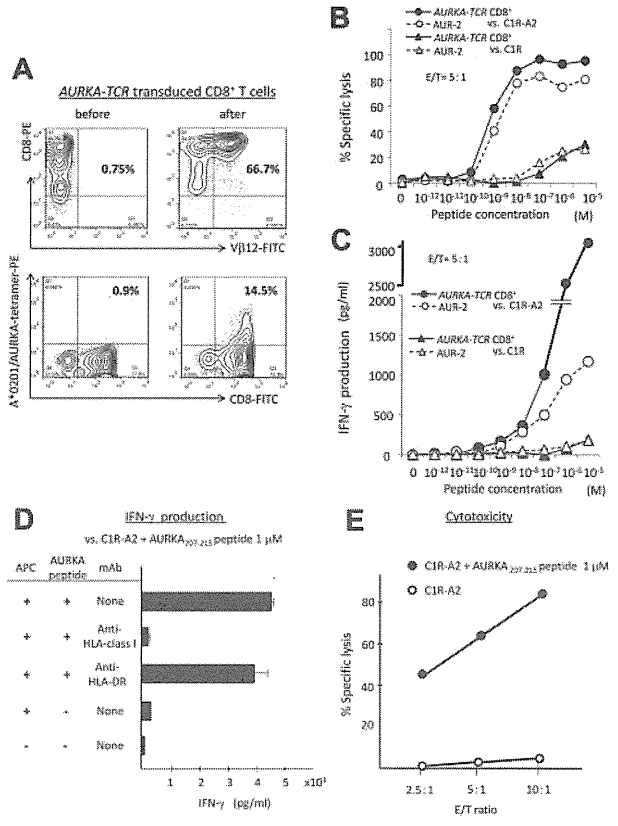

Figure 3. AURKA$_{207-215}$–specific *TCR*-transduced CD8$^+$ T cells display epitope-specific functionality. (A) Representative flow cytometry plots showing staining of AURKA$_{207-215}$–specific *TCR*-transduced CD8$^+$ T cells with anti-TCR Vβ12 mAb and HLA-A*0201/AURKA$_{207-215}$ tetramer. (B) The same AURKA$_{207-215}$ *TCR*-transduced CD8$^+$ T cells shown in panel A were tested in ^{51}Cr-release assays against C1R (negative control) and C1R-A2 cells pulsed with the indicated concentrations of AURKA$_{207-215}$ peptide. The parental AUR-2 CTL clone was tested in parallel. E/T indicates effector:target ratio. (C) IFN-γ production by AURKA$_{207-215}$–specific *TCR*-transduced CD8$^+$ T cells was measured in a similar format to that described for panel B. (D) Effects of HLA class I and class II blockade on the production of IFN-γ by AURKA$_{207-215}$–specific *TCR*-transduced CD8$^+$ T cells stimulated with cognate peptide-loaded (1 μM) C1R-A2 cells. (E) Cytotoxic activity of AURKA$_{207-215}$–specific *TCR*-transduced CD8$^+$ T cells against unpulsed or cognate peptide-loaded (1 μM) C1R-A2 cells as a function of effector:target (E/T) ratio. Error bars represent SDs.

HLA-A*0201$^+$ PBMCs (n = 3), PHA-stimulated lymphoblasts representing highly mitotic normal cells (n = 3), and normal cord blood CD34$^+$ cells (CB-CD34$^+$cells; n = 2) were not lysed by these AURKA$_{207-215}$–specific *TCR* transductants (Figure 4B). *AURKA* mRNA expression relative to K562 for each group (mean ± SD) was 0.02 ± 0.008 for PBMCs, 0.25 ± 0.005 for PHA-lymphoblasts and 0.21 ± 0.09 for CB-CD34$^+$ cells, which indicated relatively low expression levels of *AURKA* mRNA among these cells compared with K562. The cytotoxic activity of AURKA$_{207-215}$–specific *TCR*-transduced CD8$^+$ T cells against GANMO-1 and cognate peptide-loaded B-LCLs was significantly diminished by an anti-HLA class-I mAb but not by an anti–HLA-DR mAb (Figure 4C-D). To confirm recognition of the endogenously processed AURKA$_{207-215}$ epitope in the context of HLA-A*0201 expressed by leukemia cells, we retrovirally transduced the *HLA-A*0201* gene into MEG01 cells (MEG01-A2; Figure 4E). Parental MEG01 cells do not express HLA-A*0201, but abundantly overexpress both *AURKA* mRNA and AURKA protein (supplemental Figure 2). Compared with MEG01, MEG01-A2 were susceptible to the cytotoxic effects of AURKA$_{207-215}$–specific *TCR*-transduced CD8$^+$ T cells (Figure 4F). Collectively, these data indicate that the antileukemia reactivity mediated by AURKA$_{207-215}$–specific *TCR*-transduced CD8$^+$ T cells occurs through the recognition of endogenously processed and presented AURKA$_{207-215}$ peptide in the context of HLA-A*0201 on the surface of leukemia cells.

Next, the antileukemia reactivity mediated by these redirected AURKA$_{207-215}$–specific CD8$^+$ T cells was tested against freshly isolated leukemia cells in vitro (Figure 5). *AURKA* mRNA was overexpressed in all 6 leukemia samples as determined by qRT-PCR. Leukemia cells isolated from HLA-A*0201$^+$ patients (1-3), but not HLA-A*0201$^-$ patients (4-6) were lysed by the AURKA$_{207-215}$–specific *TCR*-transduced CD8$^+$ T cells. Thus, our data show that the AUR-2 TCR confers AURKA$_{207-215}$ specificity to donor CD8$^+$ T cells transduced with both TCR chains, and that normal cells, including actively cycling cells and hematopoietic progenitor cells, are not lysed by these redirected T cells.

AURKA$_{207-215}$–specific *TCR*-transduced CD4$^+$ T cells respond to cognate antigen

Next, we examined antigen reactivity in isolated populations of Vβ12$^+$ AURKA$_{207-215}$–specific *TCR*-transduced CD4$^+$ T cells (Figure 6A). AURKA$_{207-215}$–specific *TCR*-transduced CD4$^+$ T cells successfully produced IFN-γ in response to stimulation with AURKA$_{207-215}$ peptide-loaded C1R-A2 cells; this response was substantially reduced by HLA class I blockade, and a partial response reduction was also observed with HLA class II blockade (Figure 6B). These redirected CD4$^+$ T cells did not express Foxp3, which is a key molecular signature of regulatory T cells (supplemental Figure 3A), and the cognate antigen-specific proliferative response of AURKA$_{207-215}$–specific *TCR*-transduced CD8$^+$ T cells was actually enhanced in the presence of redirected CD4$^+$ T cells but not in the presence of non–gene-modified CD4$^+$ T cells (supplemental Figure 3B). Furthermore, AURKA$_{207-215}$–specific *TCR*-transduced CD4$^+$ T cells produced significant amounts of IL-2, TNF-α, and IFN-γ, but not IL-4 or IL-10 (supplemental Figure 4).

These observations suggest that AURKA$_{207-215}$–specific *TCR*-transduced CD4$^+$ T cells might be able to function as epitope-specific Th1 helper T cells, and that the interaction between T cell–expressed CD4 and target cell-expressed HLA class II molecules facilitates HLA class I–restricted AURKA$_{207-215}$–specific IFN-γ production.

AURKA$_{207-215}$–specific *TCR*-transduced CD8$^+$ T cells exhibit antileukemia reactivity in vivo

The in vivo antileukemia reactivity of AURKA$_{207-215}$–specific *TCR*-transduced CD8$^+$ T cells was assessed using the Winn assay and a therapeutic adoptive transfer model.

In the Winn assay, NOG mice were initially coinjected with GANMO-1 cells (5×10^6) and either 2.5×10^7 AURKA$_{207-215}$-specific *TCR* gene-modified or non–gene-modified CD8$^+$ T cells; 5 weekly infusions of the respective CD8$^+$ T-cell populations (5×10^6 cells per infusion) were subsequently administered. Treatment with AURKA$_{207-215}$–specific *TCR*-transduced CD8$^+$ T cells completely prohibited the engraftment and growth of inoculated leukemia cells for more than 2 months (Figure 7A), and significantly prolonged survival (Figure 7B). Similar results were obtained with AUR-2 cells in a parallel regimen (supplemental Figure 5). In contrast, non–gene-modified CD8$^+$ T cells did not prohibit leukemia growth. In a therapeutic adoptive transfer model, intravenously injected AURKA$_{207-215}$–specific *TCR*-transduced

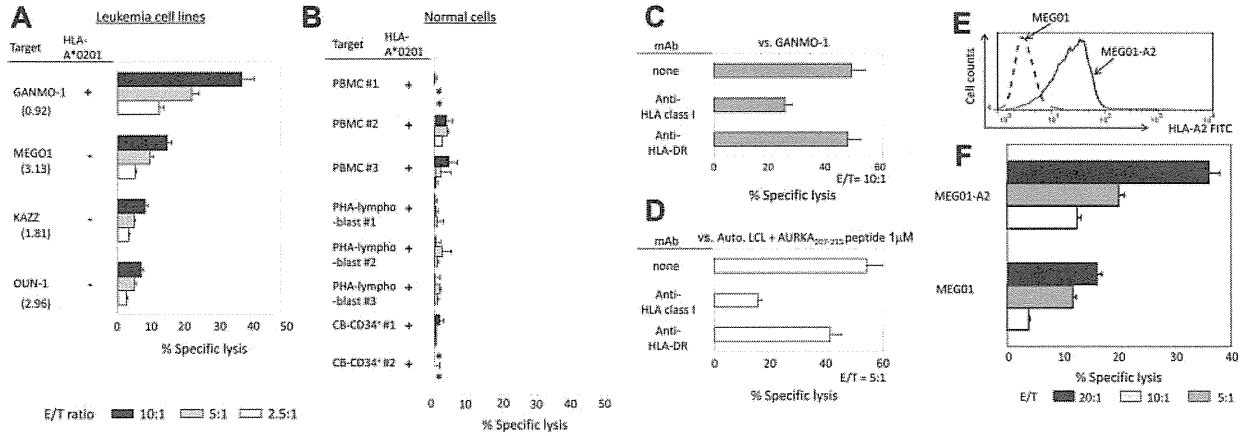

Figure 4. AURKA$_{207-215}$–specific *TCR*-transduced CD8$^+$ T cells can distinguish leukemia cells from normal cells on the basis of AURKA expression levels. (A) AURKA$_{207-215}$–specific *TCR*-transduced CD8$^+$ T cells exhibit antileukemia reactivity in an HLA-A*0201-dependent fashion. The HLA-A*0201$^+$ leukemia cell line GANMO-1 was lysed by AURKA$_{207-215}$–specific *TCR*-transduced CD8$^+$ T cells as a function of effector:target (E/T) ratio; no significant lysis was observed with the HLA-A*0201$^-$ leukemia cells lines MEG01, KAZZ and OUN-1. All of the tested leukemia cell lines overexpress *AURKA* mRNA; numbers in parentheses indicate *AURKA* mRNA expression relative to K562, and correlations with AURKA protein expression are shown in supplemental Figure 2. (B) The same AURKA$_{207-215}$–specific *TCR*-transduced CD8$^+$ T cells used in panel A at the same E/T ratios were tested in ^{51}Cr-release assays for potentially damaging effects against normal cells. No significant lysis was observed with HLA-A*0201$^+$ PBMCs (n = 3), PHA-lymphoblasts representing normal mitotic cells (n = 3) or normal cord blood–derived CD34$^+$ cells (CB-CD34$^+$) encompassing normal hematopoietic progenitor cells (n = 2). *AURKA* mRNA expression relative to K562 was 0.02 ± 0.008 for PBMCs, 0.25 ± 0.005 for PHA-lymphoblasts and 0.21 ± 0.09 for CB-CD34$^+$ cells (* indicates less than detectable). (C) Effects of HLA class I and class II blockade on the cytotoxic activity of AURKA$_{207-215}$–specific *TCR*-transduced CD8$^+$ T cells against GANMO-1 leukemia cells. E/T, effector:target ratio. (D) As for panel C, showing the effects of HLA class I and class II blockade on the lysis of autologous B-LCLs loaded with AURKA$_{207-215}$ peptide (1μM). (E) Flow cytometric confirmation of HLA-A*0201 expression by MEG01-A2 cells. (F) Enhanced lysis of MEG01-A2 cells relative to parental MEG01 cells by AURKA$_{207-215}$–specific *TCR*-transduced CD8$^+$ T cells confirms recognition of endogenously processed AURKA$_{207-215}$ peptide presented in the context of HLA-A*0201. E/T indicates effector:target ratio. Error bars represent SDs.

