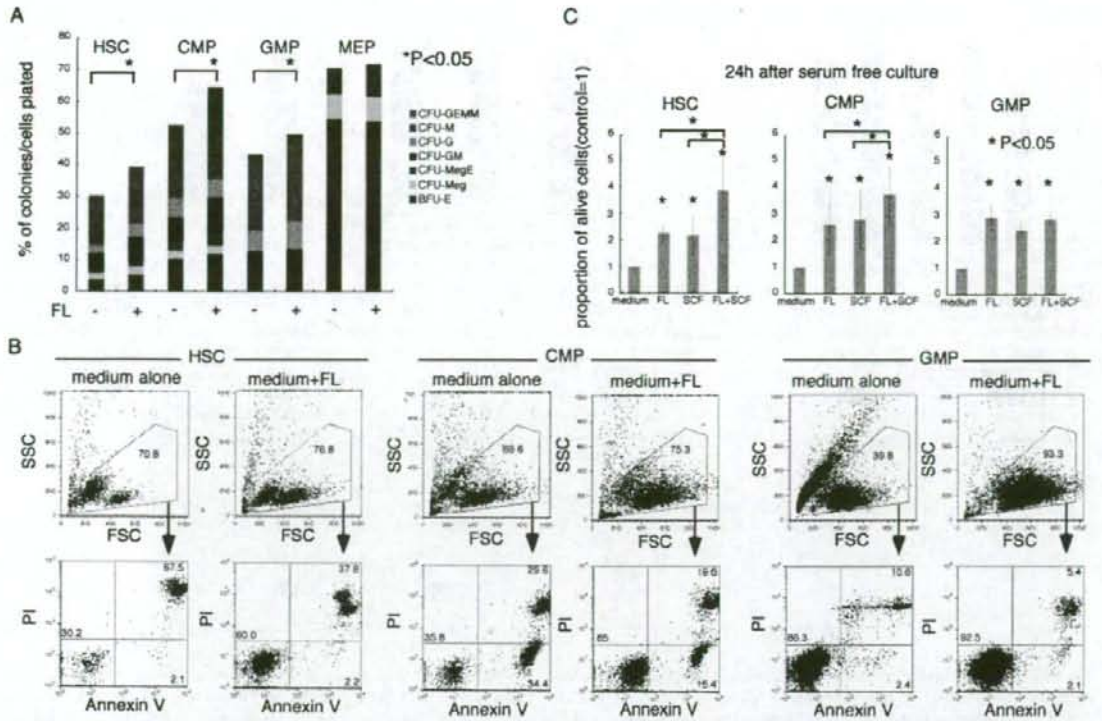

FIGURE 5. Effect of FL and SCF on the survival of purified progenitors. *A*, The effect of additional FL on colony formation of purified progenitors in methylcellulose in the presence of SCF, IL-3, IL-11, GM-CSF, Epo, and Tpo. Results from five independent experiments are shown here. Note that colony numbers are increased by the addition of FL into cultures in all hFlt3-expressing subsets including HSCs, CMPs, and GMPs but not in hFlt3$^-$ MEPs. *B*, An evaluation of apoptotic cell death in cultures of stem and progenitor cells. HSCs, hFlt3$^+$ CMPs, and GMPs were cultured in the serum-free media, with or without FL, and analyzed at 12, 18, 24, 30, 48, and 72 h after initiation of culture. A representative data obtained after 24-h culture is shown. *C*, Anti-apoptotic effects of FL and/or SCF on HSCs and Flt3$^+$ CMPs. Annexin$^-$ PI$^-$ live cells were enumerated after 24-h culture in a serum-free media. Each graph shows n-fold differences in the percentage of live cells relative to the ones without cytokine. Each bar represents the mean value and the SD of five independent samples.

and surface protein, and the distribution of Flt3 is quite different between human and mouse in early hematopoiesis.

In contrast, c-Kit was expressed at high levels in human HSCs and myelo-erythroid progenitors, while at a low level in CLPs (Fig. 1*C*). The expression pattern of c-Kit in human hematopoietic stem and progenitor cells is generally consistent with that in mouse hematopoiesis (4, 6, 7), suggesting that the c-Kit expression program is preserved in mouse and human hematopoiesis.

hFlt3 is expressed in functional hHSCs capable of reconstituting normal hematopoiesis in the NOD/SCID/IL-2 receptor γ-chain null (NOD/SCID/IL2rγ^{null}) mouse model

