検体量および採取時の苦痛には十分な配慮を行った。遺伝子解析については各種指針を遵守して、患者個人情報の保護について十分な配慮を行った。

C. 研究結果

アレイ CGH 解析では明らかな欠失は認められなかった。これまで DBA の原因として報告されているリボゾーム関連遺伝子解析を行ったが、変異は認められなかった。

Flow-FISH 法でテロメア長の測定を行ったところ、同年齢の正常対照に比べてテロメア長の短縮が認められた（図 1）。

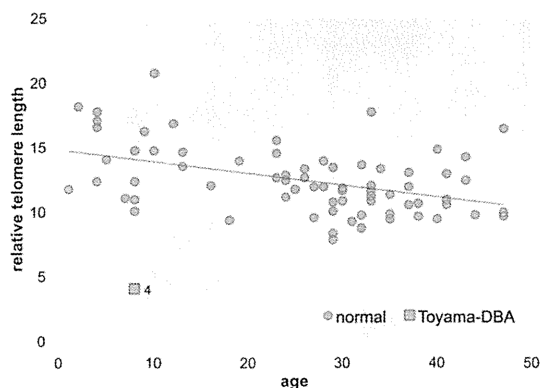

図 1 Flow-FISH 法によるテロメア長
■は患者における相対的テロメア長を示す。

そこで DKC の原因として報告されている既知のテロメア関連遺伝子の解析を行ったが、変異は認められなかった。そこで WES を行ったところ、患者固有の遺伝子変異が 48 個同定された。

D. 考察

臨床的に DBA と診断されていた患者についてリボゾーム関連遺伝子解析を行ったが、変異は同定できなかった。しかし Flow-FISH 法でテロメア長の短縮が観察され、患者はむしろ DKC に近い疾患であると考えられた。しかし既知の DKC 関連遺伝子は同定されず、WES を行った。その結果、48 個の遺伝子変異が同定されたが、リボゾームあるいはテロメア関連遺伝子は含まれておらず、これらの遺伝子に自験例の原因遺伝子が存在するかどうか現在探索中である。

E. 結論

DBA と臨床診断されていた患者でテロメア長の短縮を認め、DKC とオーバーラップする疾患と考えられる。しかしこれまで報告されている DBA ならびに DKC の既知遺伝子変異はなかった。自験例のような原因不明の疾患における WES は有力なツールではあるが、1 例のみでは原因遺伝子の探索は困難であり、類似症例の蓄積が望まれる。

F. 研究発表

なし。

G. 知的財産権の出願・登録状況

なし。

Ⅲ．研究成果の刊行に関する一覧

雑誌

発表者氏名	論文タイトル名	発表誌名	巻名	ページ	出版年
Asai D, Osone S, Imamura T, Sakaguchi H, Nishio N, Kuroda H, Kojima S, Hosoi H.	Allo-SCT in a patient with CRMCC with aplastic anemia using a reduced intensity conditioning regimen.	Bone Marrow Transplant.	47(8)	1126-7	2012
Kawabata H, Doisaki S, Okamoto A, Uchiyama T, Sakamoto S, Hama A, Hosoda K, Fujikura J, Kanno H, Fujii H, Tomosugi N, Nakao K, Kojima S, Takaori-Kondo A.	A case of congenital dyserythropoietic anemia type 1 in a Japanese adult with a CDAN1 gene mutation and an inappropriately low serum hepcidin-25 level.	Intern Med.	51(8)	917-20	2012
Kojima S.	Treatment of acquired aplastic anemia in children.	Hematology	Suppl	S11-4	2012
Jerez A, Sugimoto Y, Makishima H, Verma A, Jankowska AM, Przychodzen B, Visconte V, Tiu RV, O'Keefe CL, Mohamedali AM, Kulasekararaj AG, Pellagatti A, McGraw K, Muramatsu H, Moliterno AR, Sekeres MA, McDevitt MA, Kojima S, List A, Boultwood J, Mufti GJ, Maciejewski JP.	Loss of heterozygosity in 7q myeloid disorders: clinical associations and genomic pathogenesis.	Blood	119 (25)	6109-17	2012
Yang W, Zhang P, Hama A, Ito M, Kojima S, Zhu X.	Diagnosis of acquired bone marrow failure syndrome during childhood using the 2008 World Health Organization classification system.	Int J Hematol.	96(1)	34-8	2012
Wang X, Muramatsu H, Sakaguchi H, Xu Y, Narita A, Tsumura Y, Doisaki S, Tanaka M, Ismael O, Shimada A, Hama A, Takahashi Y, Kojima S.	Mutation in the THPO gene is not associated with aplastic anaemia in Japanese children.	Br J Haematol.	158(4)	553-5	2012
Doisaki S, Muramatsu H, Shimada A, Takahashi Y, Mori-Ezaki M, Sato M, Kawaguchi H, Kinoshita A, Sotomatsu M, Hayashi Y, Furukawa-Hibi Y, Yamada K, Hoshino H, Kiyoi H, Yoshida N, Sakaguchi H, Narita A, Wang X, Ismael O, Xu Y, Nishio N, Tanaka M, Hama A, Koike K, Kojima S.	Somatic mosaicism for oncogenic NRAS mutations in juvenile myelomonocytic leukemia.	Blood	120(7)	1485-8	2012
Sakaguchi H, Nakanishi K, Kojima S.	Inherited bone marrow failure syndromes in 2012.	Int J Hematol.	97(1)	20-9	2013
Kuramitsu M, Sato-Otsubo A, Morio T, Takagi M, Toki T, Terui K, RuNan Wang, Kanno H, Ohga S, Ohara A, Kojima S, Kitoh T, Goi K, Kudo K, Matsubayashi T, Mizue N, Ozeki M, Masumi A, Momose H, Takizawa K, Mizukami T, Yamaguchi K, Ogawa S, Ito E, Hamaguchi I.	Extensive gene deletions in Japanese patients with Diamond–Blackfan anemia.	Blood	119 (10)	2376-84	2012
Takahashi Y, Muramatsu H, Sakata N, Hyakuna N, Hamamoto K, Kobayashi R, Ito E, Yagasaki H, Ohara A, Kikuchi A, Morimoto A, Yabe H, Kudo K, Watanabe K, Ohga S, Kojima S	Rabbit antithymocyte globulin and cyclosporine as first-line therapy for children with acquired aplastic anemia.	Blood	121(5)	862-3	2013

Kawai T, Nishikomori R, Izawa K, Murata Y, Tanaka N, Sakai H, Saito M, Yasumi T, Takaoka Y, Nakahata T, Mizukami T, Nunoi H, Kiyohara Y, Yoden A, Murata T, Sasaki S, Ito E, Akutagawa H, Kawai T, Imai C, Okada S, Kobayashi M, Heike T.	Frequent somatic mosaicism of *NEMO* in T cells of patients with X-1 linked anhidrotic ectodermal dysplasia with immunodeficiency.	Blood	119 (23)	5458-66	2012
Yokoyama T, Toki T, Aoki Y, Park MJ, Kanno Y, Tanaka T, Yamazaki Y. Ito E, Hayashi Y, Nakamura T.	Identification of *TRIB1* R107L gain-of-function mutation in human acute megakaryocytic leukemia.	Blood	119 (11)	2608-11	2012
Takata K, Sato Y, Nakamura N, Tokunaka M, Miki Y, Yukie Kikuti Y, Igarashi K, Ito E, Harigae H, Kato S, Hayashi E, Oka T, Hoshii Y, Tari A, Okada H, Mohamad AA, Maeda Y, Tanimoto M, Kinoshita T, Yoshino T.	Duodenal follicular lymphoma lacks AID but expresses BACH2 and has memory B-cell characteristics.	Mod Pathol.	26(1)	22-31	2013
Yazaki M, Kamei M, Ito Y, Konno Y, Wang R, Toki T, Ito E.	A novel mutation of ribosomal protein s10 gene in a Japanese patient with diamond-blackfan anemia.	J Pediatr Hematol Oncol..	34(4)	293-5	2012
山口博樹、 檀 和夫	骨髄不全症候群における テロメア制御異常	血液フロンティア	22(6)	941-52	2012
Yabe M, Shimizu T, Morimoto T, Koike T, Takakura H, Tsukamoto H, Muroi K, Oshima K, Asami K, Takata M, Yamashita T, Kato S and Yabe H.	Matched sibling donor stem cell transplantation for Fanconi anemia patients with T-cell somatic mosaicism.	Pediatric Transplant	16	340-5	2012
Honda F, Kano H, Kanegane H, Nonoyama S, Kim ES, Lee SK, Takagi M, Mizutani S, Morio T.	The kinase Btk negatively regulates the production of reactive oxygen species and stimulation-induced apoptosis in human neutrophils	Nat Immunol	13	369-78	2012

Ⅳ．研究成果の刊行物・別刷り

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LETTER TO THE EDITOR

Allo-SCT in a patient with CRMCC with aplastic anemia using a reduced intensity conditioning regimen

Bone Marrow Transplantation (2012) **47,** 1126–1127; doi:10.1038/bmt.2011.221; published online 14 November 2011

Cerebroretinal microangiopathy with calcifications and cysts (CRMCC; Revesz syndrome)[1-3] is a rare congenital systemic disorder that was first described by Revesz *et al.* in 1992.[1] It is characterized by intrauterine growth retardation, bilateral exudative retinopathy, intracranial calcification and cysts, leukoencephalopathy, sparse hair, nail dystrophy, and skeletal defects. Approximately one-third of cases are complicated by the development of hematological disorders; in particular, aplastic anemia similar to that observed in dyskeratosis congenita (DKC).[4-6] CRMCC with aplastic anemia and DKC both belong to the congenital BM-failure disease spectrum and additional similarities between these two diseases have been reported, including shortened telomere length and mutations of the *TINF2*, which encodes TIN2 (a regulator of telomere length in human cells).[4,7,8] Similar to DKC, aplastic anemia associated with CRMCC can be cured only by hematopoietic SCT (HSCT); however, to date HSCT has not been widely used in this patient population.[4,7,8] Herein, we report a case of CRMCC associated with a *TINF2* mutation, which was successfully treated with HSCT using a fludarabine-based reduced-intensity conditioning (RIC) regimen.