CD8$^+$ T cells, but not non–gene-modified CD8$^+$ T cells, significantly suppressed the growth of inoculated leukemia cells in vivo ($P < .02$; Figure 7C). Statistically significant tumor suppression was achieved on day 65, after 10 adoptive infusions. Thereafter, all mice (n = 4) treated with non–gene-modified CD8$^+$ T cells died by day 85. 2 mice treated with AURKA$_{207-215}$–specific *TCR*-transduced CD8$^+$ T cells died from other causes (1 on day 45 and 1 on day 70); the other 2 mice in this group survived longer than 90 days and were finally euthanized because of disease progression. Collectively, these observations indicate that AURKA$_{207-215}$–specific *TCR*-transduced CD8$^+$ T cells exhibit antileukemia reactivity in vivo.

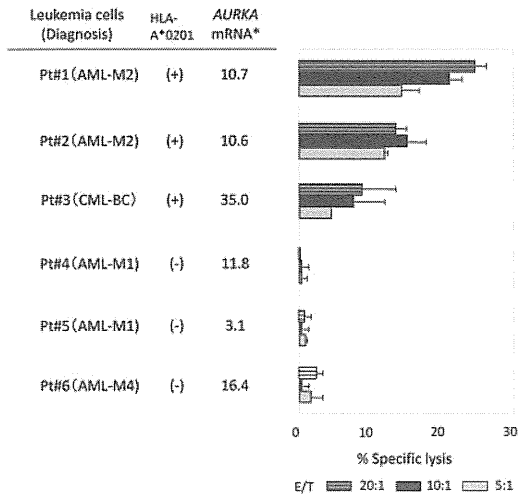

Figure 5. AURKA$_{207-215}$–specific *TCR*-transduced CD8$^+$ T cells kill freshly isolated leukemia cells in vitro. Freshly isolated HLA-A*0201$^+$ (n = 3) or HLA-A*0201$^-$ (n = 3) acute or chronic myeloid leukemia cells overexpressing *AURKA* mRNA were used as targets in ^{51}Cr-release assays with AURKA$_{207-215}$–specific *TCR*-transduced CD8$^+$ T cells at the indicated effector:target (E/T) ratios. AML, acute myeloid leukemia; BC, blast crisis; CML, chronic myeloid leukemia. M1, M2, and M4 refer to French-American-British classification subtypes (* indicates the expression of *AURKA* mRNA relative to the mean expression levels across 5 PBMC samples from healthy donors was determined by qRT-PCR and calculated using the comparative ΔCt method). Error bars represent SDs.

Discussion

In the setting of hematologic malignancies, *TCR* gene therapy targeting WT1 in leukemia,[30] and *chimeric antigen receptor* (*CAR*) gene therapy targeting CD33 in myeloid leukemias[31] and CD19, CD20, CD22, CD30, and the receptor tyrosine kinase-like orphan receptor 1 (ROR1) in B-cell malignancies,[32-38] are currently being investigated in preclinical studies or in early phase clinical trials.

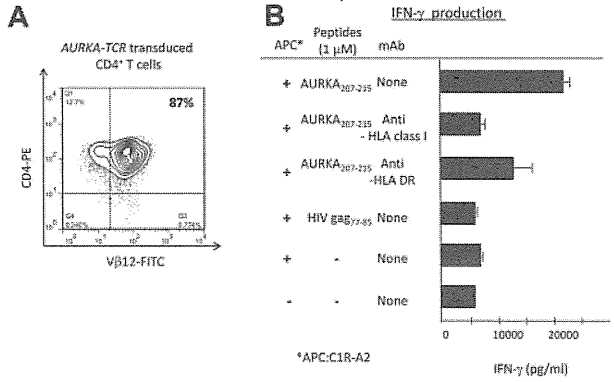

Figure 6. AURKA$_{207-215}$–*TCR* transduced CD4$^+$ T cells display antigen-specific Th1 cytokine production. (A) A representative flow cytometry plot showing surface Vβ12 expression by AURKA$_{207-215}$–specific *TCR*-transduced CD4$^+$ T cells. (B) *AURKA*$_{207-215}$–*TCR* transduced CD4$^+$ T cells produce IFN-γ in response to cognate peptide-loaded (1μM) C1R-A2 cells; unpulsed or irrelevant (HIV p17 Gag SL9) peptide-pulsed C1R-A2 cells were used as negative controls. Cognate antigen-specific IFN-γ production was reduced to background levels in the presence of anti-HLA class I blocking mAb and inhibited in the presence of anti-HLA class II blocking mAb. APC, antigen-presenting cell. Error bars represent SDs.

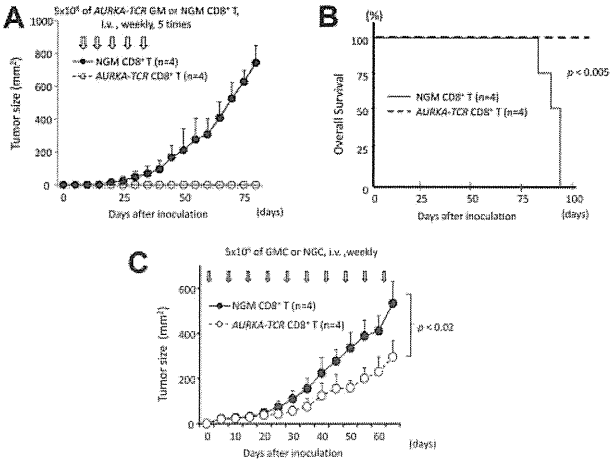

Figure 7. AURKA$_{207-215}$–specific *TCR*-transduced CD8$^+$ T cells mediate antileukemia reactivity in vivo. (A) Winn assay: tumor suppression curve. NOG mice were coinjected with GANMO-1 cells (5×10^6) and either 2.5×10^7 AURKA$_{207-215}$–specific *TCR* gene-modified (*AURKA-TCR*) or non–gene-modified (NGM) CD8$^+$ T cells (n = 4 group). Subsequently, 5 weekly infusions of the respective CD8$^+$ T-cell populations (5×10^6 cells per infusion) were administered intravenously. Tumor growth was monitored every 5 days. (B) Winn assay: survival curve. Treatment with AURKA$_{207-215}$–specific *TCR* gene-modified (*AURKA-TCR*) CD8$^+$ T cells significantly prolonged survival ($P < .005$). (C) Therapeutic adoptive transfer model. NOG mice (n = 4 per group) were inoculated with 5×10^6 of GANMO-1 cells. Intravenous administration of either 5×10^6 AURKA$_{207-215}$–specific *TCR* gene-modified (*AURKA-TCR*) or non–gene-modified (NGM) CD8$^+$ T cells commenced on the same day and was continued weekly thereafter. Therapeutic infusions of AURKA$_{207-215}$–specific *TCR* gene-modified CD8$^+$ T cells significantly suppressed tumor growth ($P < .02$). Error bars represent SDs.

Although adoptive antileukemia/lymphoma therapy with redirected T cells using tumor antigen-specific *TCR* or *CAR* gene transfer remains in its infancy, emerging evidence supports the development of such therapeutic options.

A number of preclinical and clinical studies of tumor antigen-specific *TCR* gene therapy have underscored the fact that appropriate antigen selection is essential to minimize the likelihood of on-target adverse events mediated by redirected T cell recognition of normal tissues expressing self-derived specificities.[39] This concept is further supported by a recent study of NY-ESO-1–specific *TCR* gene transfer.[19] In this report, objective clinical responses were observed in 5 of 11 patients with metastatic melanoma and 4 of 6 patients with metastatic synovial cell sarcoma without any toxicity related to engineered T cell activity.[19] Thus, the exploration of novel tumor antigens to identify safe and effective targets for *TCR* gene therapy is warranted, especially in the context of hematologic malignancies.

Previously, we reported a significant correlation between the overexpression of *AURKA* mRNA and the aggressiveness of lymphoma cells.[13] Furthermore, we found that *AURKA* mRNA is overexpressed in a large proportion of freshly isolated human leukemia cells.[6] However, in normal tissues, *AURKA* mRNA expression is largely limited to the testis.[13] Subsequently, we identified an immunogenic nonamer epitope derived from AURKA that was presented in the context of HLA-A*0201.[6] In the present study, we set out to examine the feasibility of redirected T cell–based adoptive immunotherapy for the treatment of human leukemia using a TCR derived from an HLA-A*0201–restricted AURKA$_{207-215}$–specific CD8$^+$ T-cell clone (AUR-2). Expression of this TCR in CD8$^+$ T cells conferred antileukemia reactivity both in vitro and in a xenogeneic mouse model of human leukemias. Furthermore, CD4$^+$ T cells could be redirected using this TCR to recognize the same HLA-A*0201–restricted AURKA$_{207-215}$ epitope. This represents a potentially important advantage, as the same TCR could redirect both helper (CD4$^+$) and cytotoxic (CD8$^+$) functions within the transduced T-cell population, which might sustain the antileukemia response in vivo after adoptive transfer.

Redirected CD8$^+$ T cells expressing the *TCR* cloned from AUR-2 displayed similar levels of functional sensitivity to the parental CTL clone. In vitro, AURKA$_{207-215}$–specific *TCR* gene-transduced CD8$^+$ T cells were able to lyse HLA-A*0201$^+$ human leukemia line GANMO-1 cells, which overexpress *AURKA* mRNA, and freshly isolated leukemia cells from HLA-A*0201$^+$ patients. This antileukemia reactivity was implemented through recognition of the endogenously processed AURKA$_{207-215}$ epitope presented in the context of HLA-A*0201. Importantly, these AURKA$_{207-215}$-specific *TCR*-transduced CD8$^+$ T cells did not lyse HLA-A*0201$^+$ normal PBMCs, mitotic PHA-lymphoblasts or cord blood CD34$^+$ cells; these data suggest that on-target adverse effects would be minimal in clinical applications. Furthermore, we demonstrated the efficacy of AURKA$_{207-215}$–specific *TCR*-transduced CD8$^+$ T cells in vivo by showing the inhibition of leukemia cell growth in a xenograft mouse model. As many hematopoietic progenitor cells actively proliferate and will therefore have enhanced AURKA expression levels, these cells may become targets for AURKA$_{207-215}$–specific *TCR*-transduced CD8$^+$ T cells in vivo, as is the case with selective AURKA inhibitors.[40] However, our observations suggest that redirected CD8$^+$ T cells targeting AURKA may not cause severe bone marrow failure, although further studies are needed to substantiate this point.

Notably, AURKA is overexpressed in the fraction of bone marrow cells that encompasses myeloid leukemia stem cells.[6,41] Recently, targeting leukemia stem cells has been highlighted as a treatment strategy to prevent disease progression in a durable fashion.[42] Monoclonal antibodies that target leukemia stem cell surface antigens have been proposed for this purpose. Examples of such molecules include CD123 (IL3Rα)[43] and TIM-3.[44] Cellular immunotherapy targeting antigens that are preferentially overexpressed in leukemia stem cells has also been proposed. In this regard, WT1 appears to be a particularly attractive candidate.[45] Indeed, we have cloned an HLA-A*2402–restricted WT1$_{235-243}$–specific *TCR* gene into our unique si-*TCR* vector to address the potential of this approach.[46] With respect to AURKA, we previously described that the CD34$^+$CD38$^-$ fraction of bone marrow mononuclear cells from CML patients expressed high levels of *AURKA* mRNA and that these cells were susceptible to AURKA-specific CTL-mediated lysis.[6] Thus, redirected T cell–based immunotherapy targeting AURKA might be able to suppress leukemia stem cells. Furthermore, such an approach may be synergistic with the administration of selective AURKA inhibitors, for example in the treatment of relapsed leukemia after allogeneic hematopoietic stem cell transplantation.