In the NOD/SCID/IL2rγ^{null} newborn system, hCD34$^+$hCD38$^-$ BM and CB cells are capable of reconstitution of all hematopoietic lineages for a long term (33). The entire hCD34$^+$hCD38$^-$ BM population expressed hFlt3 (Fig. 1*A*), suggesting that functional hBM HSCs possess hFlt3 on their surface. In contrast, hCD34$^+$hCD38$^-$ CB cells contained some hCD90$^-$ cells that did not express hFlt3. To formally test whether Flt3-expressing hCD34$^+$hCD38$^-$ CB cells possess LT-HSC activity, we transplanted hFlt3$^+$hCD34$^+$hCD38$^-$hCD90$^+$ CB cells in to NOD/SCID/IL2rγ^{null} newborns. As shown in Fig. 3*B*, NOD/SCID/IL2rγ^{null} mice transplanted with 1×10^3 hFlt3$^+$hCD34$^+$hCD38$^-$hCD90$^+$ CB cells reconstituted all hematolymphoid lineages for >6 mo, indicating that hFlt3 is expressed in functional hHSCs in CB as well as in BM.

Fig. 3*C* shows the phenotypic analysis of human progeny from 5×10^3 hFlt3$^+$hCD34$^+$hCD38$^-$hCD90$^+$ BM cells 6 (*upper panels*) or 15 wk (*lower panels*) after transplantation into NOD/SCID/IL2rγ^{null} newborns (33). hFlt3$^+$hCD34$^+$hCD38$^-$hCD90$^+$ BM cells differentiated into all hematopoietic lineage cells, including hCD33$^+$ granulocytes, hCD14$^+$ monocytes, hCD41$^+$ megakaryocytes, hCD19$^+$ B cells, hCD3$^+$ T cells, hCD56$^+$ NK cells (Fig. 3*C*), and hGPA$^+$ erythrocytes (not shown). Furthermore, transplanted hFlt3$^+$hCD34$^+$hCD38$^-$ HSCs purified from primary recipients developed secondary hFlt3$^+$ HSCs and hFlt3$^{-/+}$ CMPs, hFlt3$^-$ MEPs, and hFlt3$^+$ GMPs recapitulating normal human hematopoietic development. Thus, the hCD34$^+$hCD38$^-$hCD90$^+$ BM population contains cells with long-term SCID reconstitution potential as reported (33, 42), and all cells within this population express hFlt3 on their surface (Fig. 3*D*).

The up- or down-regulation of hFlt3 in the myeloid pathway is associated with GM or MegE differentiation activity, respectively

Fig. 4*A* shows the differentiation potential of purified BM progenitors in vitro in the presence of the myeloid cytokine mixture containing SCF, FL, IL-3, IL-11, Tpo, Epo, and GM-CSF. hFlt3$^+$