The patient was a Japanese boy who was born at 38 weeks gestation to non-consanguineous parents and had a birth weight of 2.57 kg (−0.8 s.d.). There was no family history of ocular or neurological disease. The neonatal period was uneventful and his development was considered normal until 1 year of age. Nail dystrophy was noted, but he displayed no signs of leukoplakia or skin disease. At the age of 15 months, his mother first noticed a right divergent squint. Subsequent ophthalmological investigation revealed bilateral exudative retinopathy and retinal detachment, consistent with bilateral Coats' disease (Figure 1a). Truncal ataxia with normal deep-tendon- and superficial-skin-reflexes was noted on neurological examination. At the age of 17 months, brain computed tomography (CT) scanning demonstrated extensive intracranial calcifications. T2-weighted magnetic resonance imaging of the brain revealed spotty high signals within the white matter of the brainstem, the deep gray nuclei, and the right frontal and parietal lobes (Figure 1b and c). A cystic mass adjacent to the thalamus was also observed. Macrocytic anemia and severe thrombocytopenia were also detected (red blood cell, 301×10^4 per μL; hemoglobin level, 9.8 g/dl; mean corpuscular volume (MCV), 98.3 fl; reticulocyte count: 37 000 per μL; platelet count: 8000 per μL). No leukopenia or neutropenia was observed (white blood cell, 5100 per μL with an ANC of 1479 per μL). BM aspiration revealed hypoplastic BM (nucleated cell count: 41 500 per μL and megakaryocytes 0 per μL, with no dysplastic features). G-banding analysis of the BM at 18 months revealed the karyotype 48, XY, +2 mar (1/16)/46, XY (15/16). A chromosomal breakage study showed no excess of mitomycin C-induced breaks, which indicated that Fanconi's anemia was not present (data not shown). Flow-FISH analysis in PBLs demonstrated shortened telomere length and DNA sequencing identified a heterozygous missense mutation (845 G > A, R282H) of the *TINF2* gene (Figure 1d). *TINF2* encodes TIN2, one of the key components of shelterin, a protein complex that stabilises telomeres.[7] Mutated amino acids were tightly clustered in the position between 280 and 290 in classic DKC or CRMCC patients.[7,8]

On the basis of these findings, a diagnosis of CRMCC was made. At the age of 17 months, the exudative retinopathy was successfully treated with laser coagulation, pars plana vitrectomy and the intra-vitreal administration of vascular endothelial growth factor inhibitors. However, at the age of 22 months, the bicytopenia became more severe and treatment with prednisolone (1 mg/kg/day) and danazol (5 mg/kg/day) was initiated. In response, the anemia improved slightly, but there was no effect on the thrombocytopenia, although bleeding tendency was not evident. Thus, we decided to perform allogeneic HSCT with an HLA-matched, related, healthy female donor. The conditioning regimen was as follows: 25 mg/m^2 fludarabine daily on days −5 to −2; 25 mg/kg CY daily on days −5 to −2 and 1.25 mg/kg anti-thymocyte globulin (Thymoglobulin) daily on days −5 to −2. Total nucleated cell and CD34+ doses were 2.48×10^8 and 2.08×10^6 per kg recipient body weight, respectively. GVHD prophylaxis consisted of CsA and short term MTX. Desired neutrophil counts (> 500 per μL) were obtained by day 12, reticulocyte counts (> 1.0%) by day 20, and platelet counts (> 5.0×10^4 per μL) by

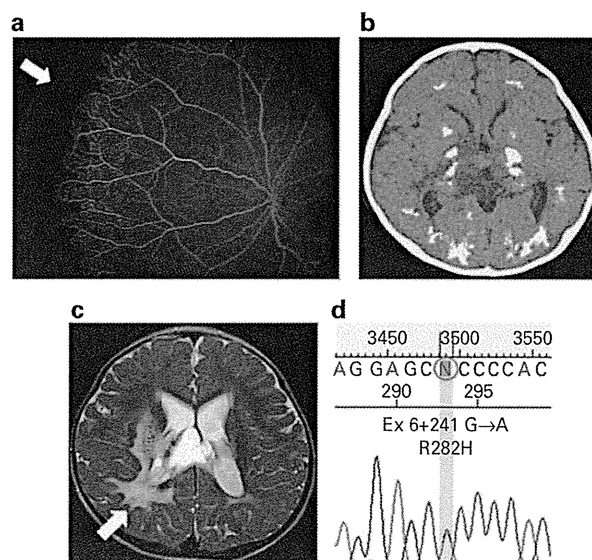

Figure 1. (**a**) Fluorescein angiogram. The arrow indicates an avascular peripheral zone in the left eye, which suggests retinal detachment. (**b**) CT of the brain. CT of the brain revealed extensive intracranial calcification, which is characteristic to CRMCC. (**c**) T2-weighted magnetic resonance imaging of the brain. The arrow indicates high signals within the white matter of the right parietal lobe, suggesting the presence of leukoencephalopathy. (**d**) Sequence tracing showing a TINF2 mutation in PBLs of the patient. Sequence tracing showing a heterozygous G to A mutation (Ex 6 +241 G→A, R282H) in *TINF2*.

day 48. Genotyping using XY-FISH analysis of a BM sample taken at day 48 revealed that 98.4% of total nucleated cells were of donor origin. The post-transplant course was uneventful with grade 1 oral mucositis being the only complication. Neither acute nor chronic GVHD was evident. The patient is currently 20 months post transplant without any immunosuppressants and transplantation-related complication.

In surveying the literature, we found that in CRMCC,[1-9] at least one of the DKC triad of dystrophic nail, abnormal skin findings and leukoplakia was noted significantly more frequently in positive vs negative hematological disorder (one of DKC triad, 10/17 vs 5/23, $P<0.01$; dystrophic nail, 9/16 vs 4/23, $P<0.01$; leukoplakia, 3/5 vs 0/9, $P<0.05$). We found two previous reports of CRMCC patients with an identical mutation in *TINF2* to that in our patient.[7,8]

Kajtar *et al.*[4] described a 2-year-old girl with CRMCC successfully treated with allogeneic BMT from an HLA-identical brother. The post-transplantation course in this patient was uneventful with only mild GVHD. Savage *et al.*[7] described a case of CRMCC who died after BMT; however, detailed information on the conditioning regimen was not available. Walne *et al.*[8] also described four cases of CRMCC with a *TINF2* mutation, of which one received BMT, but details on the conditioning regimen and transplantation courses were not reported. In DKC, circulating lymphocytes and fibroblasts exhibit an increased *in vitro* sensitivity to radiation and alkylating agents.[10] Therefore, this suggested that HSCT using myeloablative conditioning regimens in DKC patients would be associated with high regimen-related toxicity.[10] For this reason, RIC regimens are strongly recommended in HSCT for DKC patients.[11] Herein, we used a RIC regimen for our patient, which was safe and had minimal toxicity. Our experience suggests that HSCT with a RIC regimen should be considered for treatment of CRMCC patients with hematological disorders.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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REFERENCES

1 Revesz T, Fletcher S, al-Gazali LI, DeBuse P. Bilateral retinopathy, aplastic anaemia, and central nervous system abnormalities: A new syndrome? *J Med Genet* 1992; **29**: 673-675.
2 Goutieres F, Dollfus H, Becquet F, Duffer JL. Extensive brain calcification in two children with bilateral Coats disease. *Neuropediatrics* 1999; **30**: 19-21.
3 Briggs TA, Abdel-Salam GMH, Balicki M. Cerebroretinal microangiopathy with calcifications and cysts (CRMCC). *Am J Med Genet A* 2008; **146A**: 182-190.
4 Kajtar P, Mehes K. Bilateral coats retinopathy associated with aplastic anaemia and mild dyskeratotic signs. *Am J Med Genet* 1994; **49**: 374-377.
5 Duprey PA, Steger JW. An unusual case of dyskeratosis congenita with intracranial calcifications. *J Am Acad Dermatol* 1988; **19**: 760-762.
6 Gayatri NA, Hughes MI, Lloyd IC, Wynn RF. Association of the congenital bone marrow failure syndromes with retinopathy, intracerebral calcification and progressive neurological impairment. *Eur J Paediatr Neurol* 2002; **6**: 125-128.
7 Savage SA, Giri N, Baerlocher GM, Orr N, Lansdorp PM, Alter BP. *TINF2*, a component of sheltrin telomere protection complex, is mutated in dyskeratosis congenita. *Am J Hum Genet* 2008; **82**: 501-509.
8 Walne AJ, Vulliamy T, Beswick R, Kirwan M, Dokal I. *TINF2* mutations result in very short telomeres: analysis of a large cohort of patients with dyskeratosis congenita and related bone marrow failure syndromes. *Blood* 2008; **112**: 3594-3600.
9 Crow YJ, McMenamin J, Haenggeli CA, Hadley DM, Tirupathi S, Treacy EP *et al.* Coat's Plus:A progressive familial syndrome of bilateral Coat's disease, characteristic cerebral calcification, leukoencephalopathy, slow pre- and post-natal linear growth and defects of bone marrow and integument. *Neuropediatrics* 2004; **35**: 10-19.
10 M'kacher R, Laithier V, Valent A, Delhommeau F, Violot D, Deutsch E *et al.* Sensitivity to radiation and alkylation agent of peripheral lymphocytes and fibroblasts in a Hoyeraal-Hreidarsson syndrome patient. *Pediatr Hematol Oncol* 2003; **20**: 651-656.
11 Dietz AC, Orchard PJ, Baker KS, Giller RH, Savage SA, Alter BP *et al.* Disease-specific hematopoietic cell transplantation: nonmyeloablative conditioning regimen for dyskeratosis congenita. *Bone Marrow Transplant* 2011; **46**: 98-104.