Strategic options to achieve better clinical responses in the field of *TCR* gene transfer are much needed. The manipulation of helper CD4$^+$ T cells is one such approach.[24,47,48] To date, the adoptive transfer of redirected CD4$^+$ T cells concurrently with CD8$^+$ T cells expressing the same tumor-specific TCR gene has not been described; however, this is an intriguing notion that could enhance the antitumor reactivity of such adoptive transfer approaches in vivo. In recognition of this possibility, we found that AURKA$_{207-215}$ *TCR*-transduced CD4$^+$ T cells displayed Th1 cytokine production in response to the HLA-A*0201/AURKA$_{207-215}$ epitope in vitro. The effects of such activity in vivo, however, remain to be clarified. Another approach to combined immunotherapy employs peptide vaccination. Indeed, vaccination with the relevant peptide has been

shown to enhance the antitumor functionality of infused gene-modified T cells.[49] The feasibility of this combination strategy using $AURKA_{207-215}$ peptide vaccination is currently under investigation.

In summary, we have demonstrated the feasibility of antileukemia adoptive therapy using AURKA-specific *TCR* gene transfer. As AURKA is also overexpressed in diverse solid tumors,[4] the potential clinical applications of this approach are widespread. Further studies are therefore warranted to investigate the safety and utility of this novel therapy in the clinic.

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Authorship

Contribution: K.N. and T.O. performed the research and wrote the paper; H.F. designed and performed the research, wrote and edited the paper, and provided financial support; J.A., T.S., J.M., H.S., J.J.M., and E.I. discussed and interpreted the experimental results and provided materials; K.K., E.G., and D.A.P. made and supplied the tetramers and edited the paper; and M.Y. discussed and interpreted the experimental results, edited the paper, and provided financial support.

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Rapid diagnosis of FHL3 by flow cytometric detection of intraplatelet Munc13-4 protein

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Familial hemophagocytic lymphohistiocytosis (FHL) is a potentially lethal genetic disorder of immune dysregulation that requires prompt and accurate diagnosis to initiate life-saving immunosuppressive therapy and to prepare for hematopoietic stem cell transplantation. In the present study, 85 patients with hemophagocytic lymphohistiocytosis were screened for FHL3 by Western blotting using platelets and by natural killer cell lysosomal exocytosis assay. Six of these patients were diagnosed with FHL3. In the acute disease phase requiring platelet transfusion, it was difficult to diagnose FHL3 by Western blot analysis or by lysosomal exocytosis assay. In contrast, the newly established flow cytometric analysis of intraplatelet Munc13-4 protein expression revealed bimodal populations of normal and Munc13-4–deficient platelets. These findings indicate that flow cytometric detection of intraplatelet Munc13-4 protein is a sensitive and reliable method to rapidly screen for FHL3 with a very small amount of whole blood, even in the acute phase of the disease. (*Blood.* 2011;118(5):1225-1230)

Introduction

The granule-dependent cytotoxic pathway is a major immune effector mechanism used by cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells.[1] The pathway involves a series of steps, including cell activation, polarization of the lysosomal granules to the immunologic synapse, exocytosis of lytic proteins such as perforin and granzymes, and induction of apoptosis in the target cells.[2] In addition to its central role in the defense against intracellular infections and in tumor immunity, this pathway also plays an important role in the regulation of immune homeostasis. Defects in the granule-dependent cytotoxic pathway result in a catastrophic hyperinflammatory condition known as hemophagocytic lymphohistiocytosis (HLH).[1,3]

HLH is a life-threatening syndrome of immune dysregulation resulting from the uncontrolled activation and proliferation of CTLs, which leads to macrophage activation and the excessive release of inflammatory cytokines.[4,5] Clinical diagnosis of HLH is made on the basis of cardinal signs and symptoms including prolonged fever and hepatosplenomegaly, and by characteristic laboratory findings such as pancytopenia, hyperferritinemia, hypofibrinogenemia, increased levels of soluble IL-2 receptor, and low or absent NK cell activity.[5,6] HLH can be classified into primary (genetic) or secondary (acquired) forms according to the underlying etiology, although this distinction is difficult to make in clinical practice.[4,5]

Familial hemophagocytic lymphohistiocytosis (FHL) encompasses major forms of primary HLH for which mutations in the genes encoding perforin (*PRF1*; FHL2),[7] Munc13-4 (*UNC13D*; FHL3),[8] syntaxin-11 (*STX11*; FHL4),[9] and syntaxin-binding protein 2 (also known as Munc18-2) (*STXBP2*; FHL5)[10,11] have been identified to date. Perforin is a cytolytic effector that forms a pore-like structure in the target cell membrane. Munc13-4, syntaxin-11, and Munc18-2 are involved in intracellular trafficking or the fusion of cytolytic granules to the plasma membrane and the subsequent delivery of their contents into target cells.[1,12] Consequently, defective cytotoxic activity of CTLs and NK cells is one of the hallmark findings of FHL,[7,8,13-16] although NK cell activity is also decreased in some cases of secondary HLH.[15,17-20]

Prompt and accurate diagnosis of FHL is mandatory to initiate life-saving immunosuppressive therapy and to prepare for hematopoietic stem cell transplantation. Detection of perforin expression in NK cells with flow cytometry is a reliable method to screen for FHL2.[21] Another test analyzes the expression of CD107a on the surface of NK cells, which marks the release of cytolytic granules.[22] Reduced expression of CD107a implies impaired degranulation of NK cells and predicts a likelihood of FHL3.[23] However, this analysis is not available in some patients with extremely reduced NK cell numbers, such as during the acute phase of HLH.[19] In addition, NK-cell degranulation is also impaired in FHL4[24] and FHL5,[10,11] making it impossible to differentiate these disorders.

We reported previously that Munc13-4 protein is expressed in platelets and regulates the secretion of dense core granules.[25] Herein we report that Munc13-4 is expressed far more abundantly in platelets than in PBMCs. We also describe the development of a

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new method to screen for FHL3 rapidly by detecting intraplatelet Munc13-4 expression through flow cytometry.

Methods

Patients

Between January 2008 and March 2010, whole blood samples from 85 patients were screened for FHL3. The patients had been clinically diagnosed with HLH by their referring physicians and were suspected of possible FHL. Characteristics of the enrolled patients are summarized in supplemental Table 1 (available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). As a control, blood obtained from healthy adults at the time of patient sampling was shipped for screening along with the patient samples. Before the laboratory studies were performed, informed consent was obtained from the patients and their parents, in accordance with the institutional review board of Kyoto University Hospital and the Declaration of Helsinki.

Preparation of PBMCs and platelet samples

Whole blood samples treated with EDTA were centrifuged gently at 100*g* for 10 minutes, and platelets were collected from the supernatant plasma layer. Alternatively, platelets were prepared from small aliquots of blood samples by lysing red blood cells with ammonium chloride. PBMCs were obtained by Ficoll-Hypaque density gradient centrifugation from the remaining sample. $CD4^+$, $CD8^+$, $CD14^+$, $CD19^+$, and $CD45^+$ cells were separated from PBMCs using an AutoMACS Pro (Miltenyi Biotec) and magnetic bead–conjugated mAbs according to the manufacturer's instructions. Flow cytometric analysis revealed that each cell population contained > 95% $CD4^+$, $CD8^+$, $CD14^+$, $CD19^+$, and $CD45^+$ cells (data not shown).

Mutation analysis

Genomic DNA was isolated from the PBMCs of patients with defective Munc13-4 expression using standard procedures. Primers were designed for the amplification and direct DNA sequencing of the *UNC13D*-coding exons, including the adjacent intronic sequences for the identification of splice-site variants. Primer sequences are available upon request. Products were sequenced directly with an ABI3130 genetic analyzer (Applied Biosystems).

Antibodies

Rabbit polyclonal antibodies raised against the N-terminal region (residues 1-262)[25] and full-length human Munc13-4 protein were used as primary antibodies for Western blot and flow cytometric analysis, respectively. Rabbit polyclonal anti-integrin αIIb (Santa Cruz Biotechnology) and mouse polyclonal anti–β-actin (Sigma-Aldrich) antibodies were used as primary antibodies for Western blotting. The mAbs used in the flow cytometric analysis were FITC-conjugated anti-CD3 (SK7; BD Pharmingen), phycoerythrin (PE)–conjugated anti-CD41a (HIP8; BD Pharmingen); allophycocyanin-conjugated anti-CD56 (N901; Beckman Coulter), and PE-conjugated anti-CD107a (H4A3; eBioscience).

Western blot analysis

Cell extracts were fractionated by SDS-PAGE, and the fractionated proteins were electrotransferred onto polyvinylidene fluoride membranes. The membranes were blocked overnight in blocking buffer (5% skim milk) and incubated for 1 hour at room temperature with the primary antibodies, followed by HRP-conjugated anti–rabbit or anti–mouse IgG polyclonal antibodies (Santa Cruz Biotechnology). Specific bands were visualized by the standard enhanced chemiluminescence method.

Flow cytometric analysis of Munc13-4 protein

After surface staining with anti-CD41a mAbs, platelets were fixed and permeabilized by Cytofix/Cytoperm (BD Biosciences) and washed 3 times with Perm/Wash buffer (BD Biosciences). After nonspecific reactions were blocked with Chrome-Pure human IgG (Jackson ImmunoResearch Laboratories), rabbit polyclonal antibody against the full-length human Munc13-4 protein was added, followed by FITC-conjugated donkey anti–rabbit IgG (Jackson ImmunoResearch Laboratories). Platelets were gated on the basis of their appearance on forward- and side-scatter plots in log/log scale and by CD41a expression. The gated platelets were analyzed for Munc13-4 expression by flow cytometry (FACSCalibur; BD Biosciences).

Lysosomal degranulation assays

To quantify lysosome exocytosis by NK cells, 2×10^5 PBMCs were mixed with 2×10^5 human erythroleukemia cell line K562 cells and incubated for 2 hours in complete medium (RPMI 1640 medium supplemented with 2mM L-glutamine and 10% FCS) at 37°C in 5% CO_2. Cells were resuspended in PBS supplemented with 2% FCS and 2mM EDTA; stained with anti-CD3–FITC, anti-CD56–allophycocyanin, and anti-CD107a–PE mAbs; and analyzed by flow cytometry.

Platelet exocytosis of the lysosomal granules was analyzed as described previously[26] but with a minor modification. Briefly, platelets were suspended in PBS containing 2mM EDTA and PE-conjugated anti-CD107a mAb, stimulated with 5 U/mL of thrombin (Wako Pure Chemical Industries) for 10 minutes at 25°C, and immediately analyzed by flow cytometry. The degranulation index of platelets was calculated as: (mean fluorescence value of stimulated sample − mean fluorescence value of nonstimulated sample)/mean fluorescence value of nonstimulated sample.

Statistical analysis

Statistical analyses were performed with 1-way ANOVA followed by the Tukey post hoc test to compare multiple groups, with a $P < .05$ level considered to be significant.

Results

Diagnosis of FHL3 by Western blot analysis using platelets

Before screening for FHL3, the Munc13-4 expression level was compared between platelets and PBMCs. Munc13-4 expression in platelets was approximately 10 times higher than that in PBMCs (Figure 1A). $CD8^+$ cells expressed a similar level of Munc13-4 protein as other PBMC cell types (Figure 1B). Similar amounts of platelet- and PBMC-derived proteins could be obtained from a sample (data not shown). Therefore, platelets were used to perform Western blotting to screen for Munc13-4 deficiency. Of the 85 patients screened, 6 patients were diagnosed with FHL3 (Figure 1C). Munc13-4 protein was barely detected in the platelets of each FHL3 patient regardless of the gene mutation (Table 1). For each sample, no more than 1 mL of whole blood was required to perform the analysis.

