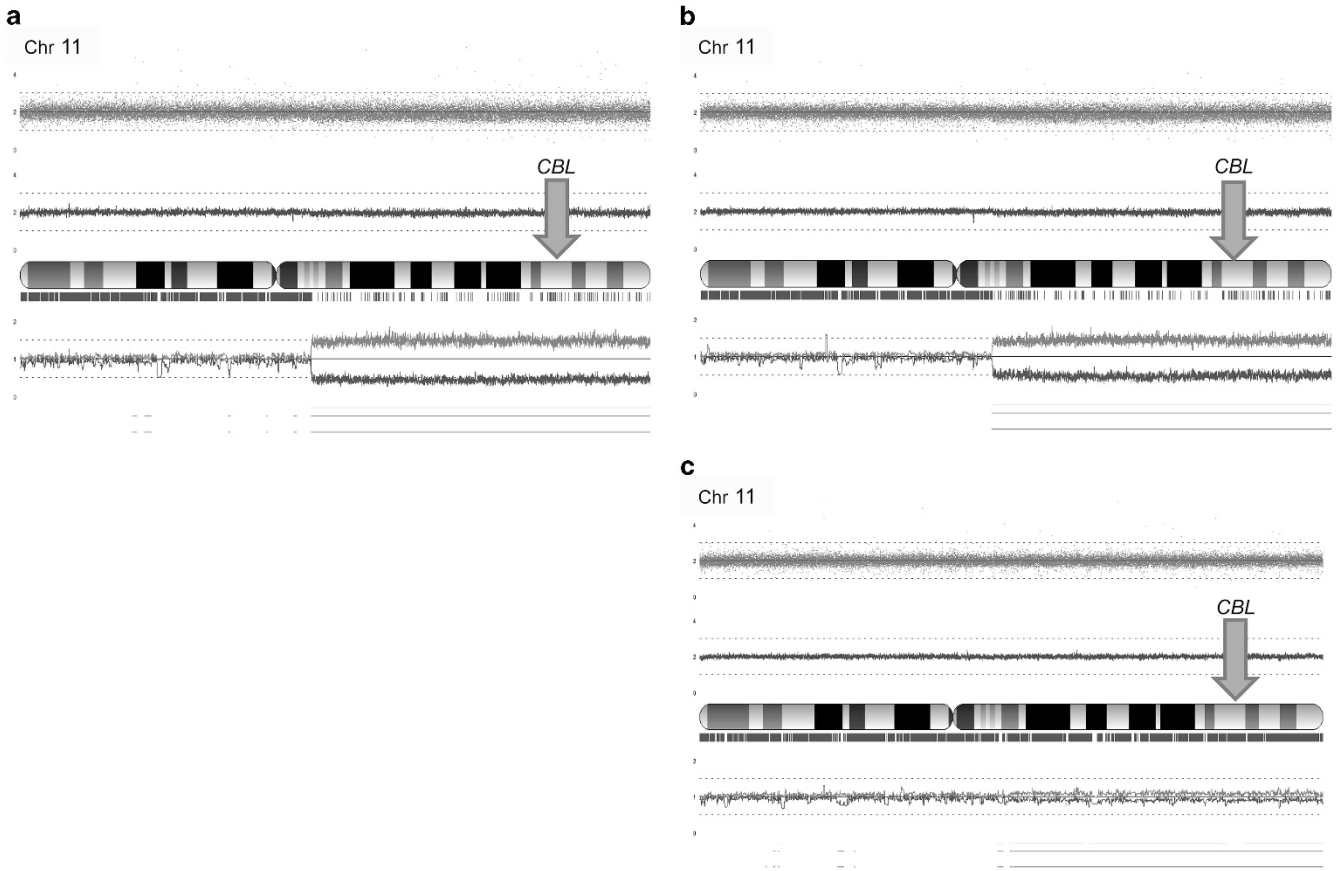

Figure 2 Results of SNP array analysis in cases 1 and 2. (**a**), and (**b**). In case 1, uniparental disomy (UPD) of chromosome 11q was observed in the first diagnostic bone marrow sample (5-month old after birth) (**a**) and in the recent peripheral blood (20-year old) (**b**). Loss of heterogeneity (LOH) of chromosome 11q including the *CBL* gene was observed and UPD was also observed in both samples. (**c**) Results of the recent peripheral blood of case 2 (31-year old). The difference is very small but UPD was found.

CBL mutation and 11qUPD has to be considered in both cases (Supplementary figure 2). Four types A, B, C and D may exist in a mixture in each patient as shown in supplementary figure 2. For example, type A and type B could be dominant in case 1 and case 2, respectively. Further large study about CBL mutation with 11q23UPD in adult cases will be needed in future.

Niemeyer *et al.*[16] suggested that germline CBL mutations have developmental, tumorigenic and functional consequences that resemble disorders that are caused by hyperactive Ras/Raf/MEK/ERK signaling and include NF1, Noonan syndrome, Costello syndrome, cardiofaciocutaneous syndrome and Legius syndrome. Therefore, these germline-mutated syndromes might complicate JMML at infancy-like transient abnormal myelopoiesis/transient myeloproliferative disorder in Down syndrome.[19]

Furthermore, *CBL* mutation syndrome was reported with or without JMML depended on the mutated site of *CBL* gene.[15,16] Interestingly, several reported cases and our two patients with the same mutation Y371H does not have any physiologic abnormalities such as hearing loss, optic atrophy, hypertension or cardiomyopathy.[4] Future study will enable to predict the prognosis of *CBL*-mutated JMML patients. Further study in long-term survivor of JMML patients will be needed in future.

In conclusion, JMML seems to show the heterogeneity due to the genetic alterations. Some *CBL*-mutated patients without typical phenomena-like Noonan syndrome might show the good clinical course after JMML remission.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Journal of Human Genetics website (http://www.nature.com/jhg)