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CASE REPORT

A Case of Congenital Dyserythropoietic Anemia Type 1 in a Japanese Adult with a *CDAN1* Gene Mutation and an Inappropriately Low Serum Hepcidin-25 Level

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Abstract

We describe the first case of genetically diagnosed congenital dyserythropoietic anemia (CDA) type 1 in a Japanese man. The patient had hemolytic anemia since he was a child, and he developed diabetes, hypogonadism, and liver dysfunction in his thirties, presumably from systemic iron overload. When he was 48 years old a diagnosis was finally made by genetic analysis that revealed a homozygous mutation of *CDAN1* gene (Pro1129Leu). His serum hepcidin-25 level was inappropriately low. We conclude that physicians should be aware of the possibility of CDA in a patient with anemia and systemic iron overload at any age.

Key words: congenital dyserythropoietic anemia, iron metabolism, hemochromatosis, hepcidin, growth differentiation factor-15

(Intern Med 51: 917-920, 2012)
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Introduction

Congenital dyserythropoietic anemia (CDA) is a rare congenital erythropoietic disorder with characteristic morphological abnormalities of the bone marrow cells, ineffective erythropoiesis and systemic iron overload (1). Three types of CDA are known: types 1, 2 and 3. The genes responsible for types 1 and 2 have recently been identified as *CDAN1* and *SEC23B*, respectively (2, 3). Both CDA types 1 and 2 are inherited recessively. The incidence of CDA is very rare, and in a recent pan-European survey, only 124 CDA type 1 cases were recorded (4). To date, several Japanese CDA type 1 cases have also been reported (5-7), but none of them has been genetically proven. Here, we describe in a Japanese adult a case of CDA type 1 with systemic iron overload that was genetically diagnosed in his late forties.

Case Report

A Japanese man was referred to the Kyoto University Hospital for hyperglycemia when he was 38 years old. He had had hemolytic anemia since he was a child, but its etiology had not been determined. He had undergone splenectomy when he was 36 years old, which ameliorated his anemia to some extent. At his first visit to our hospital, his white blood cell count was 6,400/μL; red blood cell count, 1.93×10^6/μL; hemoglobin (Hb) level, 7.5 g/dL; hematocrit level, 21.0%; mean corpuscular volume, 108.8 fL; platelet count, 365×10^3/μL; and reticulocyte count, 53×10^3/μL (Ta-

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Table 1. Laboratory Data

Features	Laboratory data at the first visit (38 years old)	Laboratory data at the time of diagnosis (48 years old)
White blood cells (per μL)	6.4×10^3	5.3×10^3
Red blood cells (per μL)	1.93×10^6	2.41×10^6
Hemoglobin (g/dL)	7.5	8.5
Hematocrit (%)	21.0	24.1
Reticulocytes (per μL)	53×10^3	—
Platelet counts (per μL)	365×10^3	312×10^3
Total bilirubin (mg/dL)	1.5	1.6
Direct bilirubin (mg/dL)	0.7	0.1
Haptoglobin (mg/dL)	<7.9	—
AST (IU/L)	49	26
ALT (IU/L)	68	16
LDH (IU/L)	302	277
Ferritin (ng/mL)	4058	186

— indicates that the tests were not performed. Abbreviations; AST, aspartate aminotransferase (reference range, 13-29 IU/L); ALT, alanine aminotransferase (reference range, 8-28 IU/L); LDH, lactate dehydrogenase (reference range, 129-241 IU/L).

ble 1). He had hepatic dysfunction, with a slightly elevated serum alanine aminotransferase level (68 IU/L), hyperglycemia (blood sugar level of 146 mg/dL and HbA1c level of 6.9%) with very low insulin secretion (serum c-peptide level, <0.1 ng/mL), and hypogonadism with a serum testosterone level lower than 0.2 ng/mL, i.e., very low (reference range, 2.7-10.7 ng/mL). His blood test results also suggested iron overload (transferrin saturation of 95.3% and serum ferritin level of 4,058 ng/mL), and the liver biopsy results revealed marked accumulation of iron in the parenchymal cells. Thus, hemochromatosis, along with liver dysfunction, diabetes and hypogonadism, was diagnosed. Insulin therapy was then started. Occasional phlebotomy was also started to remove excess iron and to gradually decrease his serum ferritin and alanine aminotransferase levels to within the reference ranges (Table 1). When he was 46 years old, a series of intensive diagnostic examinations were started. The findings of the biochemical analyses for erythrocyte membrane disorders or unstable hemoglobinopathies were all negative. The bone marrow examination revealed marked erythroid hyperplasia (the myeloid to erythroid ratio of 0.34) and remarkable dysplastic features in the erythroid cells, with megaloblastoid changes and multinuclear cells (Fig. 1A-E). However, no significant dysplasia was observed in the granulocytic or megakaryocytic series (Fig. 1A, B), and no ring sideroblasts were observed in the iron staining. When he was 48 years old, we obtained his written informed consent and approval by the ethics committee of Kyoto University to perform a genetic analysis for indicators of hereditary iron disorders in his peripheral blood cells. The results of the genetic analyses for pyruvate kinase deficiency and thalassemia syndromes were all negative. There were no mutations in the exons and the exon-intron borders of hereditary hemochromatosis genes including *HFE*, *TFR2*, *HJV*, *HAMP*, and *SLC40A1*. However, a homozygous mutation in *CDAN1* *ex26 c.3503 C>T* (Pro1129Leu) was detected, consistent with CDA type 1 (Fig. 2). When we reviewed his bone marrow specimen, internuclear bridges that connected two separate erythroblasts were occasionally observed (7 bridges in 500 erythroblasts, Fig. 1F-J). His serum hepcidin-25 level was 0.8 ng/mL [reference range, 2.3-37 ng/mL; analyzed with a quantitative liquid chromatography coupled with tandem mass spectrometry method (8)]. The growth differentiation factor-15 (GDF15) level was 8,469 pg/mL (reference range, 215-835 pg/mL; analyzed with a commercial ELISA kit from R&D, Minneapolis, MN). The patient had two siblings, a brother and a sister; both were in good health. There was no significant family history except that his mother had anemia of undetermined etiology, and his paternal grandfather had diabetes. He declined genetic analysis of his family for the *CDAN1* gene.

Discussion

We encountered an adult patient with hemolytic anemia with various symptoms caused by systemic iron overload, who turned out to have a genetic mutation consistent with CDA type 1. To our knowledge, this is the first documented case of CDA type 1 in a Japanese with *CDAN1* gene mutation. Dgany et al. identified the same *CDAN1* gene mutation as the current case in a French Polynesian family (2). *CDAN1* is located on chromosome 15q15.1-15q15.3, and it codes for a nuclear protein, codanin-1, the human homolog of discs lost (dlt) which is required for cell survival and cell cycle progression in *Drosophila* (9). The diagnosis of CDA type 1 has usually been made from clinical features together with characteristic morphological features of the bone marrow cells such as binucleated erythroblasts, and internuclear bridges between the erythroid cells. As codanin-1 is essential for proper cellular trafficking of the heterochromatin

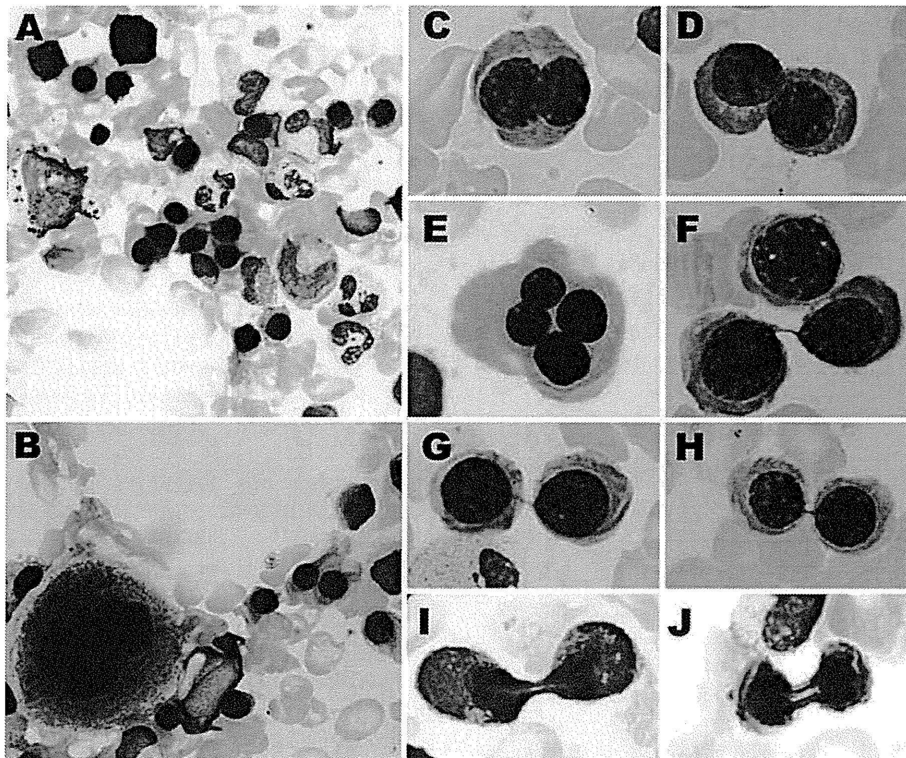

Figure 1. Bone marrow cell morphology (May-Grünwald-Giemsa staining, original magnification ×1,000; C-J, images were further magnified by photographic enlargement). A and B: Erythroid hyperplasia. No significant dysplasia was observed in the granulocytic or megakaryocytic series. C and D: Binucleated erythroblasts. These cells were found in approximately 12% of the erythroblasts. E: A few tetranucleated erythroblasts were found. F-J: Internuclear bridges between the erythroblasts were found after careful inspection.