Difficulty in diagnosing FHL3 in the acute phase of the disease

Patients in the acute phase of the disease who require screening for FHL often receive platelet transfusions because of thrombocytopenia.[4-6] To study the effect of transfused platelets on screening results, FHL3 screening was attempted in a patient receiving platelet transfusions. As expected, Western blotting using platelets could not detect Munc13-4 deficiency because of the normal expression of the protein in the transfused platelets (Figure 2A left column). Surprisingly, Western blotting using PBMCs also could not clearly identify Munc13-4 deficiency because a substantial number of platelets were present in the PBMCs obtained by the standard method (Figure 2A right column). By positively selecting $CD45^+$ cells and removing platelets, it was found that a considerable amount of the Munc13-4 protein detected in PBMC samples

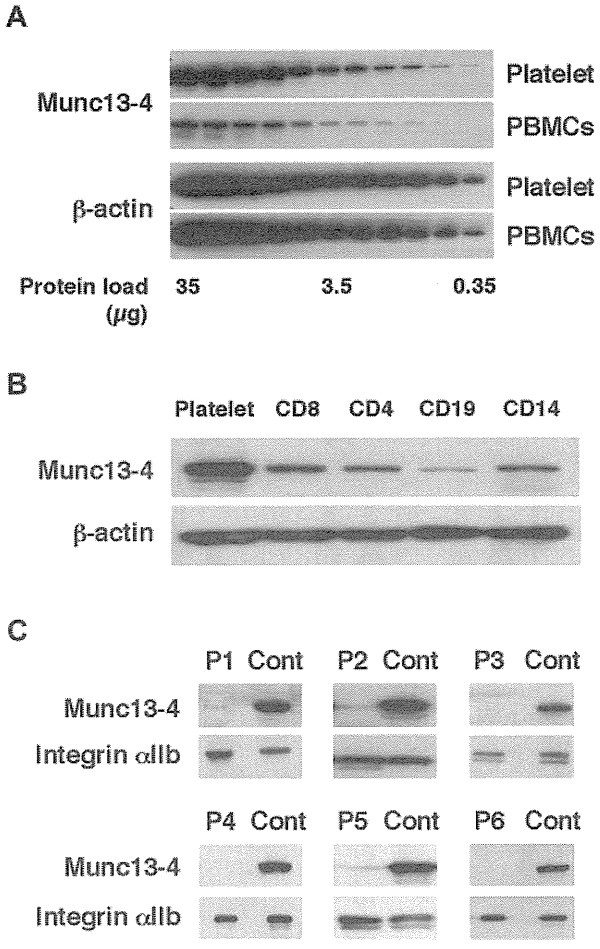

Figure 1. Diagnosing FHL3 by Western blotting using platelet protein. The amount of Munc13-4 protein expression was compared between platelets and PBMCs (A) and among platelets, $CD8^+$, $CD4^+$, $CD19^+$, and $CD14^+$ cells (B) by Western blotting. A representative result of 5 independent experiments is shown. (C) Six FHL3 patients were diagnosed by Western blotting for Munc13-4 protein using platelets.

obtained by standard density gradient centrifugation was actually derived from the contaminating platelets (Figure 2B).

We performed a NK-cell degranulation assay for every referred sample and found the assay to be defective for every FHL3 patient identified (data not shown). All of the other patients showed a normal release of lysosomal granules by NK cells; however, the analysis could not be performed in some patients because of the extremely low NK-cell number during the acute phase of the disease (data not shown).

We also examined the lysosomal granule release of platelets in 31 patients to determine whether this assay could be used as a screening method for FHL3. Lysosomal exocytosis of FHL3 platelets was partially impaired at steady state, but profound impairment was observed during the acute phase of the disease (Figure 3A-C). This profound impairment was also observed in platelets obtained from some secondary HLH patients during the acute phase (Figure 3B-C). These results indicate that it is difficult to diagnose FHL3 during the acute phase of HLH either by Western blot or by lysosomal degranulation assay.

Table 1. UNC13D gene mutations of FHL3 patients

Patient	Age at onset	Gender	Mutation	Genotype	Predicted effect
P1	14 days	Female	c.1596 + 1G → C	Homo	Splice error
P2	2 months	Male	c.322–1G → A	Hetero	Splice error
			c.990G → C	Hetero	p.Q330H
			c.3193C → T	Hetero	p.R1065X
P3	12 months	Female	c.754–1G → C	Hetero	Splice error
			c.2485delC	Hetero	p.L829fs
P4	4 months	Female	c.754–1G → C	Hetero	Splice error
			c.1799C → T	Hetero	p.T600M
			c.1803C → A	Hetero	p.Y601X
P5	2 months	Female	c.754–1G → C	Hetero	Splice error
			c.1596 + 1G → C	Hetero	Splice error
P6	5 months	Male	ND	ND	ND

Mutations were checked for single nucleotide polymorphisms using the dbSNP Build 132 database from the National Center for Biotechnology Information.

X indicates stop; fs, frame shift; and ND, not determined.

Rapid diagnosis of FHL3 by flow cytometric detection of intraplatelet Munc13-4

To overcome the difficulty in diagnosing FHL3 during the acute phase of HLH, antibodies were raised against the full-length human Munc13-4 protein (supplemental Figure 1) and a new method was developed to detect Munc13-4 protein in platelets by flow cytometry. A total of 35 patients, including 4 with FHL3 (P3-P6), were

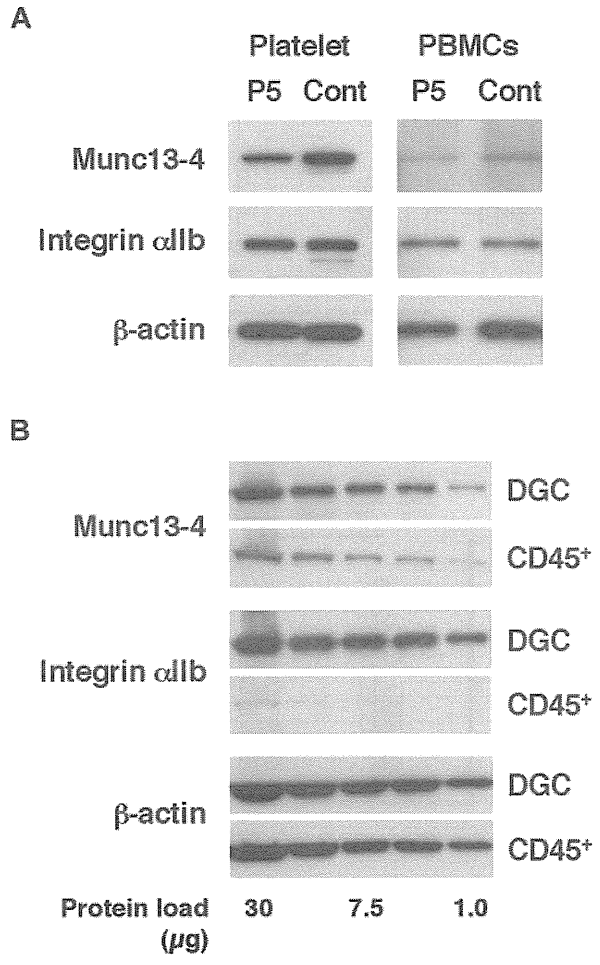

Figure 2. Effect of platelet transfusion on Western blot analysis. (A) Western blotting analysis for Munc13-4 expression using platelets and PBMCs from an FHL3 patient (P5) receiving platelet transfusions during the acute phase of the disease. (B) The expression of Munc13-4 was compared between PBMCs obtained by density gradient centrifugation (DGC) and $CD45^+$ cells obtained by magnetic sorting from healthy controls. A representative result of 3 independent experiments is shown.

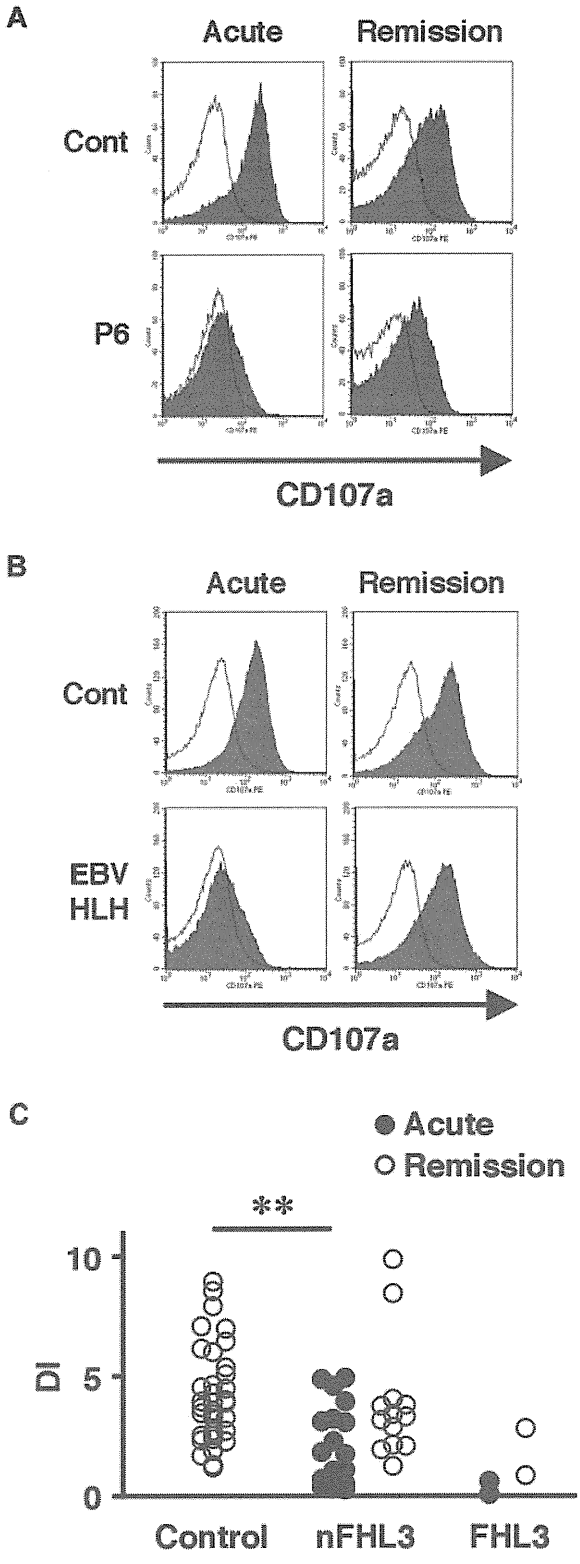

Figure 3. Analysis of lysosomal exocytosis using platelets from HLH patients. Platelets from an FHL3 patient (P6; A) and from a secondary (EBV-associated) HLH patient (B) along with healthy controls were left untreated (open histogram) or were stimulated with thrombin (closed histograms), and the surface expression of CD107a was analyzed by flow cytometry. Analysis was performed during the acute phase of the disease (left column) and after clinical remission (right column). (C) Degranulation index (DI) of platelets from HLH patients during the acute phase (●) and after clinical remission (○). HLH patients with normal NK-cell degranulation and Munc13-4 protein expression by Western blot analysis were defined as non-FHL3 (nFHL3). $^{**}P < .01$ by the Tukey post hoc test.

analyzed using this method. Munc13-4 deficiency was readily detected in all of the FHL3 patients, with a sample volume of < 100 μL of whole blood (Figure 4A-C). Munc13-4 protein was expressed at normal level in the platelets of parents and siblings of FHL3 patients carrying heterozygous *UNC13D* mutations (data not shown). In the FHL3 patient receiving platelet transfusions, flow cytometric analysis revealed bimodal populations of normal and Munc13-4–deficient platelets (P5 in Figure 4A). As shown in Figure 4B, the method was able to clearly identify Munc13-4–deficient platelets in whole blood samples stored at room temperature for 1 week.

