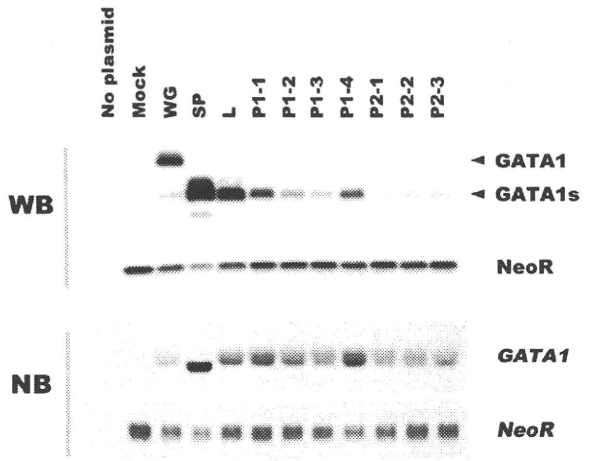

Figure 1. Effects of mutant transcripts of *GATA1* on the expression level of the truncated protein. The *GATA1* mutations observed in TAM patients are classified according to the types of transcripts. The translational efficiency of each transcript was assessed by Western blot analysis in BHK-21 cells transfected with *GATA1* cDNA expression vectors (top part of the panel) and Northern blot analysis (bottom part of the panel), respectively. WG indicates wild type GATA1; SP, splicing error mutation (Δexon 2); L, loss of first methionine mutation; P1, PTC type 1 mutation; P2, PTC type 2 mutation. The details of the *GATA1* mutations are summarized in Table 1. NeoR indicates Neomycin phosphotransferase II.

levels ($r = 0.892$, $P = .003$), but not with the long transcript containing exon2 nor total *GATA1* mRNA (supplemental Figure 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). Next, we performed RT-PCR using primers recognizing both transcripts, and calculated the ratio of Δexon 2 to the long transcript (Figure 2Bvi-vii). The intensive short transcript was detected in all samples with higher expression of GATA1s (P1-5, P1-7, P1-8, P1-2, and P1-3; Figure 2Bvii). Interestingly, most of these mutations were clustered in the 3′ region of exon 2 (Table 2, Figure 2Bvii). These results suggest that the location of the mutation predicts the efficiency of alternative splicing and GATA1s expression levels.

To examine whether differential splicing efficiency could also be observed in TAM blasts with PTC type 1 mutations, RT-PCR analysis was performed using patients' clinical samples. Intense transcription of the short form was observed in the samples from the patients who had *GATA1* mutations located on the 3′ side of exon 2 (+169 to +218 in mRNA from the ATG translation initiation codon; Figure 3A-B). We refer to them as PTC type 1-3′ and the mutations located on the 5′ side of exon 2 as PTC type 1-5′.

Correlation of the phenotype and *GATA1* mutations in TAM patients

Based on these results, *GATA1* mutations were classified into 2 groups: a high GATA1s expression group (GATA1s high group) including the loss of first methionine type, the splicing error type, and PTC type 1-3′, and a low GATA1s expression group (GATA1s low group) including PTC type 1-5′ and PTC type 2. We classified TAM patients into these 2 groups in accordance with the GATA1s expression levels estimated from the mutations and compared their clinical data. High counts of WBC and blast cells were significantly associated with the GATA1s high group ($P = .004$ and $P = .008$, respectively; Table 4). Although high WBC count was correlated with early death, there were no significant differences in the cumulative incidence of early death between the 2 groups (Figure 4). Importantly, TAM patients in the GATA1s low group had a significantly higher risk for the development of leukemia ($P < .001$; Figure 4). Of 11 TAM patients who progressed to ML-DS, 10 belonged to the GATA1s low group. Notably, 8 patients among them had PTC type 2 mutations (Tables 1, 5).

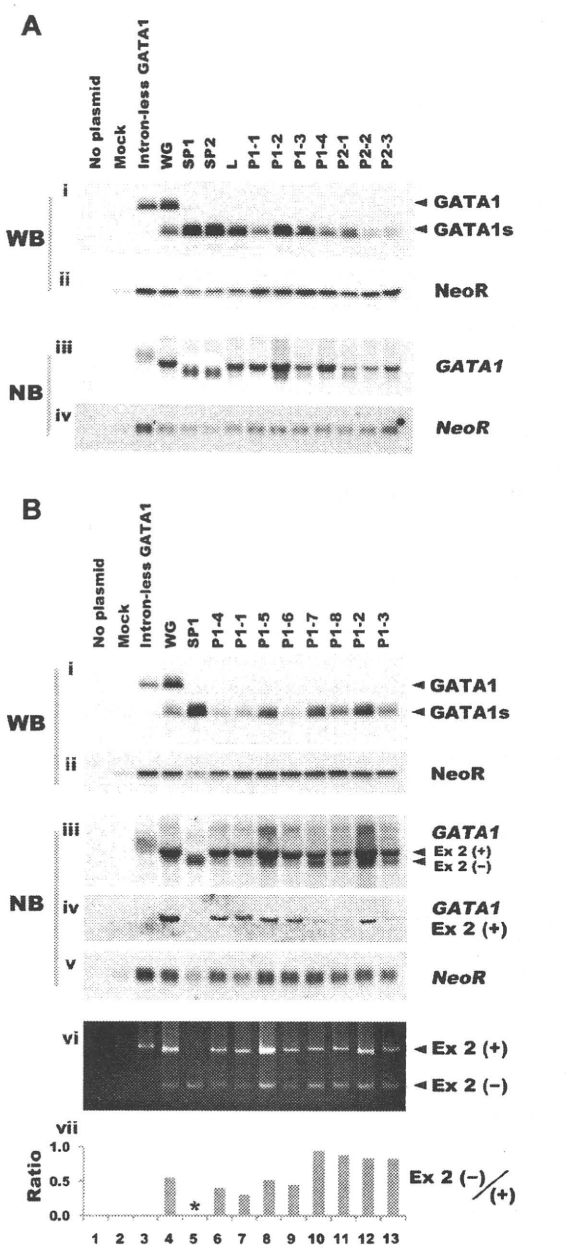

Figure 2. GATA1 mutations affect the expression level of the truncated protein. (A) The expression levels of GATA1s protein and mRNA were assessed in BHK-21 cells transfected with human *GATA1* minigene expression vectors carrying mutations observed in TAM patients. Western blot analysis was performed with anti-GATA1 (i) or anti-NeoR antibody (ii). Northern blot analysis was carried out with *GATA1* exon 3-6 fragment (iii) or *NeoR* cDNA (iv) as probe. (B) The expression levels of GATA1s protein and mRNA in BHK-21 cells transfected with human *GATA1* minigene expression vectors with PTC type 1 mutation. Levels were assessed by Western blot analysis with anti-GATA1 antibody (i), anti-NeoR antibody (ii). Northern blot analysis was performed with GATA1 exon 3-6 (iii), exon 2 (iv), or NeoR cDNA (v). To detect the transcripts derived from the human *GATA1* minigene expression construct, RT-PCR analysis was carried out using primers described in "RT-PCR" (vi). Ex 2(+) and Ex 2(−) indicate PCR products or transcripts with or without exon 2, respectively. Ratio of Ex 2(−)/(+) was calculated from the results of a densitometric analysis of the RT-PCR. The asterisk denotes unavailable data (vii).

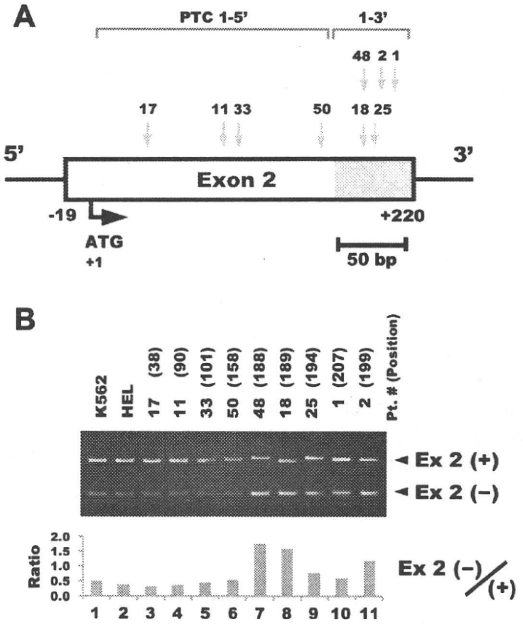

Figure 3. The location of the PTC type 1 mutation affects the efficiency of alternative splicing in TAM blast cells. (A) The location of the *GATA1* mutation in each TAM patient. Details of the mutation in each sample are described in Table 1. (B) RT-PCR analysis of *GATA1* in TAM blast cells harboring PTC type 1 mutations. RT-PCR was performed using primers recognizing both the long transcript including exon 2 and Δexon 2 (top). All of the patient samples consisted of mononuclear cells from peripheral blood. The numbers in parentheses indicate the number of nucleotides in mRNA from the translation initiation codon. Ex 2(+) and Ex 2(−) indicate PCR products with or without exon 2, respectively (middle). Ratio of Ex 2(−)/(+) was calculated from the results of a densitometric analysis of the RT-PCR (bottom). Note that the intense bands of the short form were observed in the samples from the patients who have *GATA1* mutations located on the 3′ side of exon 2 (lanes 7-11).