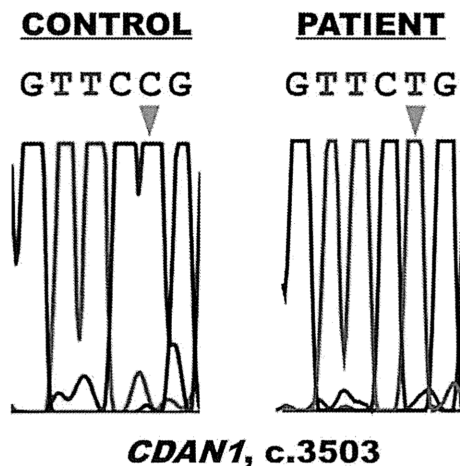

Figure 2. The homozygous mutation in *CDAN1* (*ex26 c.3503 C>T*, Pro1129Leu) detected in the patient.

protein HP1-α (10), defects of this protein may result in such morphological abnormalities. In the current case, the bone marrow examination results showed numerous binucleated erythroblasts, but the internuclear bridges, which are much more specific features of this disorder, were observed in less than 3% of the erythroblasts and were overlooked in the first inspection (Fig. 1). Therefore, making a definitive diagnosis of CDA from bone marrow cell morphology alone can sometimes be difficult.

CDA types 1 and 2 are known to be accompanied by iron overload. Similar to hereditary hemochromatosis, inappropriately low production of hepcidin, the central regulator of systemic iron homeostasis, has been proposed as the etiology of iron overload in CDA (11). As the main function of hepcidin is to downregulate the expression of ferroportin, the only known cellular iron exporter of mammals, downregulation of hepcidin results in an increase in ferroportin expression, thereby increasing iron absorption from the intestine and causing systemic iron overload. A previous report demonstrated marked increases of GDF15 in the serum of CDA type 1 patients (12). GDF15, a humoral factor belonging to the transforming growth factor-β superfamily, has been shown to suppress hepatic production of hepcidin (13). Consistent with the previous reports, systemic iron overload was induced in the current case without repeated red cell transfusions, the serum GDF15 level was remarkably elevated, and the serum hepcidin-25 level was inappropriately low. Thus, we postulate that serum hepcidin-25 and GDF15 are useful markers for CDA.

CDA is generally regarded as a pediatric disease because the initial symptoms, such as anemia, jaundice, and splenomegaly, usually appear in the first decade. However, the current case was diagnosed when the patient was in his late forties, and in the pan-European survey, CDA was diag-

nosed in a substantial proportion of patients who were middle-aged or older (4). Early diagnosis of CDA is important because iron chelation therapy (or phlebotomy if anemia is mild) should be started as early as possible to avoid iron overload, which can cause irreversible tissue damage. In addition, interferon-α is known to be effective for ameliorating anemia and iron accumulation in patients with CDA type 1, although the precise mechanism is still unknown (14). The survey data and our findings of the current case suggest that we should be aware of the possibility of CDA in patients with anemia and systemic iron overload at any age.

The authors state that they have no Conflict of Interest (COI).

References

1. Kamiya T, Manabe A. Congenital dyserythropoietic anemia. Int J Hematol **92**: 432-438, 2010.
2. Dgany O, Avidan N, Delaunay J, et al. Congenital dyserythropoietic anemia type I is caused by mutations in codanin-1. Am J Hum Genet **71**: 1467-1474, 2002.
3. Schwarz K, Iolascon A, Verissimo F, et al. Mutations affecting the secretory COPII coat component SEC23B cause congenital dyserythropoietic anemia type II. Nat Genet **41**: 936-940, 2009.
4. Heimpel H, Matuschek A, Ahmed M, et al. Frequency of congenital dyserythropoietic anemias in Europe. Eur J Haematol **85**: 20-25, 2010.
5. Kuribayashi T, Uchida S, Kuroume T, Umegae S, Omine M, Maekawa T. Congenital dyserythropoietic anemia type I: report of a pair of siblings in Japan. Blut **39**: 201-209, 1979.
6. Hiraoka A, Kanayama Y, Yonezawa T, Kitani T, Tarui S, Hashimoto PH. Congenital dyserythropoietic anemia type I: a freeze-fracture and thin section electron microscopic study. Blut **46**: 329-338, 1983.
7. Kato K, Sugitani M, Kawataki M, et al. Congenital dyserythropoietic anemia type 1 with fetal onset of severe anemia. J Pediatr Hematol Oncol **23**: 63-66, 2001.
8. Kanda J, Mizumoto C, Kawabata H, et al. Serum hepcidin level and erythropoietic activity after hematopoietic stem cell transplantation. Haematologica **93**: 1550-1554, 2008.
9. Pielage J, Stork T, Bunse I, Klämbt C. The Drosophila cell survival gene discs lost encodes a cytoplasmic Codanin-1-like protein, not a homolog of tight junction PDZ protein Patj. Dev Cell **5**: 841-851, 2003.
10. Renella R, Roberts NA, Brown JM, et al. Codanin-1 mutations in congenital dyserythropoietic anemia type 1 affect HP1α localization in erythroblasts. Blood **117**: 6928-6938, 2011.
11. Ganz T. Hepcidin and iron regulation, 10 years later. Blood **117**: 4425-4433, 2011.
12. Tamary H, Shalev H, Perez-Avraham G, et al. Elevated growth differentiation factor 15 expression in patients with congenital dyserythropoietic anemia type I. Blood **112**: 5241-5244, 2008.
13. Tanno T, Bhanu NV, Oneal PA, et al. High levels of GDF15 in thalassemia suppress expression of the iron regulatory protein hepcidin. Nat Med **13**: 1096-1101, 2007.
14. Lavabre-Bertrand T, Blanc P, Navarro R, et al. alpha-Interferon therapy for congenital dyserythropoiesis type I. Br J Haematol **89**: 929-932, 1995.

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Treatment of acquired aplastic anemia in children

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Aplastic anemia (AA) is an uncommon but serious disorder characterized by pancytopenia resulting from non-function of the bone marrow. The incidence of AA is approximately 3 fold more common in East Asia than in Europe and United States where yearly incidence rates are approximately two patients per one million.[1]

Keywords: Aplastic anemia, Children, Immunosuppressive therapy, Stem cell transplantation

Diagnosis of Childhood Aplastic Anemia

It is often difficult to distinguish hypoplastic myelodysplastic syndrome (MDS) from AA, especially in cases without cytogenetic abnormalities. Isolated anemia, which is the major presenting manifestation of refractory anemia (RA) in adults, is uncommon in children, who are more likely than adults to present with pancytopenia. In addition, hypocellularity of the bone marrow is more common in childhood RA. The new edition of the WHO classification for myeloid neoplasms outlines a provisional entity for refractory cytopenia for childhood (RCC) in which the diagnostic criteria for distinguishing RCC from AA are proposed.[2]

We studied the incidence and clinical feature of this new disease entity in Asian population with acquired bone marrow failure. A total of 100 children with cytopenia and hypocellular bone marrow (50 cases from Japan, 50 cases from China) were included in the present study. To obtain a diagnosis for cytopenia, at least two of the following must be present: (1) neutrophil count $<1.5 \times 10^9$/l; (2) hemoglobin <100 g/l; and (3) platelet count $<50 \times 10^9$/l. The severity of the disease was classified according to internationally accepted criteria.[3] AA patients exhibited no morphological changes in any of their hematopoietic cell lineages. RCC was defined as persistent cytopenia with <5% blasts in the bone marrow and <2% blasts in the peripheral blood. In addition, RCC patients had <10% dysplastic changes in >2 cell lineages, or >10% in 1 cell lineage. Patients classified as having refractory cytopenia with multilineage dysplasia (RCMD) exhibited >10% of the dysplastic changes in >2 cell lineages. Dysplastic features of bone marrow aspirate cytology and trephine biopsy sampled were evaluated according to recommendations by the French–American–British Cooperative Leukemia Working Group and the morphology group of the European Working Group of MDS in Childhood.[4,5] Final consensus for the diagnoses of 100 patients was follows: AA in 29 cases, RCC in 58 cases, and RCMD in 13 cases. The distribution of diagnoses were not different between Japanese cases and Chinese Cases; 12 : 17 in AA; 33 : 25 in RCC and 5 : 8 in RCMD, respectively. Among the three groups there were no significant differences with regard to median age at diagnosis, sex, or days from onset of symptoms to diagnosis. While eight out of 29 (28%) patients in the AA group had very severe cytopenia, only two of the 58 patients (3%) in the RCC group and none of the 13 patients in the RCMD group had very severe cytopenia. On the other hand, 45 of the 58 patients (78%) in the RCC group and 10 of the 13 patients (77%) of the RCMD group had non-severe cytopenia ($P<0.001$). Additionally, 16 out of 29 AA patients (55%) exhibited severe hypoplastic bone marrow cellularity, while only four out of 58 RCC patients (7%) and none of the RCMD patients had severe hypoplastic bone marrow. A number of the RCC/RCMD patients exhibited mild to moderate hypocellularity ($P<0.001$). Data for cytogenetic analyses were available from 75 patients. Abnormal karyotypes were detected in one patient from the AA group (47,XX,+8) and in three patients in the RCC group (47,XX,+8; 46,XY, t(x : 3)(p11.2;q13); 49,idem,+6,+21).