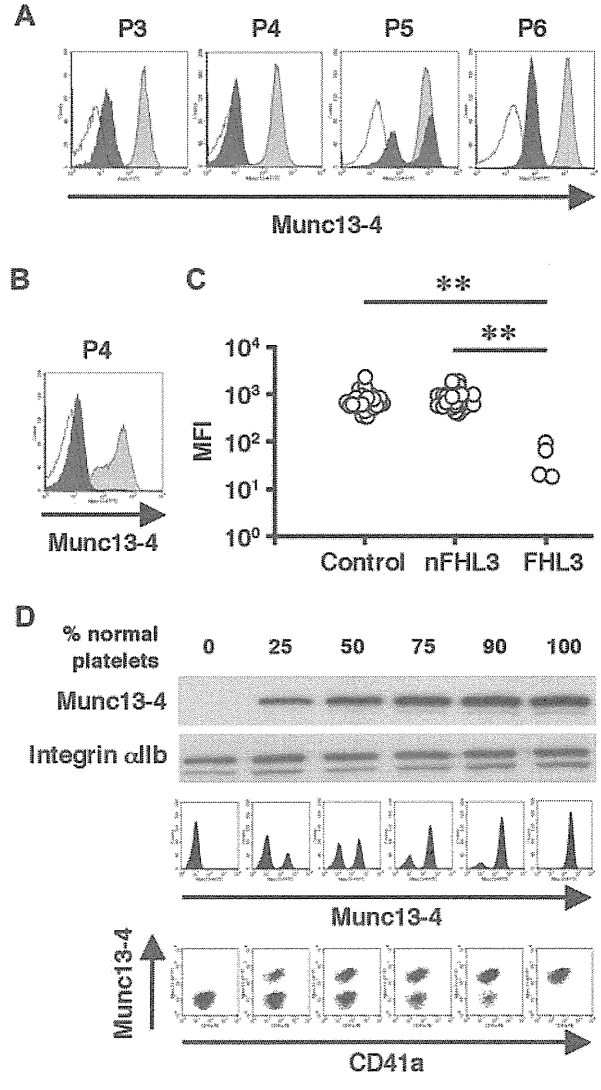

Figure 4. Flow cytometric detection of intraplatelet Munc13-4 protein. Flow cytometric analysis of intraplatelet Munc13-4 expression in 4 FHL3 patients and healthy controls using whole blood samples shipped overnight (A) and in an FHL3 patient (P4) and a healthy control using samples stored at room temperature for a week (B). Dark closed histograms represent platelets from FHL3 patients, whereas light closed histograms represent platelets from healthy controls. Open histograms represent staining with isotype controls. (C) Mean fluorescence intensity (MFI) of intraplatelet Munc13-4 staining for HLH patients and healthy controls. All of the healthy controls (n = 35) were adults. Non-FHL3 (nFHL3) patients (n = 31), as defined in Figure 3, varied in age (2 days-39 years) and included 2 patients with FHL2. Age-related variations in the MFI of Munc13-4 staining were not observed. $^{**}P < .01$ by the Tukey post hoc test. (D) The sensitivities of Western blot and flow cytometric analyses for detecting Munc13-4–deficient platelets were compared.

To determine the sensitivity of the new method, Munc13-4–deficient platelets were mixed with normal platelets at varying ratios. Western blot analysis could not detect Munc13-4–deficient platelets easily, even when the proportion of normal platelets was as low as 25% (Figure 4D). In contrast, flow cytometric analysis easily identified 10% Munc13-4–deficient platelets among 90% normal platelets (Figure 4D), which proved the high sensitivity of the method in diagnosing FHL3.

Discussion

FHL is a rare but life-threatening inherited immune disorder for which mutations in 4 genes have been identified as causative factors. *PRF1* encodes the cytolytic effector protein perforin that forms a pore-like structure in the target cell membrane.[1,12] A mutation in *PRF1* results in FHL2,[7] which accounts for 20%-50% of FHL cases.[4,5] *UNC13D* encodes the protein Munc13-4, which is crucial for the fusion of cytolytic granules to the plasma membrane and the subsequent release of perforin and granzymes.[1,12] Mutations in *UNC13D* result in FHL3,[8] which accounts for 20%-30% of FHL cases.[4,12] FHL4 is caused by mutations in *STX11*, which encodes syntaxin-11.[9] Mutations in *STXBP2*, which encodes Munc18-2, were recently reported to cause FHL5.[10,11] Syntaxin-11 and Munc18-2 also mediate the fusion of cytolytic granules to the plasma membrane.[1,5,12] The ability to screen for FHL2-5 rapidly would facilitate the initiation of life-saving immunosuppressive therapy and the preparation of FHL patients for hematopoietic stem cell transplantation.

In the present study, we found that the Munc13-4 protein is expressed abundantly in platelets (Figure 1A-B). The detection of Munc13-4 protein in platelets by Western blotting (Figure 1C) or flow cytometry (Figure 4A-B) was a reliable screening method to identify FHL3 patients. Munc13-4–deficient platelets were identified easily among normal transfused platelets by flow cytometry, which indicated that this method could be applied to patients who are receiving platelet transfusions during the acute phase of the disease (P5 in Figure 4A). Detection of intraplatelet Munc13-4 was enabled by the use of highly specific antibodies against the full-length human Munc13-4 (supplemental Figure 1).

There is a possibility that FHL3 patients with residual Munc13-4 protein expression could be overlooked by the screening methods described in this study. Most FHL3 patients have mutations that result in the absence or significant reduction of Munc13-4 protein expression,[16,23] as was the case with the patients screened in this study (Figure 1C), which suggests that the mutated Munc13-4 protein is unstable. The NK-cell degranulation assay, which was performed for every referred sample with a sufficient number of NK cells, revealed defective degranulation only in the identified FHL3 patients (date not shown). These results indicate that the majority of mutations in *UNC13D* are likely amenable to rapid detection by the new methods described in this study. Comparative studies on the *UNC13D* genotype, Munc13-4 protein expression, and the lysosomal exocytosis assay must be performed to confirm the reliability of these methods.

It was also investigated whether the analysis of lysosomal release by platelets could be used as an alternative method to screen for FHL3. Profound impairment of lysosomal exocytosis by platelets during the acute phase of the disease and restoration of this impairment after clinical remission was observed in FHL3 and in some secondary HLH patients (Figure 3). It is not clear whether this transient impairment of platelet degranulation is involved in HLH pathogenesis or if it merely reflects in vivo platelet activation by diffuse endothelial damage during the acute phase of the disease that renders them unresponsive to ex vivo stimulation. The release of lysosomal granules by Munc13-4–deficient platelets was impaired only minimally at steady state (Figure 3A and 3C), which is in contrast to a recent study showing the involvement of the Munc13-4 protein in the release of lysosomal granules in mouse platelets.[27] Although the precise reason for this discrepancy is unclear, platelet degranulation is likely to be regulated differentially between species; for example, Munc13-4–deficient mice have bruising and bleeding tendencies[27] that are not commonly associated with human FHL3. Further studies are warranted to elucidate the exocytosis pathways of platelets and their role in the pathophysiology of HLH.

With the development of tools for rapid screening, the diagnostic approach for FHL has changed over the years. Impaired NK cytotoxicity was the first reported signature clinical finding of FHL patients.[13,14] Defective CTL activity was subsequently reported as another hallmark of FHL.[7,8,16,28] However, NK-cell activity is also decreased in some cases of secondary HLH,[15,17-20] and the CTL cytotoxicity assay is not readily accessible to most clinicians. The NK-cell lysosomal exocytosis assay is a comprehensive method to identify patients with a degranulation defect.[10,11,22-24] However, this analysis is not available in some patients with extremely reduced NK-cell numbers, which are often observed during the acute phase of HLH.[19] Although CTLs can be an alternative tool to perform the lysosomal exocytosis assay,[24,28,29] it remains impossible to differentiate FHL3-FHL5.[10,11,23,24] Impairment in these assays warrants the genetic confirmation of FHL, but sequencing all of the candidate genes is not a suitable approach for rapid diagnosis. Flow cytometric detection of perforin expression in NK cells is a reliable and rapid way of identifying patients with FHL2,[21] and the new method described in this study for the detection of Munc13-4 expression in platelets would add to the rapid diagnosis of FHL3.

Platelets could also be used for the screening of FHL4 and FHL5 because they share some granule-transport mechanisms with other types of hematopoietic cells, including CTLs and NK cells.[2,30,31] Indeed, in the present study, both syntaxin-11 and Munc18-2 were expressed abundantly in platelets (data not shown). We are currently using platelet proteins to screen for FHL4-FHL5 by Western blot analysis, although no cases have been found so far because of the extreme rarity of these disorders.

In summary, platelets abundantly express Munc13-4 protein and are a useful tool to screen for FHL3. By detecting intraplatelet Munc13-4 expression by flow cytometry, it is now possible to rapidly screen for FHL3 with a very small sample of whole blood, even in the acute disease phase requiring platelet transfusion. Because platelets share some of their granule transport systems with other types of hematopoietic cells, they could also be used to screen for other types of immune disorders, including FHL4 and FHL5.

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Authorship

Contribution: T.Y., R.N., T.N., H.H., and H.T. designed the research; Y.M., K.I., and M.S. performed the Western blot and flow cytometric analyses; K.O. and O.O. performed the genetic analyses; R.S. and H.H. prepared the anti–Munc13-4 antibodies and started the FHL3 screening; Y.M., T.Y., R.S., K.I., H.S., J.A., N.T., T.K., R.N., E.I., T.N., H.H., and T.H. analyzed and discussed the results; and Y.M., T.Y., and T.H. wrote the manuscript.

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Association of viral isolates from stool samples with intussusception in children

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SUMMARY

Background: Intussusception is the most common cause of intestinal obstruction in young children. The pathogenesis of intussusception is still not well understood. In this study the pathogens from stool specimens were investigated in children with intussusception.
Methods: Patients diagnosed with primary idiopathic intussusception were enrolled. Pathogenic bacteria and viruses were detected in the stool samples by routine culture, cell culture, polymerase chain reaction, reverse transcriptase-polymerase chain reaction, enzyme immunoassay, and electron microscopy examinations.
Results: A total of 71 samples were analyzed during the 2-year study period. The patients ranged in age from 4 to 47 months. Viruses were detected in 56 of the 71 stool samples (78.9%). Adenovirus was found in 19 of 35 cases aged <2 years, whereas it was found in 17 of 21 cases aged ≥2 years. The majority of adenovirus isolates were non-enteric organisms generally associated with respiratory tract symptoms.
Conclusions: These results suggest a casual association of viral infections in children with intussusception. Adenovirus infection, especially with the primary non-enteric types, is a significant risk factor for developing intussusception in children, particularly those aged over 2 years.

1. Introduction

Intussusception is the most common cause of intestinal obstruction in young children. The majority of cases occur in children under the age of 24 months.[1,2] Several studies have demonstrated that the incidence of intussusception varies among countries and also over time. A recent report prospectively compared the incidence of intussusception between Vietnam and Australia, and showed that the incidence in Vietnam was more than four-fold higher than that in Australia.[3] The incidence of intussusception observed in Vietnam is also higher than that in any other country for which incidence data are available, including recent reports from the USA and Latin America.[4–7] Studies from China also suggest a high incidence,[8] however, those reported in the USA, Denmark, and Australia have significantly declined over the past decade.[2,4,9] These results may reflect the presence of an environmental risk factor and/or infectious etiology in developing intussusception.

The underlying cause of intussusception in children remains unknown, but it has been associated with several pathogens, including adenovirus. Previous studies have demonstrated adenoviruses in from 30% to 50% of stool specimens, and in intestinal or lymphoid tissue specimens from children with intussusception.[3,10–16] Murphy et al. reported an increased risk of intussusception after the administration of tetravalent rotavirus vaccine composed of rhesus rotavirus (RRV) and three human RRV reassortant strains, RRV-TV.[17] These results suggest an association between viral infection and the development of intussusception in young children.

The present study analyzed the association of infectious pathogens with the development of intussusception in children by a detailed examination of stool specimens during hospitalization. Results showed a high prevalence of viral isolates and a strong association with adenoviruses in patients with intussusception, particularly those over 2 years of age.

2. Materials and methods

2.1. Patients

The study was conducted at Hiroshima City Funairi Hospital, Hiroshima, Japan, over a 2-year period (August 2006 through July 2008). The study complied with the Declaration of Helsinki. The research protocol was approved by the ethics committee, and

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informed consent was obtained from the patients' guardians. Children with signs and symptoms suggestive of intussusception and seeking care for this problem were considered for enrollment into the study. The diagnosis of primary idiopathic intussusception was made according to the clinical history, physical findings, abdominal radiograph, ultrasounds, and finally an air and/or contrast enema, using the case definition of the Brighton Collaboration Intussusception Working Group.[18] All patients with intussusception were admitted to the hospital. During the same 2 years, all in-patients with uncomplicated gastroenteritis aged <4 years who were examined for the presence of viral isolates in stool specimens in order to identify the cause, were enrolled in the study.