To validate this observation, we examined the proportion of mutation types in 40 ML-DS patients observed in the same period of time as this surveillance. The results showed a significantly higher incidence of GATA1s low type mutations in ML-DS than in TAM ($P = .039$; Table 5). These results further support the present findings that quantitative differences in the mutant protein have a significant effect on the risk of progression to ML-DS.

Table 4. Correlations between patient covariates and GATA1 expression levels

	GATA1s expression group		
	High (n = 40)	Low (n = 26)	*P*
Sex: male/female, n	19/21	13/13	.843*
Gestational age, wk	37.3 (30.0-40.0)	37.9 (32.6-40.6)	.487
Birth weight, kg	2.5 (1.6-3.3)	2.5 (1.4-3.5)	.698
WBC, $\times 10^9$/L	105.65 (7.8-423.0)	39.0 (9.0-220.0)	.004
Number of blasts, $\times 10^9$/L	72.1 (0.42-301.6)	13.4 (0.45-189.2)	.008
AST, IU/L	68.5 (23-501)	46.5 (16-4341)	.113
ALT, IU/L	41.0 (5-407)	12.5 (4-653)	.075
T-Bil mg/dL	6.7 (0.6-15.3)	4.65 (1.82-46.0)	.270
Effusions, n (%)	11 of 27 (40.7)	5 of 17 (29.4)	.447†
Bleeding diatheses, n (%)	8 of 29 (27.6)	5 of 16 (31.3)	.528†

Values are given as the median (range). *P* values estimated by Mann-Whitney *U* test.

*Pearson χ^2 test.

†Fisher exact test.

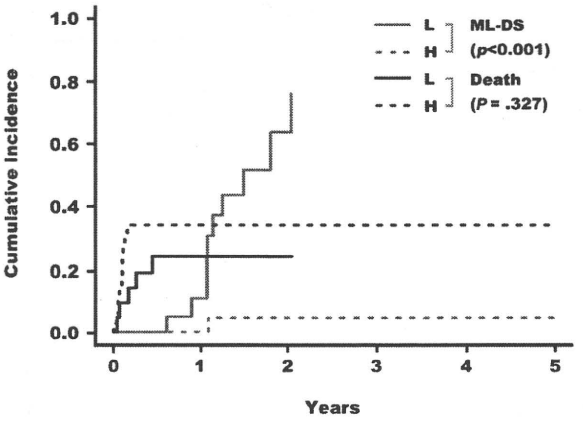

Figure 4. Cumulative incidence of early death and of ML-DS in children with TAM. Based on the estimated GATA1s expression levels, patients were classified in 2 groups: GATA1s high and low groups. TAM patients in the GATA1s low group had a significantly higher risk for the development of leukemia (P (gray) < .001).

Discussion

In TAM, *GATA1* mutations lead to the expression of proteins lacking the N-terminal transactivation domain. In addition to this qualitative change, we showed here that the mutations affect the expression level of the truncated protein. The mutations were classified into 2 groups according to the estimated GATA1s expression level. Comparison of the clinical features between the 2 groups revealed that GATA1s low mutations were significantly associated with a high risk of progression to ML-DS and lower counts of both WBC and blast cells. These results suggest that quantitative differences in protein expression caused by *GATA1* mutations have significant effects on the phenotype of TAM.

GATA1s was shown previously to be produced from wild-type *GATA1* through 2 mechanisms: use of the alternative translation initiation site at codon 84 of the full-length transcript and alternative splicing of exon 2.[12,26] However, the translation efficiencies of GATA1s from the full-length of mRNA and short transcripts have not been investigated. Our results clearly showed that the Δexon 2 transcript produced GATA1s much more abundantly than did the full-length transcript. The translation efficiencies of GATA1s from full-length transcripts containing PTC were also lower than the alternative spliced form. These results support our contention that GATA1s expression levels largely depend on the amount of the Δexon 2 transcript. Thus, one cannot predict the expression level of GATA1s protein from the total amount of the transcript.

The differences in the quantities of GATA1s proteins expressed by PTC type 1-5′ and -3′ mutations revealed the importance of the location of the mutation for splicing efficiency and protein expression. The splicing efficiency is regulated by *cis*-elements located in exons and introns (referred to as exonic and intronic splicing enhancers or silencers), and transacting factors recognizing these elements.[27,28] The PTC type 1-3′ mutations induced efficient skipping of exon 2 (Figures 2Bvi-vii, 3A-B). These mutations might affect exonic splicing enhancers or silencers located in exon 2. To predict the splicing pattern from the mutations more accurately, the elucidation of *cis*-elements and transacting splicing factors, which regulate the splicing of exon 2 of *GATA1*, will be very important.

Table 5. Summary of outcomes and *GATA1* mutation types in TAM patients

	Outcome of TAM				TAM		ML-DS	
Mutation type	CR	Early death	Evolved to ML-DS	NA	Total (n = 66)		Total (n = 40)	
High group								
Loss of 1st Met, n (%)	7	1	1	1	10 (15.2)		3 (7.5)	
Splicing error, n (%)	7	4	0	2	13 (19.7)	40 (15.2)	6 (15.0)	16 (40.0)
PTC 1-3′, n (%)	10	6	0	1	17 (25.8)		7 (17.5)	
Low group								
SPTC 1-5′, n (%)	6	4	2	3	15 (22.7)	26 (39.4)	14 (35.0)	24 (60.0)
PTC 2, n (%)	2	1	8	0	11 (16.7)		10 (25.0)	

The nonsense mediated RNA decay pathway (NMD), a cellular mechanism for detection of PTC and prevention of translation from aberrant transcripts,[29,30] might regulate the expression of GATA1s protein derived from PTC type 2 mutations, which contained PTCs after the second methionine at codon 84. We consistently detected low amounts of transcripts of *GATA1* in samples expressing PTC type 2 mutations, whereas the expression levels of *GATA1* mRNA from PTC type 1 mutations were comparable with that from wild-type *GATA1* (Figure 2Aiii). These results suggest that the location of PTC relative to alternative translation initiation sites is important for effective NMD surveillance.

Available evidence indicates that acute leukemia arises from cooperation between one class of mutations that interferes with differentiation (class II mutations) and another class that confers a proliferative advantage to cells (class I mutations).[31] Recent reports showed that introducing high levels of exogenous GATA1 lacking the N-terminus did not reduce the aberrant growth of GATA1-null megakaryocytes, but instead induced differentiation.[32,33] This observation suggested that abundant GATA1s protein functions like a class I mutation in TAM blasts. In contrast, reducing GATA1 expression leads to differentiation arrest and aberrant growth of megakaryocytic cells.[19,20] The present data suggest that GATA1s is expressed at very low levels in TAM blasts with GATA1s low mutations. These levels may not be sufficient to provoke normal maturation. Together, these findings suggest that the low expression of GATA1s might function like class II mutations in TAM blasts. Additional class I mutations or epigenetic alterations might be more effective in the development of leukemia in blast cells expressing GATA1s at low levels.

In the present study, we identified a subgroup of TAM patients with a higher risk of developing ML-DS. Of 66 children, 11 (16.7%) with TAM subsequently developed ML-DS and 10 of them belonged to the GATA1s low group harboring the PTC type 2 or PTC type 1-5′ mutations. Surprisingly, 8 of 11 patients (73%) with the PTC type 2 mutations developed ML-DS (Tables 1, 5), whereas 2 of 15 patients (13.3%) with PTC type 1-5′ mutations developed leukemia. The estimated expression levels of GATA1s from PTC type 2 mutations were lower than those from PTC type 1-5′ mutations (Figures 1, 2Ai). These results suggest that the type 2 mutations may be a more significant risk factor for developing ML-DS (supplemental Figure 2). However, our classification of *GATA1* mutations mainly rested on extrapolation from in vitro transfection experiments (Figures 1-2) and RT-PCR analyses of a small number of patient samples (Figure 3). The stability of the transcripts and the splicing efficiency of the second exon of *GATA1* will be regulated through complex mechanisms. To confirm our findings, precise mapping of the mutations that affect the expression levels of GATA1s and a prospective study with a large series of TAM patients are necessary.