The distribution of diagnoses in Japanese and Chinese children were 12 : 17 for AA; 33 : 25 for RCC and 5 : 8 for RCMD, respectively. Patients with RCC/

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RCMD were milder in disease severity and bone marrow hypocellularity, compared to those with AA. According to the WHO classification system, it is recommended that children who satisfy the criteria for RCMD should be considered as RCC until the numbers of lineages involved are fully evaluated whether it is an important prognostic discriminator in childhood MDS.[2] In the current study, 13 of the 71 MDS children (18%) were classified as RCMD. The bone marrow samples were more cellular, and dysplasia of cell morphology was more prominent in children with RCMD than those with RCC.

The most important aspect of the new proposal from the WHO classification system is whether the diagnosis has an impact on clinical outcomes including, response to treatment and incidence of late clonal diseases. Unfortunately, due to a short follow-up period and variety of treatments, we could not address this issue. To establish the new entity of RCC, future studies should prospectively compare the clinical outcomes between AA and RCC groups in a large number of patients.

First Line Treatment for Severe Aplastic Anemia in Children

Bone marrow transplantation (BMT) from an HLA-matched family donor (MFD) is the treatment of choice for severe aplastic anemia (SAA) in children. For children without an MFD, immunosuppressive therapy (IST) with a combination of antithymocyte globulin and cyclosporine has been successfully used.[6] However, this decision is based on the results of comparative studies of these two therapies mainly conducted in the 1980s, and the outcome of both BMT and IST has dramatically improved over the past three decades.[7,8] Therefore, updated evidences for treatment decision of pediatric SAA are required. We compared the outcome of children with SAA who received IST enrolled in the prospective multicenter trials of IST conducted by the Japan Childhood Aplastic Anemia Study Group[9,10] or BMT from an MFD registered to the Transplant Registry Unified Management Program conducted by the Japan Society for Hematopoietic Cell Transplantation. The influence of potential risk factors on overall survival (OS) and failure free survival (FFS) was assessed according to first line treatment, time periods of treatment (1992–1999 and 2000–2009), age and other variables related to each treatment. FFS was defined as survival with treatment response. Death, primary or secondary graft failure, relapse, and second malignancy were considered treatment failure in patients who received BMT. Death, relapse, disease progression requiring stem cell transplantation from an alternative donor or 2nd IST, clonal evolution, and evolution to paroxysmal nocturnal hemoglobinuria were considered treatment failure in patients who received IST. Between 1992 and 2009, 599 children with SAA younger than 17 years received BMT from an MFD (n=213) or IST (n=386) as a first line treatment. While the OS did not differ between patients received IST and BMT (88±2 vs 90±2% at 15 years), the FFS was significantly inferior in patients received IST than in those received BMT (54±3 vs 84±3% at 15 years, P<0.0001). There was no significant improvement of outcomes in the two time periods; OS and FFS at 10 years in 1992–1999 vs 2000–2009 were 87±2 vs 93±2% and 66±3 vs 67±3%, respectively. In multivariate analysis, age <10 years was identified as a favorable factor for OS (P=0.007) and choice of the first line IST was the only risk factor for FFS (P<0.0001). These updated data support the current algorism for treatment decision which recommends BMT when an MFD is available.[6]

HLA-mismatched Family Donors BMT

The first-line therapy for children with severe AA is allogeneic BMT from a human leukocyte antigen (HLA)-matched family donor, and IST is indicated for patients without HLA-matched family donors.[6] While the standard therapy for children who fail to respond to IST is allogeneic BMT from an HLA-matched unrelated donor, BMT from an HLA-mismatched family donor has also been indicated. Compared with unrelated donors, an HLA-mismatched family donor has some advantages especially for children who need urgent transplantation.

We analyzed the clinical outcome of 578 children (325 boys and 253 girls) with AA (age, <20 years) who received allogeneic BMT between 1990 and 2009 in Japan, and registered to the Transplant Registry Unified Management Program. The median age at transplantation was 11 years (0–19), and the donors were serological 6/6 HLA-matched related donors (MRD) (n=312), one locus-mismatched related donor (1MMRD) (n=44), 2–3 loci-mismatched related (haploidentical) donors (n=9), and HLA-matched unrelated donors (MUD) (n=213). Causes of deaths were as follows: acute graft-versus-host disease (GVHD) (n=4), chronic GVHD (n=4), acute respiratory distress syndrome (n=2), severe hemorrhage (n=7), engraftment failure (n=5), infection (n=18), idiopathic pneumonia (n=8), organ failure (n=19), secondary malignancy (n=4), and others (n=4).

The probability of severe acute GVHD (grade III–IV) in patients transplanted from 1MMRD (26.9±7.4%) was significantly higher than that in patients transplanted from MRD (4.9±1.3%) (P<0.001). The probability of five-year overall survival (5y OS) of patients transplanted from 1MMRD (93.1±3.9%)

was comparable to that of patients transplanted from MRD (93.1±1.5%) (P=not significant, NS), but it was significantly better than that of patients transplanted from haploidentical donors (66.7±15.7%, P=0.016) and MUD (79.0±2.9%, P=0.014). In the subgroup analysis of 1MMRD, no significant difference was observed between HLA class I-mismatched (n=32) and class II-mismatched patients (n=12) (5y OS; 93.8±4.3% vs 91.7±8.0%, P=NS). Comparison of the survival outcome based on the transplant period (1990–1999 vs 2000–2009) revealed that the probability of 5y OS of patients transplanted from 1MMRD was not significantly different [92.3±5.2% (n=26) vs 94.4±5.4% (n=18), P=NS], while that of patients transplanted from MUD significantly improved in the same period as we reported previously (67.1±5.5% (n=73) vs 86.1±3.1% (n=140), P=0.001).[11] In multivariate analysis, haploidentical donors (P<0.001), MUD (P<0.001), age ≥10 years (P<0.001), and transplant period (1990–1999 vs 2000–2009, P=0.006) were identified as independent covariates associated with unfavorable OS. Our analysis revealed that an HLA-mismatched related donor, especially 1MMRD, could be selected as a donor candidate for children with AA who need urgent transplantation.

Unrelated Donor BMT

Bone marrow transplantation from an unrelated donor (UBMT) is indicated as salvage therapy for patients with severe SAA who fail to respond to immunosuppressive therapy.[12] In several recent studies, the effect of HLA high-resolution matching on outcome of patients who received a UBMT has been elucidated.[13,14] However, results have been derived primarily from an analysis of patients with hematologic malignancies. Major obstacles for UBMT are different between patients with hematologic malignancies and patients with SAA. Relapse is a main cause of death for patients with hematologic malignancies, and Graft versus Leukemia effect may result in decrease in relapse rate. In contrast, graft failure is the main problem, and GVHD is the only negative effect for patients with SAA. Therefore, optimal HLA matching may be different between these two populations. Algorithms for donor selection derived from an analysis of patients with hematologic malignancies might not be useful for patients with SAA. However, a few studies have focused on the clinical significance of HLA-allele compatibility in patients with SAA.[15] In a previous study, we analyzed the clinical significance of HLA-allele mismatching in 142 patients with SAA, in whom data of high-resolution typing of HLA-A, -B, and -DRB1 were available. Mismatching of HLA-A or -B alleles between donor and recipient was a strong risk factor for acute and chronic GVHD and OS, whereas mismatching of the HLA-DRB1 allele did not have a significant effect on patient outcomes.[16] In the study from the National Marrow Donor Program, mismatching of HLA-DRB1 was the most crucial risk factor for OS.[17] On the contrary, restricting BMT to donor-recipient pairs perfectly matched at high-resolution typing reduces the chance of undergoing UBMT for many patients. Therefore, strategies for selecting a partially HLA-allele mismatched donor are required when a full matched donor cannot be identified. We report a detailed analysis of outcome in 301 patients with SAA who were typed for HLA-A, -B, -C, -DRB1, -DQB1, and -DPB1 by a molecular technique and underwent UBMT through the JMDP.[11] Of the 301 recipient/donor pairs, 101 (33.6%) were completely matched at 10 of 10 alleles, 69 (23%) were mismatched at one allele, and 131 (43.5%) were mismatched at ≥2 alleles. Subjects were classified into five subgroups: complete match group (group I); single-allele mismatch group (groups II and III); multiple alleles restricted to HLA-C, -DRB1, and -DQB1 mismatch group (group IV); and others (group V). Multivariate analysis indicated that only HLA disparity of group V was a significant risk factor for poor survival and grade II–IV acute GVHD. HLA-DPB1 mismatching was not associated with any clinical outcome. We recommend the use of an HLA 10 of 10 allele-matched unrelated donor. However, if such a donor is not available, any single-allele or multiple-allele (HLA-C, -DRB1, and -DQB1) mismatched donor is acceptable as an unrelated donor for patients with severe AA.

Conclusion

Treatment for childhood SAA has advanced in the past decade, most notably with the improvement in survival after HLA-matched UBMT and HLA-mismatched family donor BMT. Creation of an SAA outcomes registry might contribute to the improvement of outcome in children with SAA.