2.2. Assessment of infectious pathogens

Stool samples were collected from patients with intussusception during hospitalization. All samples were collected within 24 h of hospitalization and stored at 4 °C. Routine culture media were used to assay for common bacterial pathogens. Screening for enterovirus, poliovirus, echovirus, and adenovirus was performed using routine cell culture, HE, Hep-2, RD-18S and Vero cells, to second-generation of serial passage, and immunofluorescence detection.[19] Calicivirus detection was conducted using reverse transcriptase-polymerase chain reaction (RT-PCR) assays.[20] Rotavirus was tested for using an enzyme-linked immunosorbent assay (ELISA) and RT-PCR assays.[21] All patients positive by culture were examined by neutralization tests for enterovirus, poliovirus, echovirus, and adenovirus. In those patients negative by cell culture and by the other tests described above, additional examinations were performed for the detection of viral inclusion by electron microscope and using PCR assays for adenovirus, parechovirus, sapovirus, astrovirus, and aichivirus.[22,23]

2.3. Statistical analysis

Statistical significance was determined using the Chi-square test, Fisher's exact test, or Ryan's test according to the StatView software program (version 5.0l; SAS Institute, Inc., Cary, NC, USA). These tests were used to assess differences in stool findings between age groups and differences in the clinical symptoms between patients with the detection of adenovirus and those with virus other than adenovirus and negative isolation. A p-value of <0.05 was considered statistically significant.

3. Results

3.1. Study participants

During the 2-year study period, 83 children with primary idiopathic intussusception were diagnosed and admitted to the hospital. Twelve patients were excluded from the analysis because the stool specimens from these patients were not obtained during their hospitalization. Therefore, 71 patients were enrolled in the study. There was no discernible seasonal variation in the incidence of intussusception, nor was there any significant variation in the annual presentation rate over the 10-year period 1998–2008 (data not shown).

3.2. Infectious pathogens

Figure 1 shows the results of viral detection assays and age distribution in the patients. The 71 patients ranged in age from 4 to 47 months; 23 were aged <12 months (32.4%), 25 were aged 12–24 months (35.2%), and 23 were aged >24 months (32.4%). The male to female ratio of patients was approximately 2.6:1 throughout the age groups (data not shown). Fifty-six of the 71 patients (78.9%) had stool specimens positive for viruses. The rate of viral detection gradually increased with age: 15 of 23 (65.2%) aged <12 months, 20 of 25 (80%) aged 12–24 months, and 21 of 23 (91.3%) aged >24 months. Similarly, the prevalence of adenovirus isolation increased significantly with older age. Of those patients with virus detected, adenovirus was found in seven of 15 (46.7%) aged under 12 months, 12 of 20 (60%) aged 12–24 months, and 17 of 21 (81.0%) aged over 24 months. The detection of adenovirus from stool specimens of intussusception patients gradually increased with older age. Table 1 shows the number of patients with adenovirus, as well as other viral pathogens, namely rotavirus, enterovirus, parechovirus, norovirus, and poliovirus, detected in the stool specimens. Multiple viral infections occurred in one patient. Viruses causing enteritis were more frequently detected in patients under 2 years of age. A bacterial pathogen, *Campylobacter jejuni*, was simultaneously cultured in one patient (22 months of age) whose stool was positive for adenovirus type 1 (data not shown). No other bacterial pathogens were cultured from the patient stool specimens.

During the same 2 years, stool specimens from 82 patients with uncomplicated gastroenteritis aged under 48 months were screened for viral isolates (Table 2). The rate of patients with

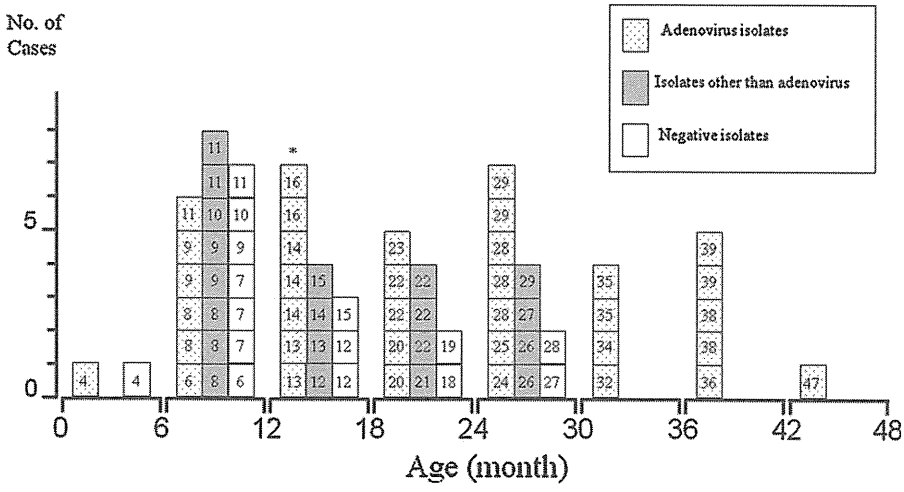

Figure 1. The number of cases with intussusception by age and viral isolate. Data represent the number of cases with adenovirus isolates ($n = 36$), isolates other than adenovirus ($n = 20$), and no isolates ($n = 15$) according to age. The numeral in the square represents the accurate age (in months) for each individual. The figure includes one case of multiple viral infections (*): a 14-month-old girl in whom both adenovirus type 5 and norovirus were detected.

Table 1
Viral isolates in children with intussusception

Virus	No. of patients with viral detections		
	Overall (n = 57)[a]	<2 years (n = 36)	≥2 years (n = 21)
Adenovirus	36	19[c]	17[c]
Poliovirus	5	5	0
Enterovirus	5	4	1
Parechovirus	4	3	1
Norovirus	4	3	1
Rotavirus	1	0	1
ND[b]	2	2	0

[a] Viruses were detected in 56 patients. This table includes a 14-month-old girl in whom adenovirus type 5 and norovirus were both detected. Three cases were detected to have adenovirus (AdV-31, 1 case; AdV41, 2 cases) and a case of parechovirus was identified by PCR.
[b] ND = viruses were present in the viral culture, but were not identified.
[c] p = 0.03 comparing age groups, determined by the Chi-square test.

positive viral isolates was 68.3%. Norovirus was identified in the majority of cases (78.6%), while others were adenovirus (7.1%), enterovirus (5.4%), parechovirus (5.4%), and rotavirus (3.6%). The detection of adenovirus in intussusception patients was significantly higher than that in patients with uncomplicated gastroenteritis ($p < 0.0001$).

3.3. Analysis of adenovirus types

Human adenoviruses are classified into 51 serotypes within six subgenera (A to F). Serotyping of 36 adenovirus isolates obtained from stool specimens was performed using various anti-adenovirus sera and PCR methods. As shown in Table 3, adenovirus serotype 1 (AdV-1) and AdV-5 were frequently detected in patients aged <2 years of age, whereas AdV-2 and AdV-3 were frequently found in those aged ≥2 years. AdV-5 was most frequently isolated in patients under 2 years of age, while AdV-3 was prominent in those over 2 years of age. The isolation of enteric types of adenovirus (AdV-31 and AdV-40/41) was found in five patients: three aged <2 years and two aged ≥2 years. The majority of adenovirus (subgenera B and C) isolates were non-enteric organisms generally associated with respiratory tract symptoms.[24–28]

3.4. Clinical manifestations

The classical clinical manifestations of intussusception, i.e., abdominal pain, vomiting, rectal bleeding/bloody stool, abdominal mass, and lethargy/irritability, were analyzed based on the age distribution and the presence of viral detections (Table 4). Abdominal pain was the most frequent presentation in patients ≥2 years of age. In contrast, lethargy/irritability and rectal bleeding/bloody stool were less frequent symptoms in older patients with intussusception. Rectal bleeding/bloody stool was a more frequent presentation in patients <2 years of age (77.1%) than in those ≥2 years of age (26.1%). No differences in the frequencies of vomiting and abdominal mass were noted between the age groups. Furthermore, there were significant differences in the primary symptoms among patients positive for adenovirus, positive for virus other than adenovirus, and negative for pathogens (Table 4). The symptom of abdominal pain was frequently seen among patients positive for adenovirus, but rectal bleeding/bloody stool was seen in less than half of these patients. However, no difference in the frequency of typical clinical manifestations was noted between those positive and negative for adenovirus isolates in each age group (data not shown).

Most patients sought medical attention and were diagnosed within 24 h after the onset of symptoms. Forty-five of 48 patients

Table 2
The number of cases with uncomplicated gastroenteritis by age and viral isolate

	No. of cases					
	Overall (%)	<1 year	≥1 to <2 years	≥2 to <3 years	≥3 to <4 years	≥4 to <5 years
Positive	56 (68.3)	16	26	8	4	2
Adenovirus	4 (4.9)	1	2	1	0	0
Type 3				1		
Type 5		1	1			
Type 31			1			
Enterovirus	3 (3.7)	2	1	0	0	0
Parechovirus	3 (3.7)	3	0	0	0	0
Norovirus	44 (53.7)	10	23	6	4	1
Rotavirus	2 (2.4)	0	0	1	0	1
Negative	26 (31.7)	13	6	2	2	3

Note. Viral isolates from stool specimens were identified in 82 patients aged under 48 months with uncomplicated gastroenteritis. The rate of patients with positive viral isolates was 68.3%; norovirus was identified in the majority of cases (78.6%), while others were adenovirus (7.1%), enterovirus (5.4%), parechovirus (5.4%), and rotavirus (3.6%).

Table 3
Types of adenovirus isolates from stool samples

Adenovirus serotype (subgenus)	No. of cases		
	Overall (n = 36)	<2 years (n = 19)	≥2 years (n = 17)
1 (C)	5	4	1
2 (C)	5	2	3
3 (B)	10	3	7
5 (C)	9	7	2
6 (C)	1	0	1
7 (B)	1	0	1
31 (A)	2	1	1
40/41 (F)	3	2	1

Note. Table 3 shows subgenus C is a dominant adenovirus species among intussusception cases. Subgenus C is a non-enteric organism generally associated with fever and upper respiratory tract symptoms. Subgenera A and F are enteric adenoviruses. With regard to age, adenovirus types 1 and 5 were dominant in cases <2 years of age, but type 3 was more frequently detected in cases ≥2 years of age.

Table 4
Clinical manifestations of intussusception cases by age and viral isolate

Symptom	No. of cases (%)[a]			No. of cases (%)[b]		
	Overall (n = 71)	<2 years (n = 48)	≥2 years (n = 23)	Adenovirus (n = 35)	Other than adenovirus (n = 20)	Negative isolation (n = 15)
Abdominal pain	36 (51)	14[c] (29)	22[c] (96)	24[d] (69)	9 (45)	2[d] (13)
Vomiting	41 (58)	31 (65)	10 (43)	18 (51)	13 (65)	9 (60)
Rectal bleeding/bloody stool	45 (63)	38[c] (79)	7[c] (23)	17[e] (49)	16[e] (80)	12[e] (80)
Lethargy/irritability	53 (75)	43[c] (90)	10[c] (43)	22 (63)	17 (85)	13 (87)
Abdominal mass	27 (38)	17 (35)	10 (43)	13 (37)	10 (50)	4 (27)

[a] Data represent the number of cases presenting the symptoms. Data show that in cases under 2 years of age, there was a high frequency of lethargy/irritability and rectal bleeding/bloody stool; in cases over 2 years of age, there was a high frequency of abdominal pain.
[b] Data represent the number of cases presenting the symptoms, except for one case of multiple viral infections. The symptom abdominal pain was frequently seen in cases where adenovirus was detected, but rectal bleeding/bloody stool was observed in less than half of them.
[c] $p < 0.0001$ comparing age groups, determined by Chi-square test.
[d] $p < 0.01$.
[e] $p < 0.05$, determined by the Chi-square test and Ryan's test, when comparing adenovirus cases with those in whom a virus other than adenovirus was detected and those in whom virus was not detected.

aged <2 years and 18 of 23 patients aged ≥2 years were diagnosed in less than 24 h. None of the patients enrolled in this study required surgical intervention.