Finally, we proposed the hypothesis that the quantitative differences in GATA1s protein expression caused by mutations have a significant effect on the phenotype of TAM. The observations described here provide valuable information about the roles of *GATA1* mutations on multistep leukemogenesis in DS patients. Moreover, the results might have implications for management of leukemia observed in DS infants and children. Because the blast cells in both TAM and subsequent ML-DS appear highly sensitive to cytarabine,[34-39] the preleukemic clone could be treated with low-dose cytarabine without severe side effects, and elimination of the preleukemic clone might prevent progression to leukemia.

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Authorship

Contribution: R.K. and T. Toki designed, organized, and performed research, analyzed data, and wrote the paper; K.T. designed research and collected and analyzed clinical data; G.X. and R.W. performed mutation screening; A.S., H.K., K. Kawakami, M.E., D.H., K. Kogawa, S.A., Y.I., S.I., T. Taga, Y.K., and Y.H. provided clinical samples and data; A.H. and S.K. performed mutation screening and provided clinical samples and data; and E.I. designed and organized research, analyzed data, and wrote the paper.

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Correspondence: Etsuro Ito, Department of Pediatrics, Hirosaki University Graduate School of Medicine, 5 Zaifu-cho, Hirosaki, Aomori, 036-8563, Japan; e-mail: eturou@cc.hirosaki-u.ac.jp.

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Brief report

Relapse of leukemia with loss of mismatched HLA resulting from uniparental disomy after haploidentical hematopoietic stem cell transplantation

Itzel Bustos Villalobos,[1] Yoshiyuki Takahashi,[1] Yoshiki Akatsuka,[2,3] Hideki Muramatsu,[1] Nobuhiro Nishio,[1] Asahito Hama,[1] Hiroshi Yagasaki,[1] Hiroh Saji,[4] Motohiro Kato,[5] Seishi Ogawa,[6] and Seiji Kojima[1]

[1]Department of Pediatrics, Nagoya University Graduate School of Medicine, Nagoya; [2]Division of Immunology, Aichi Cancer Center Research Institute, Nagoya; [3]Department of Hematology and Oncology, Fujita Healthy University, Aichi; [4]Human Leukocyte Antigen Laboratory, Nonprofit Organization, Kyoto; [5]Department of Pediatrics, Graduate School of Medicine, University of Tokyo, Tokyo; and [6]21st Century Center of Excellence Program, Graduate School of Medicine, University of Tokyo, Tokyo, Japan

We investigated human leukocyte antigen (HLA) expression on leukemic cells derived from patients at diagnosis and relapse after hematopoietic stem cell transplantation (HSCT) using flow cytometry with locus-specific antibodies. Two of 3 patients who relapsed after HLA-haploidentical HSCT demonstrated loss of HLA alleles in leukemic cells at relapse; on the other hand, no loss of HLA alleles was seen in 6 patients who relapsed after HLA-identical HSCT. Single-nucleotide polymorphism array analyses of sorted leukemic cells further revealed the copy number-neutral loss of heterozygosity, namely, acquired uniparental disomy on the short arm of chromosome 6, resulting in the total loss of the mismatched HLA haplotype. These results suggest that the escape from immunosurveillance by the loss of mismatched HLA alleles may be a crucial mechanism of relapse after HLA-haploidentical HSCT. Accordingly, the status of mismatched HLA on relapsed leukemic cells should be checked before donor lymphocyte infusion. (*Blood.* 2010;115(15):3158-3161)

Introduction

Human leukocyte antigen (HLA) molecules expressed on the cell surface are required in presenting antigens to T cells. The HLA class I antigens are vital in the recognition of tumor cells by tumor-specific cytotoxic T cells. The loss of HLA class I molecules on the cell surface membrane may lead to escape from T-cell immunosurveillance and the relapse of leukemia. Previously, loss of HLA class I haplotype has been described in solid tumors.[1-3] However, there are few reports concerning HLA-haplotype loss in leukemia.[4,5]

We examined HLA class I expression in leukemic blasts from patients who relapsed after hematopoietic stem cell transplantation (HSCT) to analyze whether the loss of HLA on leukemic cells was related to the relapse after HLA-identical or haploidentical HSCT.

Methods

Patients and transplantation procedure

We identified 9 children with acute leukemia who relapsed after HSCT. Their leukemic samples were cryopreserved both at the time of the initial diagnosis and of relapse. The patients' characteristics are summarized in supplemental Table 1 (available on the *Blood* website; see the Supplemental Materials link at the top of the online article). Three patients received HSCT from an HLA-haploidentical family donor, and the other 6 patients received HSCT from an HLA-matched donor (4 siblings and 2 unrelated donors).

Written informed consent was given by the parents according to the protocol approved by the ethics committee of Nagoya University Graduate School of Medicine in accordance with the Declaration of Helsinki.

HLA class I expression on leukemic cells

Samples were collected at diagnosis and post-transplantation relapse. HLA expression of leukemic blasts and normal cells was analyzed by flow cytometry as previously reported.[6] Anti-HLA A2-FITC (cloneBB7.2) and anti-HLA A24-FITC (clone17a10) monoclonal antibodies were purchased from Medical & Biological Laboratories; HLA-A11 (IgM), HLA-A30, HLA-31 (IgM), HLA-25, HLA-26 (IgM), HLA-Bw6 (IgG3), and HLA-Bw4 (IgG3) antibodies were purchased from One Lambda. For leukemic cell markers, CD13-PE (IgG1) were purchased from Immunotech and CD34-APC (IgG1) were purchased from BD Biosciences. Samples were analyzed with FACSCalibur cytometer and CellQuest software. The method of genomic HLA typing was previously reported.[7]

Isolation of DNA and single nucleotide polymorphism analysis

The $CD13^+/CD34^+$ leukemic blasts were sorted by flow cytometry from bone marrow cells at the time of diagnosis and of relapse. Genomic DNA was extracted from leukemic cells sorted by a fluorescence-activated cell sorter as well as from phytohemagglutinin-stimulated patient-derived T cells and subjected to single nucleotide polymorphism (SNP) array analysis using GeneChip NspI arrays (Affymetrix) according to the manufacturer's protocol. Allele-specific copy number was detected using Copy Number Analyzer for GeneChip software as previously described.[8]

Limiting dilution-based CTLp frequency assay

The frequencies of cytotoxic T-lymphocyte precursor (CTLp) specific for the recipient-mismatched HLA molecules were analyzed using a standard limiting dilution assay.[9]