References

1 Young NS, Issaragrasil S, Chieh CW, Takaku F. Aplastic anaemia in the Orient. Br J Haematol. 1986;62:1–6.
2 Baumann I, Niemeyer CM, Bennett JM, Shannon K. Childhood myelodysplastic syndrome. In: Swerdlow SH, *et al.*, editors. WHO classification of tumors of haematopoietic and lymphoid tissues. Lyon: International Agency for Research on Cancer; 2008. p. 104–7.
3 Camitta BM, Thomas ED, Nathan DG, Gale PR, Kopecky KJ, Rappeport JM, *et al.* A prospective study of androgens and bone marrow transplantation for treatment of severe aplastic anemia. Blood. 1979;53:504.
4 Bennett JM, Orazi A. Diagnostic criteria to distinguish hypocellular acute myeloid leukemia from hypocellular myelodysplastic syndromes and aplastic anemia: recommendations for a standardized approach. Haematologica. 2009;94:264–8.
5 Rajnoldi AC, Fenu S, Kerndrup G, van Wering ER, Niemeyer CM, Baumann I, *et al.* Evaluation of dysplastic features in myelodysplastic syndromes: experience from the morphology group of the European Working Group of MDS in Childhood (EWOG-MDS). Ann Hematol. 2005;84:429–33.

6 Davies JK, Guinan EC. An update on the management of severe idiopathic aplastic anaemia in children. Br J Haematol. 2007;136:549–64.
7 Locasciulli A, Oneto R, Bacigalupo A, Socié G, Korthof E, Bekassy A, *et al.* Outcome of patients with acquired aplastic anemia given first line bone marrow transplantation or immunosuppressive treatment in the last decade: a report from the European Group for Blood and Marrow Transplantation (EBMT). Haematologica. 2007;92:11–8.
8 Meyers G, Maziarz RT. Is it time for a change? The case for early application of unrelated allo-SCT for severe aplastic anemia. Bone Marrow Transplant. 2010;45:1479–88.
9 Kojima S, Hibi S, Kosaka Y, Yamamoto M, Tsuchida M, Mugishima H, *et al.* Immunosuppressive therapy using antithymocyte globulin, cyclosporine, and danazol with or without human granulocyte colony-stimulating factor in children with acquired aplastic anemia. Blood. 2000;69:2049–54.
10 Kosaka Y, Yagasaki H, Sano K, Kobayashi R, Ayukawa H, Kaneko T, *et al.* Prospective multicenter trial comparing repeated immunosuppressive therapy with stem-cell transplantation from an alternative donor as second-line treatment for children with severe and very severe aplastic anemia. Blood. 2008;111:1054–9.
11 Yagasaki H, Kojim S, Yabe H, Kato K, Kigasawa H, Sakamaki H, *et al.* Acceptable HLA-mismatching in unrelated donor bone marrow transplantation for patients with acquired severe aplastic anemia. Blood. 2011;118:3186–90.
12 Passweg JR, Pérez WS, Eapen M, Camitta BM, Gluckman E, Hinterberger W, *et al.* Bone marrow transplants from mismatched related and unrelated donors for severe aplastic anemia. Bone Marrow Transplant. 2006;37:641–9.
13 Morishima Y, Sasazuki T, Inoko H, Juji T, Akaza T, Yamamoto K, *et al.* The clinical significance of human leukocyte antigen (HLA) allele compatibility in patients receiving a marrow transplant from serologically HLA-A, HLA-B, and HLA-DR matched unrelated donors. Blood. 2002;99:4200–6.
14 Lee SJ, Klein J, Haagenson M, Baxter-Lowe LA, Confer DL, Eapen M, *et al.* High-resolution donor-recipient HLA matching contributes to the success of unrelated donor marrow transplantation. Blood. 2007;110:4576–83.
15 Maury S, Balère-Appert ML, Chir Z, Boiron JM, Galambrun C, Yakouben K, *et al.* Unrelated stem cell transplantation for severe acquired aplastic anemia: improved outcome in the era of high-resolution HLA matching between donor and recipient. Haematologica. 2007;92:589–96.
16 Kojima S, Matsuyama T, Kato S, Kigasawa H, Kobayashi R, Kikuta A, *et al.* Outcome of 154 patients with severe aplastic anemia who received transplants from unrelated donors: the Japan Marrow Donor Program. Blood. 2002;100:799–803.
17 Deeg HJ, Seidel K, Casper J, Anasetti C, Davies S, Gajeweski JL, *et al.* Marrow transplantation from unrelated donors for patients with severe aplastic anemia who have failed immunosuppressive therapy. Biol Blood Marrow Transplant. 1999;5:243–52.

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Loss of heterozygosity in 7q myeloid disorders: clinical associations and genomic pathogenesis

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Loss of heterozygosity affecting chromosome 7q is common in acute myeloid leukemia and myelodysplastic syndromes, pointing toward the essential role of this region in disease phenotype and clonal evolution. The higher resolution offered by recently developed genomic platforms may be used to establish more precise clinical correlations and identify specific target genes. We analyzed a series of patients with myeloid disorders using recent genomic technologies (1458 by single-nucleotide polymorphism arrays [SNP-A], 226 by next-generation sequencing, and 183 by expression microarrays). Using SNP-A, we identified chromosome 7q loss of heterozygosity segments in 161 of 1458 patients (11%); 26% of chronic myelomonocytic leukemia patients harbored 7q uniparental disomy, of which 41% had a homozygous *EZH2* mutation. In addition, we describe an SNP-A–isolated deletion 7 hypocellular myelodysplastic syndrome subset, with a high rate of progression. Using direct and parallel sequencing, we found no recurrent mutations in typically large deletion 7q and monosomy 7 patients. In contrast, we detected a markedly decreased expression of genes included in our SNP-A defined minimally deleted regions. Although a 2-hit model is present in most patients with 7q uniparental disomy and a myeloproliferative phenotype, haplodeficient expression of defined regions of 7q may underlie pathogenesis in patients with deletions and predominant dysplastic features. (*Blood*. 2012;119(25):6109-6117)

Introduction

Complete loss of chromosome 7 (monosomy 7) or partial deletion involving its long arm [del(7q)] are highly recurrent chromosomal aberrations in myeloid disorders, including myelodysplastic syndrome (MDS), acute myeloid leukemia (AML), and juvenile myelomonocytic leukemia (JMML).[1,2] The International Prognostic Scoring System (IPSS), the most validated score for predicting the evolution of patients with MDS, does not discriminate among chromosome 7 anomalies, uniformly assigning these patients to the poor-risk karyotype group.[3] Other metaphase cytogenetic (MC) studies have consistently associated lesions involving the long arm of chromosome 7 with inferior survival in AML cases.[1,4] However, there is a contention that monosomy 7 and del(7q) are not equivalent in prognosis and disease phenotype spectrum.[5,6]

In the traditional genetic view, loss of heterozygosity (LOH) for 1 tumor suppressor gene (TSG) allele increases the chance of inactivation of the remaining allele and total loss of function for a cancer-protective locus. In accordance to this 2-hit model, we and other groups found loss-of-function hypomorphic homo- and hemizygous mutations in a variety of genes, including *TP53*, *CBL*, or *TET2*.[7-10] However, there is growing evidence that haploinsufficient TSGs also lead to hastened tumorigenesis, showing dramatic phenotypes with loss of only a single allele.[11,12] The haploinsufficient model is supported by recent studies in the context of myeloid disorders harboring a deletion of the long arms of chromosome 5 or chromosome 20,[13-15] and it is possible that monosomy 7/del(7q) cases are associated with a similar mechanism.

To better address the genomic and clinical complexity of myeloid malignancies associated with 7q abnormalities, we analyzed a large series of cases with single nucleotide polymorphism array (SNP-A)–based karyotyping, direct and next-generation sequencing (NGS), and microarray expression platforms to (1) examine the association of different SNP-A 7q lesions with certain clinical features and other genomic aberrations, (2) define a commonly deleted region or regions (CDRs) and search for recurrent tumor suppressor mutations, and (3) test the haploinsufficiency hypothesis.

Methods

Patients

Informed consent was obtained following the Declaration of Helsinki according to protocols approved by the review boards and ethics committees of the participating institutions. Presentation bone marrow (BM)

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aspirates from 1458 patients with myeloid malignancies were studied using SNP-A, including 200 AML cases analyzed by SNP-A and NGS 300 through The Cancer Genome Atlas project (TCGA; http://tcga-data.nci.nih.gov/tcga/tcgaHome2.jsp). Microarray expression data were available on a cohort of 183 patients with MDS and 17 healthy controls.[16]

Diagnosis of hypocellular myelodysplastic syndrome (hMDS) was made based on the presence of dysplastic features and the overall clinical presentation, including the presence of cytopenias, the absence of an excess of blasts (5% in BM or 2% in blood), and a decreased cellularity of the marrow of less than or equal to 20%. When indicated based on clinical suspicion, immunohistochemical staining for CD34 was performed to rule out, or find, collections of immature cells.

Metaphase cytogenetics

Chromosome preparations were G-banded using trypsin and Giemsa, and karyotypes were described according to the International System for Human Cytogenetic Nomenclature.[17]

SNP-A analysis

Genome-Wide Human SNP 6.0 and GeneChip Human Mapping 250K arrays (Affymetrix) were used for SNP-A analysis of bone marrow DNA as described previously.[18] Germ-line encoded copy number variants and nonclonal areas of uniparental disomy (UPD) were excluded from further analysis by a bioanalytic algorithm, based on lesions identified by SNP-A karyotyping in an internal control series (n = 1003) and reported in the Database of Genomic Variants (http://projects.tcag.ca/variation/). Size and location criteria (telomeric > 8.7 Mb and interstitial, > 25 Mb) were used for identification of somatic UPD. In 11 patients, a 7q microdeletion (median size, 0.3 Mb; range, 0.1-0.7 Mb) was detected but testing of germ line DNA was not possible because of lack of appropriate samples. These patients were excluded from analysis as the pathophysiologic significance of such small lesions is not clear. None of those 7q microdeletions was included in any of the CDRs described subsequently.