4. Discussion

The underlying cause of intussusception in young children is unknown. However, it has been associated with several pathogens, including adenovirus and rotavirus.[3,10–16,28–32] In the current study various viruses from stool specimens were isolated in 78.9% of patients with intussusception during their hospitalization. In addition, 63.2% of the pathogens were adenovirus and the others were various pathogens commonly observed in patients aged under 2 years. The isolation of adenovirus was significantly increased to 73.9% in patients over 2 years of age. Among the patients with uncomplicated gastroenteritis, adenovirus was detected in only four cases, thus suggesting that adenovirus infection had not reached epidemic proportions.

In this report, we compared the rate of virus isolation from stool specimens between patients with intussusception and those with uncomplicated gastroenteritis. A study of viral isolates from healthy children is required to conclusively determine the involvement of viral infections, including adenovirus infections, in intussusception. However, we were unable to examine the viral isolates in stool specimens from healthy subjects (children) as a control group because of the difficulty in obtaining informed consent from such individuals. Nevertheless, our results show an apparent high number of adenovirus isolates. It would also be helpful to identify other studies that have searched for such adenoviruses in healthy controls.

The causative incidence of adenovirus isolation ranges from 30% to 50%.[3,10–16] Adenovirus is frequently seen in the mucosa and hyperplasic lymph nodes at the lead point and in the appendices of intussusception patients.[30,33] In the current study the detection rate of adenovirus (36 of 71, 50.7%) was remarkably high in comparison to previous reports. A recent study comparing Vietnam and Australia showed that 34% of patients were positive for adenovirus in stool in Vietnam and 40% in Australia. All patients were aged ≤25 months. Bines et al. reported that the significantly increased isolation rate in comparison to that of healthy controls in each country strongly supports an association.[3] Similarly in a recent study from Taiwan, 44% of patients with intussusception shed adenovirus in the throat or rectal specimens in comparison to only 3.8% of healthy controls. Furthermore, in another study, acute primary viral infections were identified in 65% of intussusception patients for whom paired sera were available (39.5% adenovirus, 9.3% human herpes virus 6, 11.6% human herpes virus 7, 4.7% Epstein–Barr virus).[14] The adenovirus genome was detected in four of nine mesenteric lymph nodes; 75% of the patients in whom a primary adenovirus infection was confirmed by seroconversion were aged >1 year.[14] These reports reduce the likelihood that shedding of adenovirus in intussusception patients is coincidental. The frequency of adenovirus isolates in this study was almost comparable to the data from Vietnam and Australia in patients <2 years of age. Therefore, almost 40% of the patients with intussusception aged below 2 years may have adenovirus infections.

The majority of patients with intussusception are under 1 year of age.[1] The present study in Hiroshima shows a different age distribution in patients with intussusception in comparison to other studies: 32.4% were aged <1 year, 35.2% were aged 1–2 years, and 32.4% were aged >2 years (Figure 1). Furthermore, another remarkable finding was the increased frequency of adenovirus isolates in those patients aged over 2 years. No precise evidence for the prevalence of adenovirus infection in the city of Hiroshima was noted during the study period. There were no significant variations in the annual presentation rate and the age distribution of intussusception patients over the 10-year period (data not shown). Therefore, it appears that the reason for the difference in age distribution of intussusception patients in Hiroshima is not due to an epidemic of adenovirus infection over the last 2 years. Alternatively, adenovirus infection may be a major risk factor for intussusception in children over 2 years of age.

Adenoviruses cause acute respiratory disease in children plus a wide array of other syndromes, including pharyngoconjunctival fever, epidemic keratoconjunctivitis, myocarditis, hemorrhagic cystitis, acute diarrhea, intussusception, and encephalomyelitis.[25] Respiratory tract infections are the most common manifestation of adenovirus infections in children and are caused by the respiratory types: AdV-1, 2, 3, 5, and 6 (subgenera B and C).[24,26] AdV-31 and AdV-40/41 (subgenera A and F), which are the important serotypes causing enteritis,[24,27] were detected by PCR in the stool specimens of only five patients. The current study showed AdV-1 and AdV-5 (species C) to be dominant in the age group <2 years, and AdV-3 and AdV-7 (species B) to be dominant in the age group ≥2 years. Schmitz et al. reported an age predilection for adenovirus.[25] The highly significant age predilections were subgenus A (AdV-12, 18, 31) in infants and subgenus C (AdV-1, 2, 5, 6) in infants and small children. Regarding subgenus B, AdV-3 is common in school children, AdV-7 is common in school children and adults, and AdV-4 and AdV-8 and other species of subgenera B and D are common in adults. The current results show an age distribution of adenovirus isolates similar to the above age predilections of adenovirus. Salvaraj et al. reported the intussusception-associated adenovirus

isolates to be similar to the circulating non-intussusception-associated strains, and the host immune response may be the key determinant of the clinical course of infection after adenovirus colonization.[32] In the current study, the common adenovirus types causing respiratory tract infection were detected at a high frequency associated with the development of intussusception in children. An evaluation of host responses to adenovirus, together with an exploration of adenovirus virulence determinants remains a priority area of investigation into the most common cause of acute bowel obstruction in young children.

Recently, Blanch et al. reported that the classic picture of intussusception might frequently not be present in the majority of current cases.[34] The incidences of the typical clinical manifestations in the cases in this study were also less frequent (Table 4) and the presence of the classic triad of vomiting, abdominal pain, and rectal bleeding/bloody stool was rare (data not shown). Furthermore, it was found in the present study that the primary clinical manifestations were different in the two different age groups (Table 4). Particularly, the presence of rectal bleeding/bloody stool and lethargy/irritability was frequently observed in cases <2 years of age. Lethargy/irritability was particularly frequent in the cases <2 years of age; in contrast abdominal pain was frequent in the cases ≥2 years of age. Furthermore, there were significant differences in the primary symptoms among cases positive and negative for adenovirus (Table 4). No difference in the frequency of typical clinical manifestations was noted in patients positive and negative for adenovirus isolates in each age group (data not shown). It appears that the age factor rather than adenovirus infection may result in the differences in primary symptoms. Blanch et al.[34] suggested that the early diagnosis of intussusception may decrease the frequency of the classical clinical presentations. In this study, the fact that no cases required surgical treatment may support the possibility of early diagnosis of intussusception.

In this study a high prevalence of viral isolates was found in the stools of patients with intussusception during early childhood. Adenovirus was significantly isolated in patients with intussusception aged ≥2 years, thus suggesting that adenovirus is an important risk factor for the development of intussusception in young children, particularly those ≥2 years of age.

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Institutional report - Thoracic oncologic

Repeat resection of pulmonary metastasis is beneficial for patients with osteosarcoma of the extremities

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Abstract

Pulmonary metastasectomy in osteosarcoma can lead to long-term survival, but the role for repeat pulmonary metastasectomy is undefined. To confirm the value of repeat pulmonary resection of recurrent pulmonary metastases, we herein reviewed our institutional experience. Between 1989 and 2007, 25 patients with pulmonary metastases from osteosarcomas of the extremities underwent pulmonary resection, and 14 patients underwent repeat pulmonary metastasectomy. Ten of 14 patients underwent complete resection. Various perioperative variables were investigated retrospectively in these patients to confirm a role for repeat metastasectomy and analyze prognostic factors for overall survival (OS) after repeat pulmonary metastasectomy. OS rate after repeat pulmonary metastasectomy was 43% at two years and 19% at five years. On multivariate analysis, patients with complete resection presented significantly favorable OS ($P=0.02$). Interestingly enough, survival curve of patients with complete resection after the first pulmonary metastasectomy was almost the same as that of patients with complete resection after the second pulmonary metastasectomy. In conclusion, patients with complete resection for recurrent pulmonary metastasis show a significantly better prognosis after repeat pulmonary metastasectomy. Our data imply that repeat pulmonary metastasectomy might be beneficial because it can salvage a subset of patients with osteosarcoma who retain favorable prognostic determinants.

Keywords: Lung; Metastasectomy; Osteosarcoma; Recurrence; Repeat resection

1. Introduction

Surgical resection has been consistently shown to prolong survival among patients with pulmonary metastases [1], and similar results are obtained for lung metastases from osteosarcoma [2–7]. The unique characteristics of osteosarcoma, including metastatic affinity for the lung, make pulmonary metastasectomy a central component of therapy for this disease [1]. Approximately 50% of patients with osteosarcoma develop synchronous or metachronous metastatic lung diseases, and only 20–40% of those found to have pulmonary metastases will survive five years [2–7]. Although the prognosis for patients with osteosarcoma has recently improved dramatically, those who develop metastatic pulmonary disease still continue to pose a particularly difficult challenge.

There are several reports about the survival and the prognostic factors for patients with pulmonary metastasectomy for osteosarcoma [2–7], but there are few data in the literature on patients who have undergone repeat pulmonary metastasectomy for osteosarcoma [8, 9]. Thus, we reviewed the clinical data of patients with osteosarcoma treated with pulmonary metastasectomy in our hospital to determine the long-term results and the prognostic predictors of survival in this subset of patients.

2. Patients and methods

From December 1989 to November 2007, according to our medical records, 25 patients had received pulmonary resection for the first time due to metastases of osteosarcoma of the extremities. Twenty-three of them underwent complete resection. Furthermore, 17 patients presented recurrence after the first metastasectomy and 14 patients underwent repeat pulmonary metastasectomy (Fig. 1). The patients were 10 males and 4 females with a median age of 22 years (range, 10–54 years) at the time of repeat pulmonary metastasectomy. Multimodality treatment consisting of surgery and chemotherapy were conducted in all cases. Diagnosis of metastatic pulmonary nodules was made by X-rays and computed tomography that was routinely examined at several months interval after the first diagnosis of primary tumor. Further examinations were also performed to exclude extrapulmonary metastases.

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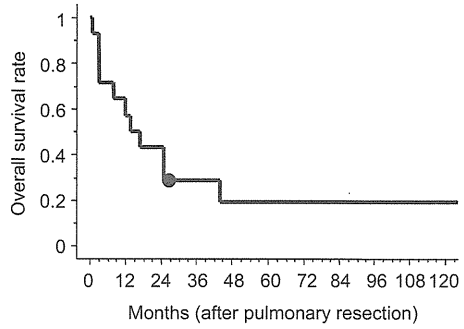

Fig. 1. Overall survival of patients following repeat pulmonary metastasectomy ($n=14$).

All patients who underwent resection of their pulmonary metastases at any time met the following criteria: (1) pulmonary lesions were deemed resectable not only by radiological examinations but also by the patients' general conditions, (2) metastatic disease was limited to the lungs, and (3) locoregional control of their primary cancer was obtained or obtainable. Complete resection was defined as no tumor cell at the surgical margin of the resected lung that was examined macroscopically and histologically. All the visible and palpable nodules were resected during the surgery and subjected to histological examination for confirmation of the diagnosis of metastases. The surgical approach was chosen according to the location and number of pulmonary nodules.

Therapeutic control for patients with lung metastases was based on the surgery in combination with pre- and/or postoperative multiagent chemotherapy. As to the treatment of osteosarcoma in the original site, all the patients undertook the surgery in combination with neoadjuvant and/or adjuvant chemotherapy consisting of multiagents. The detailed regimens of chemotherapy were different among patients; however, cisplatin, adriamycin, etoposide, methotrexate, and isofamide were used as antitumor drugs for chemotherapy pre- and postoperatively.

All patients were retrospectively analyzed for age, gender, detection of the first pulmonary metastasis, recurrence of primary tumors before pulmonary metastases, disease-free interval (DFI), number of pulmonary metastases, location of metastases, complete resection, and number of metastasectomy for pulmonary recurrence, regarding long-term survival. The endpoint was overall survival (OS) after the repeat pulmonary metastasectomy. DFI-1 was defined as the duration from the resection of the primary tumor to the initial diagnosis of the metastatic pulmonary tumor. DFI-2 was defined as the duration from the first pulmonary metastasectomy to the diagnosis of the recurrent pulmonary metastasis. OS was defined as the time between the repeat resection of pulmonary metastasis and the date of the last follow-up or death.