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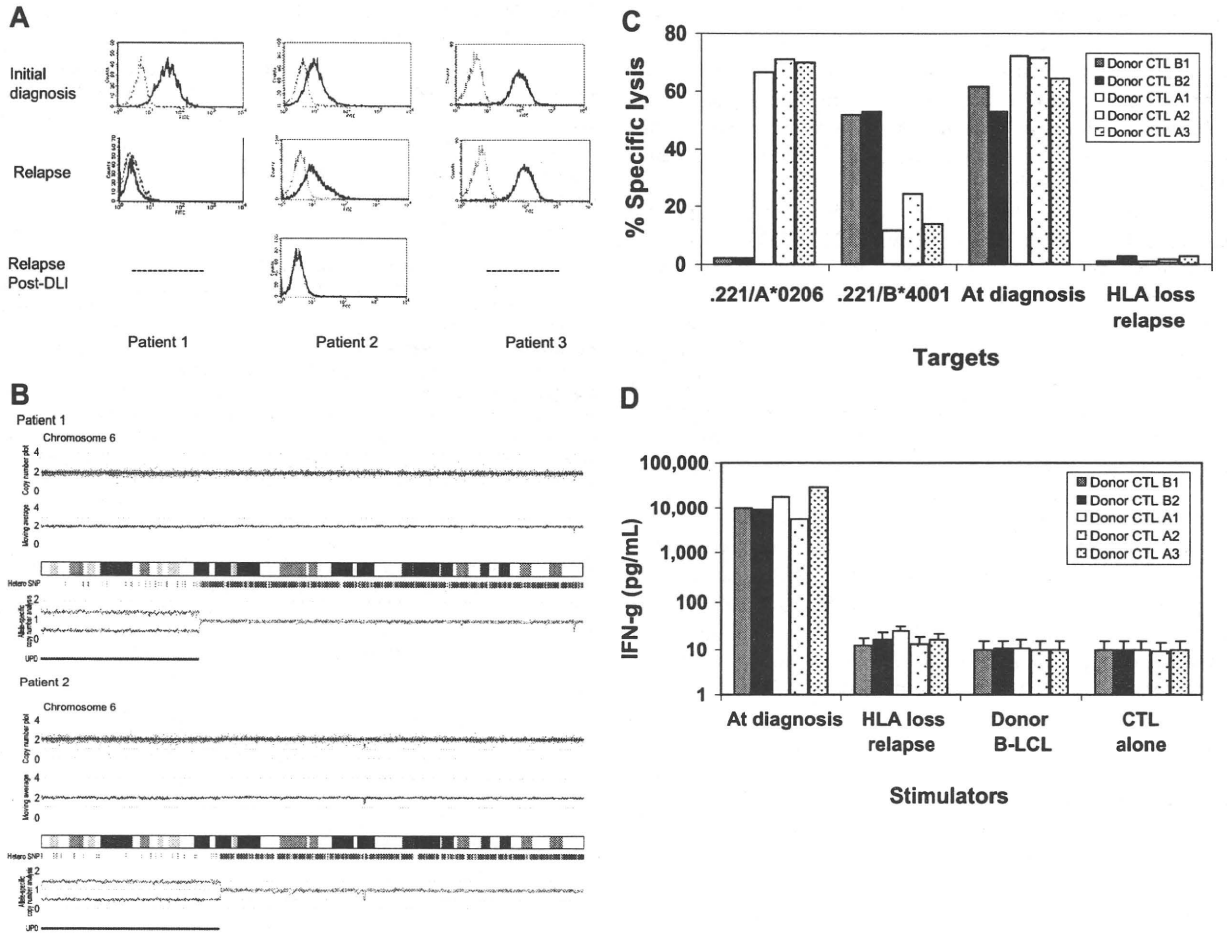

Figure 1. The loss of mismatched HLA expression on leukemic blasts caused by uniparental disomy on chromosome 6p impaired recognition and killing of donor's alloreactive cytotoxic T lymphocytes. (A) Leukemic blasts at the time of initial diagnosis and at the time of relapse after hematopoietic stem cell transplantation (HSCT) and donor lymphocyte infusion (DLI) were gated by $CD34^+$ and $CD13^+$, and then the surface expression of mismatched human leukocyte antigen (HLA) alleles was examined with anti–HLA-A2 antibodies. In 3 patients with acute myelogenous leukemia (AML) who experienced relapse after HLA-haploidentical HSCT, HLA-A2 expression was lost in patient 1 at relapse 15 months after HSCT and lost in patient 2 at second relapse 6 months after DLI. (B) Single nucleotide polymorphism (SNP) array analyses of sorted leukemic cells with the loss of an HLA allele revealed that the short arm of chromosome 6 shows copy number-neutral loss of heterozygosity or acquired uniparental disomy as detected by dissociated allele-specific copy number plots (red and blue lines at the bottom), resulting in the total loss of the mismatched HLA haplotype in both patient 1 and patient 2. The presence of acquired uniparental disomy is also indicated by normal total copy numbers with missing heterozygous SNPs (green bars) in the distal part of the short arm. (C) Recipient alloantigen-specific cytotoxic T-lymphocyte (CTL) clones were generated by a conventional cloning method from cytotoxicity-positive wells obtained in the limiting dilution assays using the donor $CD8^+$ cells as responders. Donor CTL clones A1, A2, and A3 were specific for HLA-A*0206. Donor CTL clones B1 and B3 were specific for HLA-B*4001, all of which recognize mismatched HLA alleles between the donor and recipient. Those 5 representative CTL clones were tested for HLA specificity and recognition of leukemic blasts obtained at the time of the initial diagnosis and at the time of HLA loss relapse after DLI by a standard ^{51}Cr-release assay at the effector/target ratio of 30:1. (D) Their interferon-γ production was also assessed against leukemic blasts collected at the time of diagnosis and at the time of HLA-loss relapse.

Cytotoxic assay of CTL clones against leukemic blasts and a mismatched HLA cDNA-transfected B-lymphoblastoid cell line

The remaining cells of several cytotoxicity-positive wells used for the CTLp assay for the donor were used to obtain allo-HLA–restricted CTLs. CTL clones were isolated by standard limiting dilution and expanded as previously described.[10,11]

The HLA class I–deficient 721.221 B-lymphoblastoid cell line was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2mM L-glutamine, and 1mM sodium pyruvate. Retroviral transduction was conducted as previously described.[12]

The cytotoxicity of CTL clones against target cells was analyzed by conventional chromium 51 (^{51}Cr) release assay as previously reported.[13]

CTL clones (10^4 cells/well) were mixed with the indicated stimulator cells (10^4 cells/well) in 96-well, round-bottom polypropylene plates and spun at 1200*g* for 3 minutes before overnight incubation in 200 μL of RPMI 1640 medium supplemented with 10% fetal bovine serum. On the next day, 50 μL of supernatant was collected and interferon-γ was measured by enzyme-linked immunosorbent assay with 3,3′,5,5′-tetramethylbenzidine substrate (Sigma-Aldrich).

Results and discussion

Three children with high-risk acute myelogenous leukemia (AML) received haploidentical grafts from their parents but relapsed 8, 14, and 15 months after HSCT. Patient 2 received 3 courses of donor lymphocyte infusion (DLI) for relapsed leukemia after haploidentical HSCT. After the third unmanipulated DLI (10^7 $CD3^+$/kg), she experienced acute grade-III graft-versus-host disease and achieved complete remission. However, she experienced a second relapse 6 months later. To monitor residual disease in those patients, we used flow cytometric analysis with antibodies specific for the mismatched HLA alleles between the donor and patient. Surprisingly, we found total loss of

HLA-A2 expression on $CD13^+/CD34^+$ leukemic cells from bone marrow in 2 of 3 patients who underwent HLA-haploidentical HSCT, whereas microscopic analysis showed relapse (Figure 1A). To test whether HLA class I molecules could be up-regulated, samples were cultured for 48 hours in medium supplemented with tumor necrosis factor-α or interferon-γ and measured again; however, no restoration was observed (data not shown).

Next, to examine the potential loss of genes encoding the undetectable HLA alleles, we sorted $CD13^+/CD34^+$ leukemic blasts and performed DNA genotyping. We found that, in addition to the HLA-A locus, the HLA-B, -C, and -DR loci were not encoded; only the mismatched haplotype was lost in both patients (supplemental Table 2). We then questioned whether this phenomenon would also occur in HLA-matched HSCT settings using anti-HLA class I antibodies. We did not observe any loss of HLA class I expression in any of the patients at the time of relapse (supplemental Figure 1). These results suggest that loss of HLA class I haplotype at the time of posttransplantation relapse is uncommon in HLA-matched HSCT.

To elucidate the mechanism of the loss of the mismatched HLA haplotype, we performed an SNP array analysis of genomic DNA extracted from leukemic blasts at the time of diagnosis and of relapse. Genomic DNA from patient-derived T cells was used as a reference. Leukemic cells at the time of relapse showed copy number-neutral loss of heterozygosity or an acquired uniparental disomy (UPD) of the short arm of chromosome 6 encompassing the HLA locus, whereas no allelic imbalance was identified at the time of diagnosis (Figure 1B). Loss of one allele from one parent and duplication of the remaining allele from the other parent led to UPD.[14]

In patient 2, we examined whether the number of CTLp had changed during the posttransplantation course. Limiting dilution analysis with a split-well ^{51}Cr-release assay was carried out to compare the CTLp frequencies specific for the mismatched antigens between the recipient and donor. Interestingly, the CTLp frequencies were recovered after DLI (Table 1). Restoration of CTLp after 3 DLIs could eradicate such leukemic cells, lasting for 6 months thereafter.

Next, we generated allo-HLA–restricted CTLs from $CD8^+$ cells obtained at day 520 in patient 2 and tested with the 721.221 B-lymphoblastoid cell line transfected with 1 of 3 mismatched HLA alleles (Figure 1C-D).