Direct sequencing

Sanger technique was used for sequencing all exons of 36 candidate genes, included in our SNP-A–defined CDRs, screening a subset of 50 7q LOH patients [UPD(7q), n = 7; del(7q), n = 31; monosomy 7, n = 12]. Samples from this cohort were not used for NGS.

Next-generation sequencing

Two NGS approaches were used in this study. We generated exome chromosome 7 libraries that were enriched for the content of chromosome 7 coding sequences using the SureSelect capture synthetic biotinylated RNA probes from Agilent Technologies, tiling all the coding regions from chromosome 7. Libraries were subjected to high-throughput sequencing on a Genome Analyzer IIx (Illumina) and applied to 11 7q LOH patients [del(7), n = 6; del(7q), n = 2; UPD(7q), n = 3].

The second approach involved the sequencing of 15 paired bone marrow mononuclear cells and $CD3^+$ lymphocytes (used as germ line controls) from 15 patients with different myeloid disorders and SNP-A findings. Among them, we included 2 patients with 7q LOH [UPD(7q) and del(7q)]. A rational bioanalytic algorithm was applied to identify candidate nonsynonymous alterations. First, nonredundantly mapped reads were used for whole exome assembly using the reference genome hg19. Next, the software algorithm called all the positions that vary from the reference genome. The candidate alterations were subtracted by the results of $CD3^+$ lymphocyte-derived DNA (double-checked by direct and simultaneous visualization using DNAnexus Site) and subsequently validated using Sanger sequencing. Moreover, gene mutations affecting 7q LOH were screened using whole exome sequencing results available through TCGA.

Microarray data analysis

Previously published microarray expression data were obtained on a cohort of 183 MDS patients [monosomy 7/del(7q), n = 9].[16] Cell intensity calculation and scaling was performed using GeneChip Version 1.40 operating software (Affymetrix). Affymetrix CEL files were preprocessed using robust multiarray average. Data from 17 healthy controls were used to obtain patient and control expression ratios.

Statistical analysis

Comparisons of proportions and ranks of variables between groups were performed by the χ^2 test, Fisher exact test, Student t test or Mann-Whitney U test, as appropriate. We used the Kaplan-Meier and the Cox method to analyze overall survival (OS) and progression-free survival, with a 2-sided P less than or equal to .05 determining significance. In Cox models, examination of log (−log) survival plots and partial residuals was performed to assess that the underlying assumption of proportional hazards was met.

Results

Patient cohorts

Using SNP-A karyotyping, LOH segments involving 7q were identified in 161 of 1458 patients (11%), consisting of 9% MDS, 28% MDS/myeloproliferative neoplasms (MDS/MPN), 11% AML, 14% JMML, and 16% Fanconi anemia subsets (Figure 1A). MC identified 7q LOH in each of the cases detected by SNP-A except for 26 UPD cases and 11 patients in whom no interpretable metaphases were obtained (Figure 1B). In addition, in 7 cases a balanced translocation with 7q material was noted by MC; in all instances, SNP-A analyses detected a small deletion (> 1 and < 5 Mb) affecting the boundaries of the translocation. In 16 of 67 monosomy 7 cases by MC, SNP-A detected retained chromosome 7 material, probably contributing to marker chromosomes found by MC analysis. With increased resolution, there was a shift toward identification of more complex karyotypes and of additional lesions among the patients with isolated MC 7q aberrations (Figure 1C). By SNP-A, previously cryptic lesions were identified in 45% of the patients who otherwise showed a singular 7q LOH lesion by MC.

The 7q LOH cohort included men (57%) and women (43%) with a median age of 65 years (interquartile range, 59-73 years). The distribution of disease subsets and associated genomic lesions among the 3 classes of chromosome 7 lesions [UPD7q, del(7q) and monosomy 7] is shown in Figure 2, and Table 1 shows clinical characteristics at baseline.

Clinical and genomic correlates of monosomy 7/del(7q) patients

Compared with cases of partial deletions, those patients with del(7) were characterized by a lower number of genomic lesions per patient (1.2 vs 4.8; $P < .001$); the most remarkable the absence of 17p LOH cases among MDS patients.

Of 26 patients with monosomy 7 by SNP-A and a diagnosis of MDS, 20 (77%) fulfilled the diagnostic criteria of hMDS. Of note, these 20 patients had no other lesion detectable by SNP-A (supplemental Figure 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). That strong correlation between hMDS and the presence of an isolated monosomy 7 could not be established by MC, because no-growth was obtained in 8 hMDS-MC analyses and 4 high-risk MDS patients were described to harbor an isolated monosomy 7 by MC, whereas SNP-A found additional lesions in all of them. When patients with hMDS with or without monosomy 7 were compared, those with monosomy 7 showed a worse prognosis, with a higher transformation to leukemia ($P = .02$; hazard ratio, 3.4, 95%

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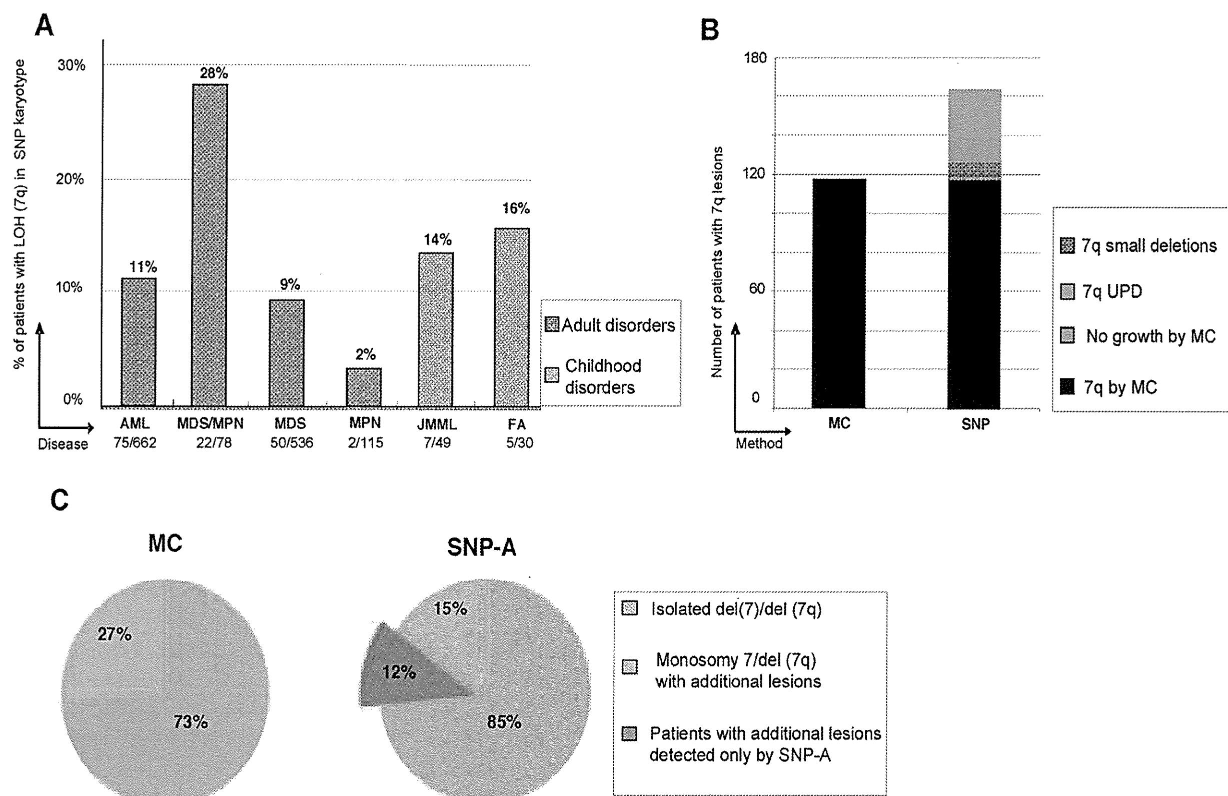

Figure 1. Frequency of detection of 7q and additional abnormalities by SNP-A. (A) Distribution of 7q LOH among the 1458 SNP-A–tested patients with myeloid malignancies, according to World Health Organization disease classification. (B) Number of patients with 7q LOH seen on MC and SNP-A. Lesions were observed in 117 of 1458 and 161 of 1458 patients when using MC and SNP-A, respectively. The additional 7q lesions found by SNP-A included those found in patients with no growth of MC cultures, small deletions affecting balanced translocation boundaries,[11] and UPD undetectable by MC.[26] (C) Percentage of patients with a sole 7q lesion versus accompanied by other abnormalities as identified by MC and SNP-A. SNP-A indicates single nucleotide polymorphism array;MDS/MPN, myelodysplastic syndrome/myeloproliferative neoplasm; AML, acute myeloid leukemia; MPN myeloproliferative neoplasms; JMML, juvenile myelomonocytic leukemia; FA, Fanconi anemia; UPD, uniparental disomy; MC, metaphase cytogenetics; monosomy 7, deletion of whole chromosome 7; and del(7q), partial deltion involving 7q.

confidence interval, 1.2%-9.7%; Figure 3A). Interestingly, 3 cases of monosomy 7 AML had a history of antecedent aplastic anemia. All the chromosome 7 lesions detected in 79 pediatric patients were monosomies; monosomy 7 was detected in 14% and 16% of patients with JMML and FA, respectively.