2.1. Statistical analysis

Statistical analysis was performed using the StatView (version 4.5) software package (Abacus Concepts, Berkeley, CA). The postoperative survival rate was analyzed by the Kaplan–Meier method. The prognostic influence of variables on survival was analyzed using the log-rank test for univariate analyses and the Cox's proportional hazards model for multivariate analyses. Differences were considered significant when $P<0.05$.

3. Results

Fourteen patients with recurrent pulmonary metastases from osteosarcomas underwent a thoracotomy and resection. The sites of primary tumors were the femur in eight cases, the tibia in five cases, and the humerus in one case (Table 1). In all cases, the primary tumors were resected completely. DFI-1 varied from 0 month to 9 years, including one case (7%) with simultaneous detection of the first pulmonary metastasis and the primary tumor. DFI-2 varied from 3 months to 8 years. In four patients (29%), lung metastases were found to be bilateral, while 10 patients (71%) had a solitary pulmonary metastasis. Ten of 14 patients underwent complete surgical resection. As of four patients with incomplete resection, two patients had bilateral metastatic lesions and were planned to undergo a staged bilateral thoracotomy; however, in all cases the

Table 1
Patient characteristics

Age	10–54 years (median 22 years)
Gender	
Male	10
Female	4
Location of primary tumors	
Femur	8
Tibia	5
Humerus	1
Detection of the first lung metastasis	
At initial presentation	1
During chemotherapy	3
After treatment	10
Recurrence of primary tumors ahead of lung metastases	
Yes	3
No	11
DFI-1	0–108 months (median 22 months)
DFI-2	3–95 months (median 7 months)
Number of recurrent pulmonary metastases	
<5	11
≥ 5	3
Location of recurrent metastases	
Unilateral	10
Bilateral	4
Surgical operation	
Wedge resection	11
Lobectomy	2
Pneumonectomy	1
Complete resection	
Yes	10
No	4
Number of surgery	
2	8
3	3
4	1
5	2

DFI (disease-free interval)-1: the duration from the resection of the primary tumor to the initial diagnosis of the metastatic pulmonary tumor. DFI-2: the duration from the first pulmonary metastasectomy to the diagnosis of the recurrent pulmonary metastasis.

Table 2
Univariate analysis for overall survival

Variables	Number of patients	2-year OS (%)	5-year OS (%)	Univariate analysis *P*-value
Age				
<20 years	6	33.3	16.7	0.74
≥20 years	8	50.0	12.5	
Gender				
Male	10	50.0	20.0	0.30
Female	4	25.0	0.0	
Pulmonary metastases identified during chemotherapy				
Yes	3	0.0	0.0	0.0036
No	11	54.5	18.2	
Recurrence of primary tumors ahead of lung metastases				
Yes	3	0.0	0.0	0.017
No	11	54.5	24.2	
DFI-1				
<1 year	6	16.7	0.0	0.029
≥1 year	8	62.5	25.0	
DFI-2				
<1 year	11	45.5	18.2	0.65
≥1 year	3	33.3	0.0	
Number of pulmonary metastases				
<5	11	54.5	18.2	0.25
≥5	3	0.0	0.0	
Location of metastases				
Unilateral	10	50.0	20.0	0.46
Bilateral	4	25.0	0.0	
Complete resection				
Yes	10	60.0	20.0	0.0058
No	4	0.0	0.0	
Number of metastasectomies for pulmonary recurrence				
2	8	37.5	12.5	0.36
≥3	6	50.0	16.7	

OS (overall survival): the time between the repeat resection of pulmonary metastasis and the date of the last follow-up or death. DFI (disease-free interval)-1: the duration from the resection of the primary tumor to the initial diagnosis of the metastatic pulmonary tumor. DFI-2: the duration from the first pulmonary metastasectomy to the diagnosis of the recurrent pulmonary metastasis.

disease exacerbated after unilateral metastasectomy. The remaining two patients had unilateral metastatic lesions, but one of them found pleural dissemination at the time of thoracotomy, and the other presented a new extrathoracic metastatic lesion at the perioperative time. Ten patients with unilateral tumors underwent a unilateral thoracotomy for resection of their metastases. One of four patients with bilateral tumors underwent simultaneous bilateral thoracotomy, whereas three patients had planned a staged bilateral thoracotomy for bilateral lesions; however, two patients were forced to abandon the contralateral surgery as described before. As for the number of surgeries, staged bilateral thoracotomies were counted as one operation. An attempt was made to conserve as much lung tissue as possible. This was reflected by the fact that wedge resections were the most common procedures performed. No patients died directly of surgery.

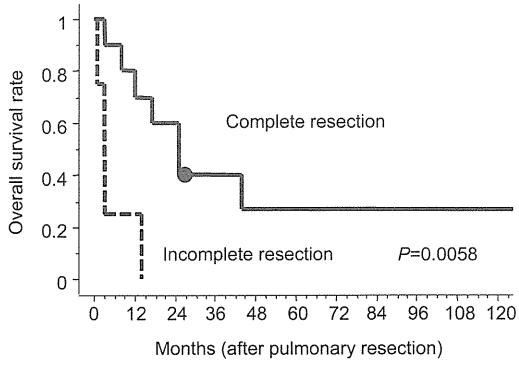

Fig. 2. Overall survival for patients concerning completeness of resection. A better overall survival was observed for patients with complete resection of recurrent pulmonary metastases ($P=0.0058$).

The median time at follow-up examination was 16 months (range 1–208 months). Of 10 patients with complete resection, three patients (30%) remained free of disease after repeat pulmonary metastasectomy, and seven patients (70%) developed recurrences, the majority of which were located in the chest. Seven of eight recurrent patients (88%) underwent a third metastasectomy for the re-recurrent pulmonary metastases. Three and two patients underwent fourth and fifth metastasectomy, respectively.

The OS rate was 42.9% at two years and 19.0% at five years after pulmonary resection (Fig. 1). Three (21%) patients are currently alive without evidence of disease and no patient is alive with disease. Eleven patients (79%) died of disease, and none died of other causes. Univariate analysis showed that a better OS was observed for patients without lung metastases identified during chemotherapy ($P=0.0036$), without recurrence of primary tumors ahead of lung metastases ($P=0.017$), with DFI-1 <1 year ($P=0.029$), and with complete resection ($P=0.0058$) (Table 2, and Fig. 2). However, on multivariate analysis, patients with complete resection presented significantly favorable OS ($P=0.021$) (Table 3). Interestingly enough, survival curve of patients with complete resection after the first pulmonary metastasectomy was almost the same as that of patients with complete resection after the second pulmonary metastasectomy (Fig. 3).

4. Discussion

Pulmonary metastasectomy has become the standard therapy for various metastatic malignancies to the lungs and pulmonary metastasectomy in osteosarcoma is also thought to lead to long-term survival [1, 2]. Unlike epithelial cancers, around 50–60% of patients with osteosarcoma relapse only in the lung, making pulmonary metastasectomy a viable option for treatment [8]. Furthermore, 40% of patients who relapse after pulmonary resection again

Table 3
Multivariate analyses for overall survival

Variables	Hazard ratio	95% CI	*P*-value
Pulmonary metastases identified during chemotherapy			
Yes	20.84	0.81–537.12	0.067
DFI-1			
<1 year	1.54	0.21–11.29	0.67
Recurrence of primary tumors ahead of lung metastases			
Yes	2.40	0.29–20.28	0.42
Complete resection			
Incomplete	16.64	1.53–180.60	0.021

CI, confidence interval. DFI (disease-free interval)-1: the duration from the resection of the primary tumor to the initial diagnosis of the metastatic pulmonary tumor.

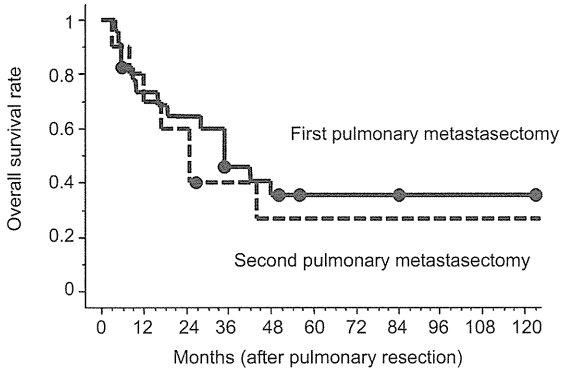

Fig. 3. Comparison with overall survival after the first pulmonary metastasectomy with complete resection and that after the second pulmonary metastasectomy with complete resection.

exhibit recurrence in the lung [10]. There are several encouraging studies about repeated surgical interventions for recurrent metastases from various primaries [11–13]; however, few data are available in the literature on patients who have undergone repeat pulmonary metastasectomy for osteosarcoma [8, 9]. Therefore, we decided to report our experience with repeat pulmonary metastasectomy in patients with osteosarcomas and to evaluate its role in their treatment focusing on OS. The 5-year OS rate for patients who underwent first pulmonary metastasectomy for osteosarcomas was up to 40% in several reports [3–7]. Since Fig. 3 showed that the 5-year OS rate for patients who underwent first pulmonary metastasectomy was 36%, our attitude toward the first pulmonary metastasectomy for osteosarcomas appeared to be acceptable. Biccoli et al. showed that patients who have a second metastasectomy have the same probability of disease-free survival as those operated upon the first time [8]. Bielack et al. stated in their most recent and largest series that five-year OS for the second recurrence was 16%, while it went up to 32% when a renewed surgical remission was achieved [9]. On the other hand, we demonstrated that patients who have a second metastasectomy with complete resection have the same probability of OS as those who have a first metastasectomy with complete resection.

To date, various parameters, such as DFI, completeness of the resection, timing of metastases, number of metastases, tumor size, and laterality of metastases have been reported as prognostic factors for the first metastasectomy [2–7], but there are few reports about the repeat pulmonary metastasectomy for osteosarcoma [9]. Bielack et al. described several parameters, such as shorter interval for recurrence, multiple lesions, failure to achieve a macroscopically complete surgical remission, and no chemotherapy administered for recurrence, as adverse prognostic factors for OS after the second recurrence on multivariate analysis [9]. In this study, several prognostic factors for the repeat pulmonary metastasectomy were found on univariate analysis, but a better OS was observed for patients with complete resection on multivariate analysis. Complete resection has been reported to be a better prognostic factor for OS after the first pulmonary metastasectomy in many studies [3, 6, 14]. Furthermore, complete resection has also been shown as a favorable prognostic factor for reoperative pulmonary metastasectomy for osteosarcoma and sarcomatous pediatric histologies [9, 15]; however, we should keep in mind that our study consisted of a small number of patients, so the accumulation of cases is necessary to evaluate a prognostic factor properly and to determine the selection criteria for resection. It is interesting that whether complete resection is performed or not is only determined after the surgical intervention is done. In this sense, our results support the idea that survival benefit will be obtained if pulmonary metastasectomy is performed aggressively and repetitively [2, 4, 8, 9].

There are several limitations to our analysis. The retrospective design is the most practical way of addressing our question because of the incidence of osteosarcoma, but the results should be interpreted carefully. Chemotherapeutic regimens have evolved substantially and rapidly over the last three decades and continue to be highly individualized based on unique patient and tumor characteristics, but we could not analyze our patients in this study because of a lack of complete data. It could be hypothesized that long-term survivors have biologically different and less aggressive tumors. In addition, since our results were based on the small number of patients in one institution, we needed a long study period to increase the number of patients in our study; however, several factors related to this could affect the results, such as the fact that a diagnostic modality had been changed due to the introduction of PET scan. Therefore, a prospective, large-scale study with multiple institutions would be inevitable in the future to reconfirm the current results.

In conclusion, patients with complete resection for recurrent pulmonary metastasis show a significantly better prognosis after repeat pulmonary metastasectomy. Our data imply that repeat pulmonary metastasectomy might be beneficial because it can salvage a subset of patients with osteosarcoma who retain favorable prognostic determinants.

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