Despite high transplantation-related mortality resulting from severe graft-versus-host disease and posttransplantation infections, haploidentical HSCT has been widely used with the expectation of a strong graft-versus-leukemia effect.[15] However, our observation provides a possible limitation of this strategy. Indeed, 2 of 3 patients showed genomic loss of the recipient-specific HLA-haplotype, which led to escape from the graft-versus-leukemia effect and relapse of the disease.

Vago et al also reported a similar observation in 5 of 17 (29.4%) patients whose disease relapsed after haploidentical HSCT.[16] Relapsed leukemic cells may possess genomic instability that elicits genetic diversity.[17] Immunologic pressure by alloreaction to major HLA antigens may select leukemic variants of HLA class I loss, which results in the survival and proliferation of these variants.

In haploidentical HSCT, the importance of natural killer (NK)–cell alloreactivity is emphasized to achieve the graft-versus-leukemia effect.[18,19] HLA loss on leukemic blasts may in turn enhance the NK-cell alloreactivity. Our 2 patients with HLA loss had a group 1 homozygous HLA-C locus that is a suppressive killer immunoglobulin-like receptor (KIR) for NK cells and a KIR-matched donor (supplemental Table 2). Because UPD does not change the total copy number of the gene, donor NK cells should have been suppressed even after UPD occurred in these patients. Interestingly, the remaining patient who experienced relapse without HLA loss after HLA-haploidentical HSCT had a KIR-mismatched donor, so alloreactive NK cells were possibly enhanced to kill leukemic blasts with HLA loss.

Although one limitation of our study is an insufficient number of cases, our results combined with those in a recent report[16] suggest that leukemic cells occasionally escape from immunosurveillance through the loss of the mismatched HLA haplotype by the mechanism of UPD after haploidentical HSCT. DLI for relapsed AML is less effective than that for chronic myelogenous leukemia after HLA-matched HSCT.[20] However, DLI is effective even for the relapse of AML after haploidentical HSCT.[21] Evaluation of loss or down-regulation of HLA on relapsed leukemic blasts after HLA-haploidentical HSCT should be considered because DLI would probably be ineffective in patients whose leukemic cells lose HLA class I antigen.

Table 1. The CTLp frequency reactive to the recipient alloantigen in the recipient after transplantation and the donor

Samples	Maximum $CD8^+$ input*	No. of growing wells†	CTLp frequency^{-1} (95% confidence interval)
Donor	33 300	8	8.6×10^5 (1.49×10^6-5.0×10^5)
Day 100	35 500	0	UD
Day 180	17 700	0	UD
Day 300‡	86 000	0	UD
Day 520§	95 000	7	4.3×10^5 (7.2×10^5-2.5×10^5)

Purified $CD8^+$ T cells from the peripheral blood mononuclear cells obtained after transplantation from patient 2 and her donor were cultured at 2- or 3-fold serial dilutions with 33 Gy-irradiated 3×10^4 leukemic blasts cryopreserved at the time of initial diagnosis in 96-well, round-bottom plates in advanced RPMI 1640 medium supplemented with 4% pooled human serum, interleukin-6 (IL-6), and IL-7 (10 ng/mL; both from R&D Systems). The IL-2 (50 U/mL) was added on day 7 with a half medium change. For each dilution, there were at least 12 replicates. On day 14 of culture, a split-well analysis was performed for recipient-specific cytotoxicity against ^{51}Cr-radiolabeled recipient T-cell blasts, donor T-cell blasts, and leukemic blasts harvested at the time of initial diagnosis and at the time of relapse after DLI if indicated. The supernatants were measured in a γ counter after 4-hour incubation. The wells were considered to be positive for cytolytic activity if the total counts per minute released by effector cells was more than 3 SD above the control wells (mean counts per minute released by the target cells incubated with irradiated stimulator cells alone). The CTLp frequency was calculated using L-Calc software (StemCell Technologies). The CTLp frequencies reactive with recipient T-cell blasts in $CD8^+$ T cells obtained around days 100, 180, and 300 (4 months before relapse) were undetectable, whereas the CTLp frequency obtained at day 520 (1 month after the third DLI or 2 weeks after remission confirmed by bone marrow aspirate) was close to the CTLp frequency in the donor $CD8^+$ cells. Complete remission and more than 99% donor chimerism were confirmed on those days

CTLp indicates CTL precursor; and UD, undetermined because no growing wells are present.

*Number of input $CD8^+$ T cells seeded at the highest number per well.

†Number of wells out of 12 wells that received the highest $CD8^+$ cells and showed detectable growth.

‡Corresponds to 4 months before relapse.

§Corresponds to 1 month after the third DLI or 2 weeks after complete remission was confirmed by bone marrow aspirate.

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Authorship

Contribution: I.B.V. performed experiments and wrote the manuscript; Y.T. designed the research, analyzed data, and wrote the manuscript; Y.A., H.S., M.K., and S.O. performed experiments, analyzed data, and wrote the manuscript; S.K. supervised this work and wrote the manuscript; and all other authors were responsible for clinical work and critically reviewed the manuscript.

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Correspondence: Seiji Kojima, Department of Pediatrics, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, 466-8550, Japan; e-mail: kojimas@med.nagoya-u.ac.jp.

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Recent progress in dyskeratosis congenita

Nobuhiro Nishio · Seiji Kojima

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Abstract Dyskeratosis congenita (DC) is an inherited disease associated with nail dystrophy, abnormal skin pigmentation, oral leukoplakia, bone marrow failure and a predisposition to cancer. DC is a disease of defective telomere maintenance and patients with DC have very short telomeres. To date, mutations in six genes of telomerase and telomere components have been identified in patients with DC. Recently, mutations in telomerase and telomere components were also identified in patients with aplastic anemia, pulmonary fibrosis, and liver diseases who did not have mucocutaneous manifestations. These findings imply that defective telomere maintenance may cause not only classical DC but also a broad spectrum of diseases previously thought to be idiopathic, and have led to a new concept of diseases, termed "syndromes of telomere shortening". An understanding of the role of telomeres in these diseases is indispensable for diagnosis, genetic counseling and clinical management.

Keywords Dyskeratosis congenita · Telomere · Telomerase · Bone marrow failure

1 Introduction

Elizabeth Blackburn, Carol Greider, and Jack Szostak were awarded the 2009 Nobel Prize in Physiology or Medicine for their work describing telomeres and telomerase [1, 2]. Telomeres are DNA–protein structures that protect chromosome ends, which consist of a TTAGGG repeat bound by a cap protein, shelterin. Telomeres cannot be replicated by standard polymerase but only by a specialized transcriptase, called telomerase.

Dyskeratosis congenita (DC) is a rare inherited multisystem bone marrow failure syndrome characterized mainly by mucocutaneous abnormalities including nail dystrophy, mucosal leukoplakia, and abnormal skin pigmentation, along with a predisposition to cancer. Patients with DC have very short germ-line telomeres compared with normal individuals due to a defect of telomere maintenance. DC has been receiving increased attention because "telomere maintenance" is closely associated with life events, including aging and cancer predisposition. Recently, mutations in telomerase and telomere components were also identified in patients with aplastic anemia (AA), pulmonary fibrosis, and liver diseases who did not have mucocutaneous manifestations [3–13]. These findings implicate that defective telomere maintenance causes not only classical DC but also a broad spectrum of diseases previously thought to be idiopathic, and have lead to a new concept of diseases, termed "syndromes of telomere shortening".

In this review, we will discuss recent progress in the understanding of the pathophysiology of DC and other telomere diseases, as well as treatment for these diseases including stem cell transplantation.

2 Dyskeratosis congenita

The incidence of classic DC is approximately 1/1,000,000 individuals [14]. Classic DC presents with a triad of mucocutaneous abnormalities in around 80–90% of patients; abnormal skin pigmentation, nail dystrophy and oral leukoplakia [15]. Skin pigmentation and nail changes

N. Nishio · S. Kojima (✉)
Department of Pediatrics, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Shouwa-ku, Nagoya 466-8550, Japan
e-mail: kojimas@med.nagoya-u.ac.jp

usually appear in childhood followed by oral leukoplakia and bone marrow failure, which develop by the age of 20 years. Other clinical manifestations, including non-mucocutaneous abnormalities, have also been reported. Non-mucocutaneous features such as bone marrow failure and pulmonary fibrosis occasionally precede mucocutaneous abnormalities, making it difficult to diagnose patients with DC based on clinical features alone. The diagnostic criteria for DC proposed by Vulliamy [16] include one or more of the three classic mucocutaneous features combined with hypoplastic bone marrow and at least two other somatic features known to occur in DC. The main causes of death in patients with DC are bone marrow failure/immunodeficiency (60–70%), pulmonary complications (10–15%), and malignancy (10%) [17, 18].