The subset of patients with del(7q) presented with an elevated percentage of high-risk disease (88% had AML or high-risk/intermediate-2 IPSS MDS) and a higher number of associated genomic lesions per patient. Del(5q) was the most common del(7q)-associated lesion found, occurring in 35 of 72 patients, all of which had AML or higher risk MDS. Of note, all del(5q) segments, except in 4 cases, involved either the centromeric or the telomeric extremes of the long arm of chromosome 5. The high frequency of del(5q) was followed closely by LOH 17p, seen in 14 of 72 patients, of which 5 of 14 were UPDs. Similar to patients with 5q, all patients had advanced stages of MDS or AML at diagnosis. All patients with LOH 17p spanned TP53; somatic mutations were present in 77% of cases tested.

The del(7q) MDS and MDS/MPN cohort had a shorter OS and time to leukemia transformation compared with patients with UPD(7q) or monosomy 7 (Figure 3B). In contrast, OS was similar in AML cases with monosomy 7 and del(7q) (Figure 3C), with both showing a significantly worse survival than in those patients with AML but without 7q LOH.

Clinical and genomic correlates of UPD(7q) patients

The UPD(7q) subset consisted of 26 patients, of which 17 were diagnosed with chronic myelomonocytic leukemia (CMML). Interestingly, 2 cases of AML and UPD(7q) also had history of antecedent CMML. The number of associated genomic lesions in the UPD(7q) cohort was lower than in the monosomy 7 and del(7q) subsets ($P = .03$ and $P < .001$, respectively), with a predominant presence of other regions of somatic copy neutral LOH rather than unbalanced defects. UPD(7q) was not associated with 5q or 17p LOH segments.

Comparing the 17 CMML patients to 55 CMML patients without UPD(7q) by SNP-A analysis, we found a trend toward worse survival. Those CMML patients with UPD(7q) showed a trend toward a shorter median OS (460 vs 730 days ($P = .2$; Figure 3D) and a higher rate of transformation to leukemia; whereas 26% of UPD(7q) patients progressed to higher-risk MDS or AML, advanced disease was observed in 13% of CMML patients without UPD(7q) ($P = .001$).

To test the prognostic validity and independence from known clinical variables of chromosome 7 SNP-A findings in patients' MDS and CMML, we developed a multivariate model for each cohort (Table 2). In the MDS model, the absence or presence of del(7q) or monosomy 7 kept the independent prognostic value when analyzed controlling for the clinical variables from the IPSS, ie, bone marrow blast percentage and number of cytopenias retained, whereas in the CMML model, the presence or absence of UPD 7q showed a trend toward statistical significance ($P = .1$) when tested together with the variables included in the score described by Onida et al,[19] that is, hemoglobin level below 12 g/dL, presence of circulating immature myeloid cells,

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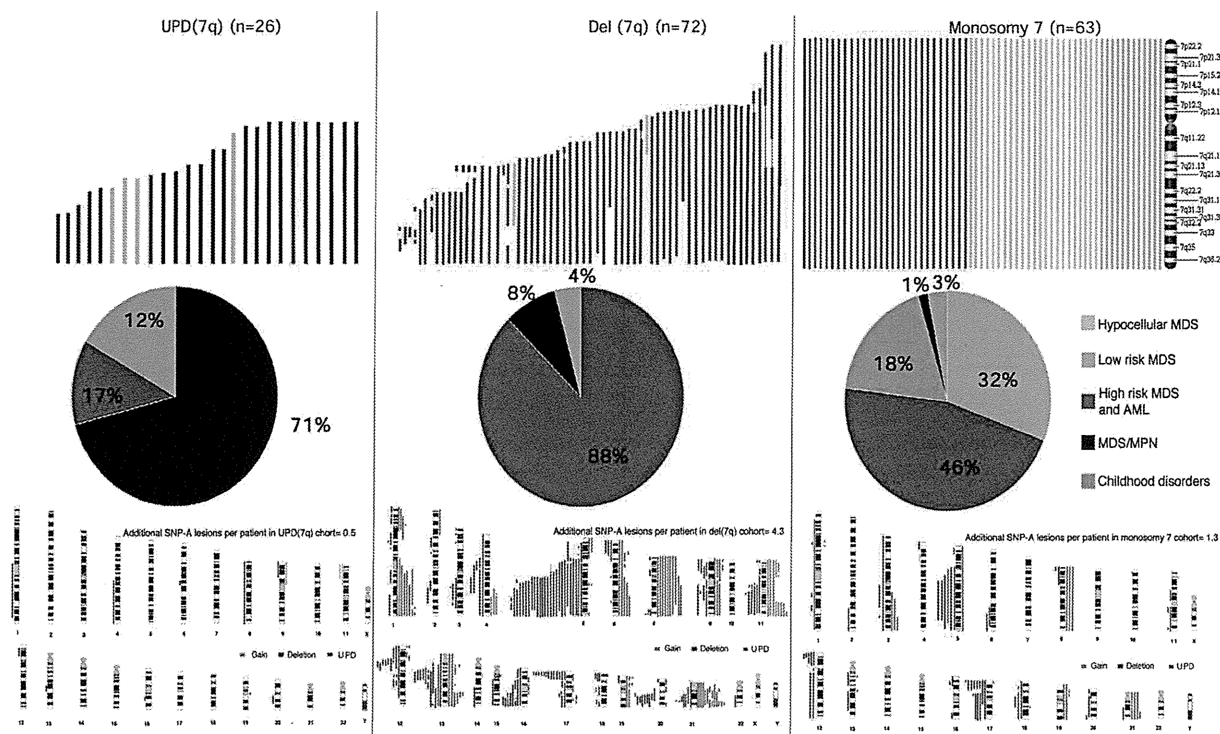

Figure 2. Distribution of disease subsets and associated genomic lesions among the 3 classes of chromosome 7 lesions. (Top) Distribution of LOH detected by SNP-A in the cohort, separated according to the nature of the lesion ([UPD(7q), del(7q), monosomy 7]. Patients have been grouped as follows: red, AML + high risk and intermediate-2 MDS; gray, low risk and intermediate-1 MDS; blue, hypocellular MDS; black, MDS/MPN; and green, Fanconi anemia and JMML. (Middle) Distribution of disease status in patients with 7 LOH separated according the nature of the lesion. (Bottom) Additional SNP-A–detected genomic lesions separated according the same criteria as stated herein. MDS indicates myelodysplastic syndrome; AML, acute myeloid leukemia; UPD, uniparental disomy; monosomy 7, deletion of whole chromosome 7; del(7q), partial deletion involving 7q; and MDS/MPN, myelodysplastic syndrome/myeloproliferative neoplasm.

absolute lymphocyte count greater than 2.5 × 10/L,[9] and marrow blasts greater than 10%.

Comparative analysis of SNP-A and MC

To provide a more detailed analysis about the added information that these karyotyping techniques could offer, Figure 4 illustrates how the distribution of disease subsets and outcome associations would be according to the lesion found by MC. Leaving aside those 44 patients with 7q LOH not detected by MC (26 UPD, 11 no growth, 7 small deletions in balanced translocations), the allocation of entities among MC del(7q) and monosomy 7 did not show a significant change. In fact, when only MC informative cases were considered, a multivariate model including both SNP-A and MC 7q lesions in MDS resulted in the variables cancelling each other (P = .7). No SNP-A–defined monosomy 7 was defined as a partial deletion by MC, because of which the strong association among monosomy 7 and hMDS and its high rate of transformation compared with hMDS without monosomy 7 remains unaltered. However, we must remark that this subgroup of MDS patients showed a higher frequency of no-growth MC analysis (in 8 hMDS patients, half of them harbored a monosomy 7 by SNP-A).

As a result of SNP findings, 16 patients were erroneously assigned to the monosomy 7 group by MC. SNP-A revealed that these samples instead had partial deletions and thus had been misclassified as high-risk patients. Probably, "relocation" of patients led to the lack of statistical difference noted in survival between MC-defined del(7q) and monosomy 7 patients (although a trend is still noted, P = .07).

Table 1. Comparative of clinical characteristics of patients at baseline according to the SNP-A–detected lesion nature

	Deletion(7), n = 63 (A)	Deletion(7q), n = 72 (B)	UPD(7q), n = 26 (C)	P (only significant comparisons reported)
Median age, y (range)	58 (27-70)	64 (56-72)	68 (63.2-77)	A vs B (P = .01) A vs C (P = .009)
Sex				
Male, %	59	56	57	
Female, %	41	44	43	
White blood cell count, × 10^9/L, median (IQR)	5.5 (2.7-25.7)	4.3 (2.1-13.5)	10.5 (8.1-37.9)	C vs A (P = .028) C vs B (P = .01)
Hemoglobin, g/dL, mean ± SD	9.1 ± 1.9	9.2 (8.9-10)	9.1 ± 2.1	
Mean corpuscular volume, median (IQR)	91 (85-99)	90 (86-104.8)	90 (85-103)	
Platelets, × 10^9/L, median (IQR)	47 (22-71)	50 (24-86)	46 (20.2-146.2)	
BM cellularity, median (%)	35 (22-61)	75 (45-90)	76(55-100)	A vs B (P = .03) A vs C (P = .036)

IQR indicates interquartile range; BM, bone marrow; UPD, uniparental disomy; and CMML, chronic myeloid leukemia..