Until now, mutations in six genes involved in telomere maintenance have been identified in patients with DC. Figure 1 shows the schema of telomerase and shelterin complex. *DKC1* gene, encoding dyskerin, is the first gene identified in X-linked DC patients [19]. Dyskerin has a close association with the RNA component of telomerase (TERC), and mutations in dyskerin cause a reduction in accumulation of TERC and reduced telomere length [20]. In addition to its role in the biogenesis of telomerase RNA, dyskerin is involved in ribosomal RNA biogenesis. Dyskerin catalyzes uridine to pseudouridine, which is a critical step for ribosomal RNA maturation and function. These findings imply that both telomere and ribosomal defects may occur in patients with *DKC1* mutations. Subsequently, heterozygous *TERC* mutations were found in autosomal dominant DC patients [21]. Mutation screening demonstrated mutations of other components of telomerase complex including telomerase reverse transcriptase (*TERT*) [22, 23], *NOP10* [24], and *NHP2* [25] in patients with rare autosomal recessive DC. Mutations of *TERT* were also reported in the autosomal dominant family [8]. More recently, heterozygous mutations of *TINF2* encoding TIN2, main component of shelterin which protects telomeres, have been identified in ~11% of DC patients [5, 26].

3 Gene mutations of telomere maintenance in aplastic anemia and other bone marrow failure syndromes

Patients with DC have disease diversity in terms of age at onset, symptoms, and severity; this diversity occurs even among the patients with the same gene mutation. Bone marrow failure sometimes precedes mucocutaneous manifestations in patients with DC, and a substantial proportion of patients with AA have shorter telomeres compared with normal individuals [27, 28]. These observations prompted screening for gene mutations responsible for telomere maintenance in patients with AA and other bone marrow failure syndromes. This screening identified mutations in *TERC* and *TERT* in 3% of patients with AA [7, 9] (Table 1). We also identified *TERT* mutations in 2 of 96 Japanese children with AA, but no patient had a *TERC* mutation [6]. Patients with *TERC* or *TERT* mutations have very short telomeres in blood cells. Recently, Du et al. [4] found that 6 (5.5%) of 109 pediatric patients with severe AA had mutations of *TINF2*. We also screened for mutations of *TINF2*, but none of 96 pediatric patients with AA showed mutations of this gene (unpublished data).

Among three methods of measuring telomere length, including southern blot, real-time polymerase chain reaction, and flow cytometry and fluorescence in situ

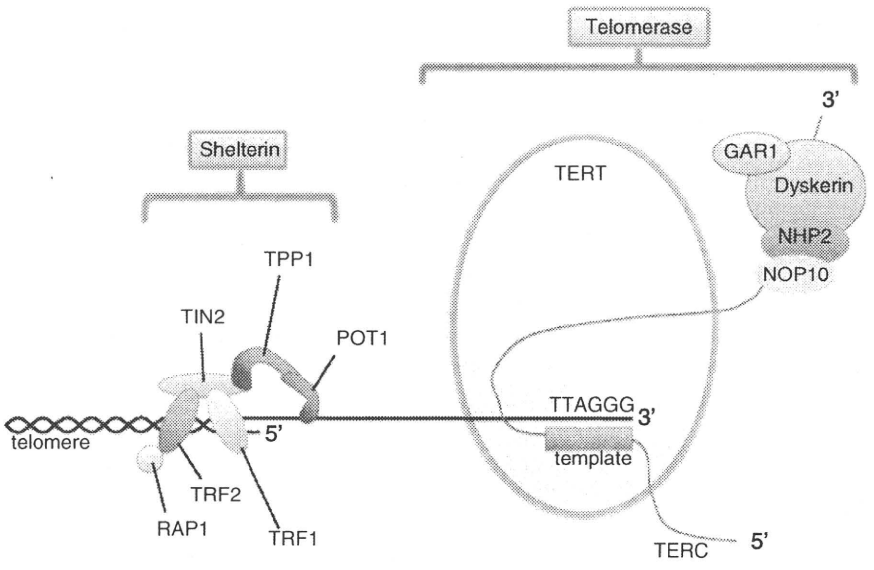

Fig. 1 Schema of telomerase and shelterin complex. Telomerase complex consists of the enzyme telomerase transcriptase (TERT), RNA component (TERC), and dyskerin protein complex (dyskerin, NOP10, NHP2, and GAR1). TERT adds new telomeres (TTAGGG repeats) onto the chromosome end by using the template provided by TERC. The shelterin complex consists of six proteins (TRF1, TRF2, RAP1, POT1, TPP1, and TIN2) and protects telomeres and regulates telomerase

Table 1 Mutations of genes associated with telomere maintenance identified in patients with aplastic anemia

References	Gene	Number of mutated and screened patients
Vulliamy et al. [10]	*TERC*	2/17 (12%)
Vulliamy et al. [8]	*TERT*	2/80 (2.5%)
Yamaguchi et al. [9]	*TERC*	2/150 (1.3%)
Yamaguchi et al. [7]	*TERT*	7/200 (3.5%)
Savage et al. [50]	*TERF1*	1/47 (2.1%)
	TERF2	1/47 (2.1%)
Liang et al. [6]	*TERT*	2/96 (2.1%)
Walne et al. [51]	*TINF2*	2/111 (1.8%)
Du et al. [3]	*TERT*	4/199 (2.0%)
Du et al. [4]	*TINF2*	6/109 (5.5%)

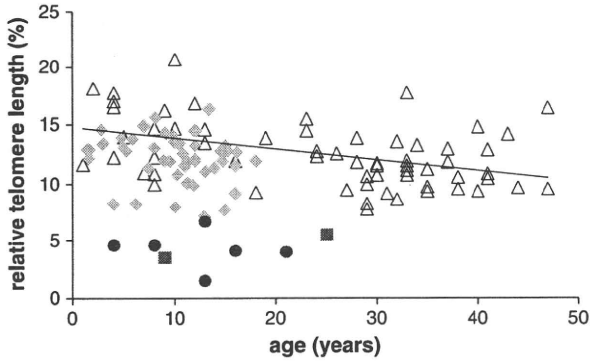

Fig. 2 Relative telomere length in peripheral blood lymphocytes from patients with dyskeratosis congenita (*filled circles*), patients with aplastic anemia harboring *TERT* mutations (*filled squares*), patients with idiopathic aplastic anemia (*filled argyles*) and normal individuals (*open triangles*). Telomere lengths were measured by flow cytometry-fluorescent in situ hybridization (flow-FISH). Relative telomere length was calculated as the ratio between the telomere signal of each sample and the telomere signal of the control cell line (cell line 1301). These data are from the Department of pediatrics, Nagoya University Graduate School of Medicine

hybridization (flow-FISH), flow-FISH is the most appropriate for "prospective" screening [29, 30]. As shown in Fig. 2, patients with DC and AA with the *TERT* mutation demonstrated very short telomeres as compared with idiopathic AA patients and normal individuals. Given the finding that a small subset of patients with apparently idiopathic AA carry telomere gene mutations and recognizing these patients is critical to treatment decisions, it is desirable to screen telomere gene mutations routinely in patients with AA before starting treatment. However, because screening of gene mutations is laborious and time-consuming, we have adopted screening of telomere length in blood cells instead of gene mutations.

It should be noted that short telomeres are not specific for patients with DC but are also seen in patients with bone marrow failure syndromes. Although short telomeres are also found in patients with other congenital bone marrow failure syndromes, such as Shwachman–Diamond syndrome and Fanconi anemia, telomere length in patients with DC is the shortest compared with other bone marrow failure syndromes. In fact, telomere length in most patients with DC is below the first percentile of telomere length found in healthy controls [31].

Family members of patients with DC should receive genetic counseling to rule out if they are silent carriers. In particular, genetic counseling is necessary during the proband search for a donor for hematopoietic stem cell transplantation. Sometimes, telomere length analysis in families with DC demonstrates that mutated carriers with clinical signs of bone marrow failure have the short telomeres. However, telomere length cannot predict the presence or absence of a mutation in family members with bone marrow failure. There are rare cases that show normal telomere length even though they harbor the same mutation as the proband, suggesting that mutation alone does not sufficiently shorten the telomeres [3].

4 Telomere diseases other than bone marrow failure syndromes

Clinical manifestations in patients with DC include not only bone marrow failure, but also other organ failures. Progressive pulmonary fibrosis develops in around 10–15% of patients with DC [17, 18], and is the second most common cause of death. Respiratory failure is also a common fatal complication after hematopoietic stem cell transplantation. Idiopathic pulmonary fibrosis (IPF) is an adult-onset, progressive scarring of the lung of unknown etiology that ultimately leads to respiratory failure. From 2 to 20% of patients with IPF have a family history of the disease that is inherited as an autosomal dominant trait with variable penetrance [12, 32]. Because some individuals in a pedigree of DC had the IPF phenotype, Armanios et al. [12] hypothesized that *TERC* or *TERT* may be candidate genes for familial IPF. They screened 73 probands of IPF and found 6 (8%) had heterozygous mutations in *TERT* or *TERC*. Tsakiri et al. [11] also independently found three missense mutations and one deletion of *TERT* genes in 44 probands of familial IPF and an additional single mutation in 44 sporadic cases of IPF. These mutant telomerase resulted in short telomeres. However, these patients did not show any classic mucocutaneous manifestations of DC.

Liver diseases have been also described as one of the clinical presentations in patients with DC. Some patients with DC develop severe liver complications after hematopoietic stem cell transplantation even if they have a normal liver function at the time of transplant [33]. In parallel with

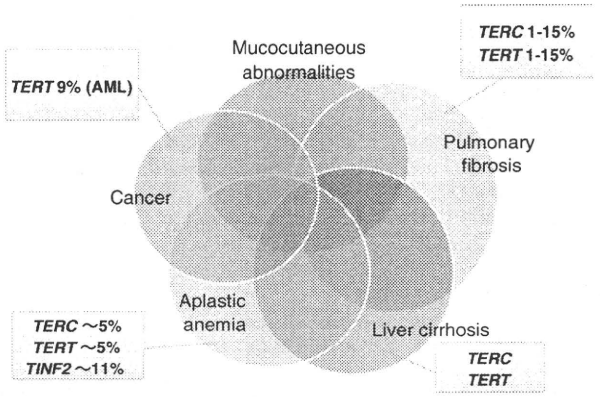

Fig. 3 Schema of phenotypic variations and identified gene mutations in defective telomere maintenance

reports of familial IPF, Calado et al. [13] reported that many relatives of patients with AA and a telomerase mutation had liver diseases, including pathologic fibrosis with inflammation and nodular regenerative hyperplasia. These patients did not present symptoms in childhood or display the characteristic physical abnormalities of DC, but had very short telomeres. These authors proposed that these disorders be collectively considered as "syndromes of telomere shortening". Figure 3 shows the schema of phenotypic variations and identified gene mutations in defective telomere maintenance.

5 Telomere shortening, chromosome instability and cancer predisposition

Patients with DC are prone to hematological malignancies and other solid tumors [17]. The defect of telomere maintenance and telomere attrition leads to chromosomal instability such as loss or gain of chromosomes and end-to-end fusion in in vitro studies and mouse models [34, 35]. Alter et al. recently reported that the expected cancer risk is 11-fold higher in patients with DC compared with the general population. The most frequent solid tumors were head and neck squamous cell carcinomas followed by skin and anorectal cancer [36].

Even outside DC, telomere attrition appears to cause chromosomal instability and cancer predisposition. Calado et al. [37] recently reported that patients with AA with shorter telomeres at diagnosis had a sixfold higher probability of developing clonal malignant disease following immunosuppressive therapy than patients with longer telomeres. They also showed that cultured bone marrow cells of patients with short telomeres in the presence of cytokines and high-dose granulocyte-colony stimulating factor (G-CSF) demonstrated increased telomere-free chromosomal ends and aneuploidy and translocations, including Robertsonian translocations.

Because patients with DC have been thought to be prone to myeloid malignancy, a screening for *TERT* and *TERC* mutations in patients with acute myeloid leukemia (AML) was conducted by the NIH group [38]. The authors found constitutional *TERT* mutations in 9% of patients with AML and a strong association of *TERT* mutations with the risk of cytogenetic abnormalities including trisomy 8 and inversion 16. None of the AML patients with *TERT* mutations had physical abnormalities that led to a suspicion of DC.

In addition, short telomeres have been linked to tumorigenesis of several solid tumors, including esophageal cancer, colorectal cancer, gastric cancer [39], and lung cancer [40]. Recent genome-wide studies demonstrated a higher frequency of *TERT* gene polymorphism in these patients than in normal individuals [41, 42].

6 Treatment of bone marrow failure

Bone marrow failure and immune deficiency are the most common causes of death in up to 60–70% of patients with DC. Androgen (e.g. oxymetholone) has been used to improve cytopenia in patients with DC since the 1960s. However, the mechanism of action of androgen has not been well understood until recently. Calado et al. [43] showed that in vitro exposure of normal peripheral blood cells to androgen produce higher *TERT* mRNA levels, and cells from patients who had heterozygous mutation of telomerase restored their low baseline telomerase activity to normal levels. As telomere shortening is closely associated with malignant disease, androgen therapy might prevent or postpone the development of various types of cancers. Erythropoietin and/or G-CSF combined with androgen has occasionally provided transient hematopoietic recovery to poor responders to androgen alone [44]. However, this combination should be used with caution because severe splenic peliosis and fatal rupture have been reported in two patients with DC who received simultaneous administration of androgen and G-CSF [45].

Allogeneic hematopoietic stem cell transplantation is the only curative treatment for bone marrow failure in patients with DC. However, the outcome in previous reports has been disappointing because of unacceptable transplant-related toxicities, including severe pulmonary/liver complications, especially in transplants from an alternative donor [36, 46]. To avoid these complications, non-myeloablative conditioning regimens have been recently used in several cases. Dietz et al. [47] reported encouraging results of six patients with DC who received a fludarabine-based non-myeloablative regimen. Four patients are alive, three of whom were recipients of unrelated grafts. Non-myeloablative transplants are expected to provide improvement in short-term survival. However,

longer-term follow-up is necessary because the late effects of conditioning agents and allogeneic immune responses within the recipient's organs, such as the lung and liver, remain to be clarified.

7 Future direction

Since the review article concerning DC was published by Walne et al. [14] in 2005 in this journal, many advances have occurred in the understanding of DC; however, many unsolved issues remain. Six causative genes have been identified, but mutations of these genes have been found in only half of patients with DC. Telomere-related gene mutations have been identified in patients with not only DC but also in patients with idiopathic AA, pulmonary fibrosis, and liver disease. These findings indicate that telomere-related diseases have a broad spectrum and may represent a new disease entity. A recent study demonstrated that exogenous expression of *TERC* alone can increase telomere activity and create growth potential and longevity in both *TERC* mutant and *DKC1* mutant cells [48]. More recently, Agarwal et al. [49] established induced pluripotent stem cells derived from a patient with DC and showed that the reprogrammed DC cells overcome a critical limitation in TERC levels to restore telomere maintenance and self-renewal. These findings indicate that drugs or gene therapy that can upregulate TERC activity have attractive therapeutic potential in patients with DC. Multicenter prospective studies are warranted to establish appropriate conditioning regimens aimed at reducing transplant-related mortality. We should improve not only short-term outcomes, such as hematological recovery, but also long-term effects on vital organs, especially the lungs and liver, following stem cell transplantation.

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Autoimmune Lymphoproliferative Syndrome Like Disease With Somatic *KRAS* Mutation

Masatoshi Takagi[1*], Kunihiro Shinoda[2], Jinhua Piao[1], Noriko Mitsuiki[1], Mari Takagi[2], Kazuyuki Matsuda[3], Hideki Muramatsu[4], Sayoko Doisaki[4], Masayuki Nagasawa[1], Tomohiro Morio[1], Yoshihito Kasahara[5], Kenichi Koike[6], Seiji Kojima[4], Akira Takao[2], Shuki Mizutani[1*]

[1]Department of Pediatrics and Developmental Biology, Graduate School of Medicine, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo, 113-8519, Japan

[2]Department of Pediatrics, Gifu Municipal Hospital
7-1 Kashimacho, Gifu-shi, Gifu, 500-8513, Japan

[3]Department of Laboratory Medicine, Shinshu University School of Medicine, 3-1-1 Asahi, Matsumoto-shi, Nagano, 390-8621, Japan

[4]Department of Pediatrics, Nagoya University, Graduate School of Medicine, 65 Tsurumaicho, Showa-ku, Nagoya-shi, Aichi, 466-8550, Japan

[5]Department of Laboratory Sciences, Kanazawa University School of Health Sciences, 5-11-80 Kodatsuno, Kanazawa, Ishikawa, 920-0942, Japan

[6]Department of Pediatrics, Shinshu University School of Medicine, 3-1-1 Asahi, Matsumoto-shi, Nagano, 390-8621, Japan

[*]Correspondence

Masatoshi Takagi and Shuki Mizutani

Department of Pediatrics and Developmental Biology, Graduate School of Medicine, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo, 113-8519, Japan

Abstract

Autoimmune lymphoproliferative syndrome (ALPS) is classically defined as a disease with defective FAS-mediated apoptosis (Type I–III). Germline *NRAS* mutation was recently identified in Type IV ALPS. We report two cases with ALPS like disease with somatic *KRAS* mutation. Both of the cases were characterized by prominent autoimmune cytopenia and lymphoadenopathy/splenomegaly. These patients did not satisfy the diagnostic criteria for ALPS or juvenile myelomonocytic leukemia (JMML), and are likely to be defined as a new disease entity of RAS associated ALPS like disease (RALD).

Introduction

ALPS is a disease characterized by dysfunction of the FAS-mediated apoptotic pathway[1,2], currently categorized as Type Ia, germline *TNFRSF6/FAS* mutation; Type Ib, germline *FAS ligand* mutation; Type Is, somatic *TNFRSF6/FAS* mutation; and Type II, germline *Caspase 10* mutation. Patients exhibit lymphadenopathy, hepatosplenomegaly, and autoimmune diseases such as immune cytopenia and hyper-γ-globulinemia. An additional subclassification has been proposed that includes Types III and IV, whereby Type III has been defined as that with no known mutation but with a defect in FAS-mediated apoptosis, and Type IV as one showing germline *NRAS* mutation[3]. Type IV is considered exceptional because the FAS-dependent apoptosis pathway is not involved in the pathogenesis, and this subclass is characterized by a resistance to IL-2 depletion-dependent apoptosis. Recent updated criteria and classification of ALPS suggested type IV ALPS as a RAS associated leukoproliferative disease [4].

JMML is a chronic leukemia in children. Patients show lymphadenopathy, hepatosplenomegaly, leukocytosis associated with monocytosis, anemia, thrombocytopenia, and occasional autoimmune phenotypes. About 80% of patients with JMML have been shown to have a genetic abnormality in their leukemia cells including mutations of *NF1*, *RAS* family[5], *CBL*, or *PTPN11*. The hallmarks of the laboratory findings of JMML include spontaneous colony formation in bone marrow (BM) or peripheral blood (PB) mononuclear cells (MNC) and hypersensitivity to granulocyte-macrophage colony-stimulating factor (GM-CSF) of CD34 positive BM-MNC[6].

Germline RAS pathway mutations cause Costello (*HRAS*), Noonan (*PTPN11*, *KRAS*, and *SOS1*), and cardio-facio-cutaneous (CFC) syndromes (*KRAS*, *BRAF*, *MEK1*, and *MEK2*). Patients with Costello and Noonan syndromes have an increased propensity to develop solid and hematopoietic tumors, respectively[7],

among these tumors the incidence of JMML in patients with germline mutation of *NF1* or *PTPN11* is well known.

We present two cases with autoimmune cytopenia and remarkable lymphadenopathy and hepatosplenomegaly, both of which were identified as having a somatic KRAS G13D mutation without any clinical features of germline *RAS* mutation such as CFC or Noonan syndrome.

Patients and Methods

All studies were approved by the ethical board of Tokyo Medical and Dental University.

Case 1

A 9-month-old boy had enormous bilateral cervical lymphadenopathy and hepatosplenomegaly (Supplemental data 1 Fig. 1a, b). Blood test revealed presence of hemolytic anemia and autoimmune thrombocytopenia. hyper-γ-globulinemia with various auto-antibodies was also noted. ALPS and JMML were nominated as the diseases to be differentially diagnosed. Detailed clinical history and laboratory data are provided as Supplemental data 1. The patient did not satisfy the criteria for the diagnosis of ALPS or JMML as discussed in results and discussion section.

Case 2

A 5-month-old girl had a fever, massive hepatosplenomegaly (Supplemental data 1 Fig. 1d). She was initially diagnosed with Evans syndrome based on the presence of hemolytic anemia and autoimmune thrombocytopenia with hyper-γ-globulinemia and auto-antibodies. Spontaneous colony formation assay and GM-CSF hypersensitivity of BM-MNC showed positivity. Then, tentative diagnosis of JMML was given, even though she showed no massive

monocytosis or increased HbF. Detailed clinical history and laboratory data is provided as Supplemental data 1.

Detailed methods for experiments are described in Supplemental data 2

Results and Discussion

Case 1 showed a high likelihood of being a case of ALPS according to the symptoms and clinical data presented (Supplemental data 1, Table 1) except for number of Double-negative T (DNT) cells which was only 1.4% of TCRαβ cells (Fig. 1a). JMML was also nominated as a disease to be differentiated, because remarkable hepatosplenomegaly with thrombocythemia and moderate monocytosis was noted. However, no hypersensitivity to GM-CSF as determined by colony formation assay for BM-MNC (data not shown) or phospho STAT5 staining (data not shown) was observed. DNA sequence for JMML associated genes such as *NRAS, KRAS, HRAS, PTPN11* and *CBL* was determined, and KRAS G13D mutation was identified (Fig. 1b). The mutation was seen exclusively in the hematopoietic cell lineage and no mutation was seen in the oral mucosa or nail-derived DNA. Granulocytes, monocytes, T cells, and B cells were all positive for KRAS G13D mutation (data not shown). The proportion of mutated cells in each hematopoietic lineage was quantitated by mutation allele specific quantitative PCR methods, which revealed mutated allele was almost equally present in granulocytes, T cells and B cells (Fig 1c). CD34-positive hematopoietic stem cells (HSC) was also positive for KRAS G13D mutation, and 60% of CFU-GM colonies developed from isolated CD34 cells carried KRAS G13D mutation (data not shown). These observations suggest that the mutation occurred at the HSC level, and HSCs consists of wild type and mutant HSCs.

NRAS mutated Type IV ALPS was previously characterized by apoptosis

resistance of T-cells in IL-2 depeletion[3]. Then, activated T cells were subjected to an apoptosis assay by FAS stimulation or IL-2 depletion. Remarkable resistance to IL-2 depletion but not to FAS-dependent apoptosis (Fig. 1d and e) was seen. This was in contrast to T cells from FAS mutated ALPS type 1a which showed remarkable resistance to FAS dependent apoptosis and normal apoptosis induction by IL-2 withdrawal (Fig. 1d and e). Western blotting analysis of activated T cells or Epstein-Barr virus-transformed B cells showed reduced expression of Bim (Fig. 1f).

In case 2, autoimmune phenotype and hepatosplenomegaly were remarkable as shown in Supplementary data 1. The patient was initially diagnosed as Evans Syndrome based on presence of hemolytic anemia and autoimmune thrombocytopenia. DNT cells were 1.1% of TCRαβ cells in the peripheral blood, which did not meet with the criteria of ALPS. Although spontaneous colony formation was shown in PB- and BM-MNC, and GM-CSF hypersensitivity was demonstrated in BM-MNC derived CD34 positive cell (Supplemental data 1 Table2), she showed no massive monocytosis or increased HbF. Thus the diagnosis was less likely to be ALPS or JMML. DNA sequencing of JMML related genes such as *NRAS, KRAS, HRAS, PTPN11*, and *CBL* identified somatic but not germline KRAS G13D mutation (Fig. 1b). KRAS G13D mutation was detected in granulocytes and T cells. Mutation was not identified in B cells by conventional DNA sequencing (data not shown). Mutant allele specific quantitative PCR revealed mutated allele was almost equally present in granulocytes and T cells, but barely in B cells (Fig. 1c). Activated T cells showed resistance to IL-2 depletion but not to FAS-dependent apoptosis (Fig. 1d and e). Both of our cases were characterized by strong autoimmunity, immune cytopenia and lymphadenopathy or hepatosplenomegaly with partial similarity with ALPS or JMML. However, they did not meet with the well defied diagnostic