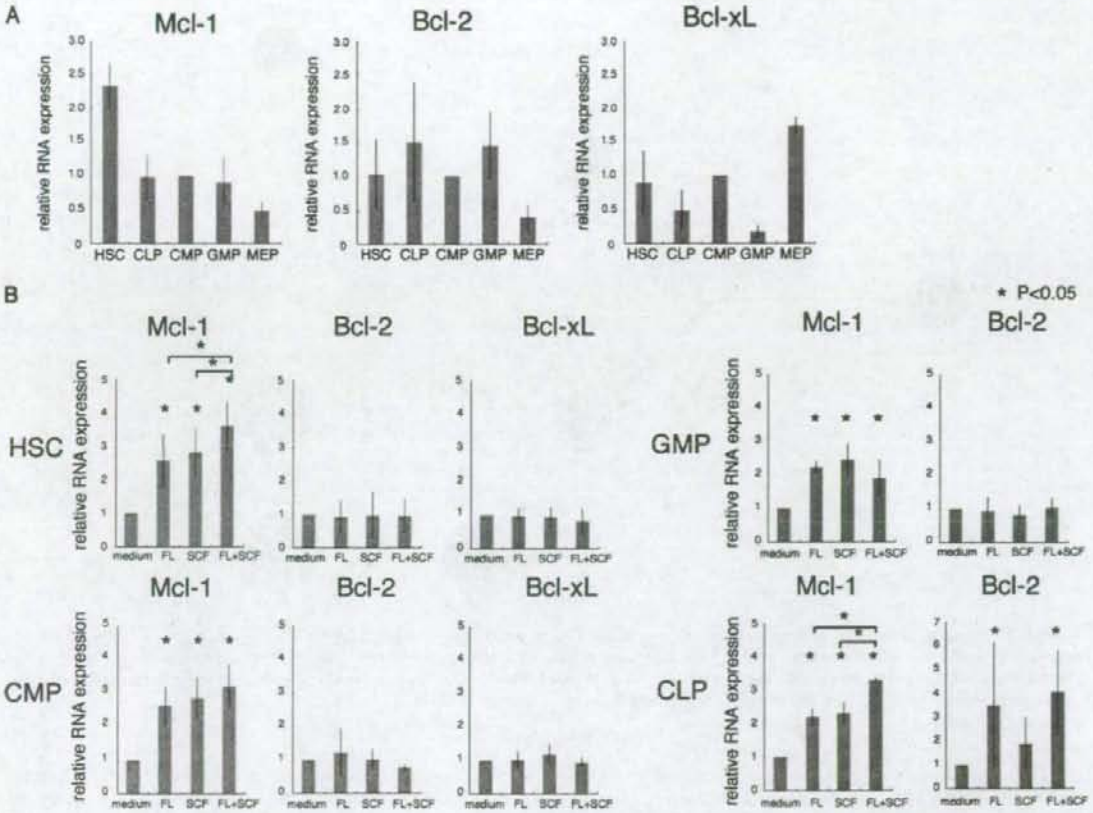

FIGURE 6. *A*, Quantitative RT-PCR assays for human anti-apoptotic genes such as Mcl-1, Bcl-2, and Bcl-x_L in purified HSCs and each progenitor population. Each bar represents an *n*-fold difference in the amount of anti-apoptotic gene expression relative to that in Flt3$^+$ CMPs. Note that Mcl-1 expression level is highest in HSCs, whereas Bcl-2 and Bcl-x_L expression is most pronounced in GMPs and MEPs, respectively. *B*, Changes in anti-apoptotic gene expression in each progenitor after incubation with FL and/or SCF. Significant up-regulation of Mcl-1 mRNA was seen in HSCs, Flt3$^+$ CMPs, GMPs, and CLPs after incubation with FL and/or SCF. Each bar represents the mean value and the SD of sixe independent samples.

CMPs formed a variety of myelo-erythroid colonies including clonogenic CFU-granulocyte/erythroid/macrophage/megakaryocyte (CFU-GEMM), whereas hFlt3$^-$ CMPs did not form CFU-GEMM, but preferentially differentiated into the MegE lineage. Since GMPs (hFlt3$^+$) and MEPs (hFlt3$^-$) exclusively gave rise to GM- and MegE-related colonies, respectively, hFlt3 expression could be associated with GM lineage development. These results suggested that hFlt3$^+$ CMPs might differentiate into MEPs via hFlt3$^-$ CMPs. We thus directly tested the lineage relationship of these purified myelo-erythroid progenitor populations (Fig. 4*B*). hFlt3$^+$ and hFlt3$^-$ CMPs were purified and cultured in vitro. Then, 72 h after the initiation of culture, hFlt3$^+$ CMPs gave rise to hFlt3$^-$ CMPs, hFlt3$^+$ GMPs and hFlt3$^-$ MEPs, whereas hFlt3$^-$ CMPs did not up-regulate hFlt3, differentiating only into hFlt3$^-$ MEPs. Such phenotypically defined secondary myeloid progenitors displayed differentiation activity consistent with their phenotypic definition (Fig. 4*C*). These data suggest that multipotent hFlt3$^+$ CMPs can differentiate into both GMPs and MEPs, whereas hFlt3$^-$ CMPs represent a transitional stage into MEPs.

Flt3 signaling protects human hematopoietic stem and progenitor cells from apoptotic cell death

We wished to elucidate the role of Flt3 signaling in human hematopoiesis. We first tested the effect of Flt3 signaling on the differentiation of HSCs, CMPs, and GMPs. Purified hFlt3$^+$ HSCs, CMPs, and GMPs were cultured in methylcellulose in the presence of the myeloid cytokine mixture, with or without hFL. As shown in Fig. 5*A*, the addition of FL in the culture did not affect the percentage of GM, MegE, or mix colonies in any of these populations. Interestingly, however, the colony numbers significantly increased in all cases when FL was added to the culture. This effect was dose-dependent, and the stimulatory activity of FL reached its peak at a concentration of 5 ng/ml (not shown). The plating efficiencies of hFlt3$^+$ HSCs, CMPs, and GMPs cultured with the cytokine mixture containing FL (20 ng/ml) were significantly higher than those cultured without FL, suggesting that FL signaling may enhance the viability of cells (Fig. 5*A*). We then directly tested the viability of HSCs, CMPs, and GMPs 24 h after the initiation of culture in serum-free media, with or without FL. The live, apoptotic, and dead cells after culture were enumerated by the Annexin/PI staining (43). In this staining, live cells are Annexin$^-$/PI$^-$, whereas Annexin$^+$/PI$^-$ and Annexin$^+$/PI$^+$ cells are apoptotic and dead cells, respectively (Fig. 5*B*). Without FL, a considerable proportion of purified HSCs, CMPs, and GMPs rapidly became Annexin$^+$/PI$^-$ and Annexin$^+$/PI$^+$ cells undergoing apoptotic cell death. The addition of FL significantly blocked apoptotic cell death in all of these populations, indicating that FL plays a critical role in human hematopoietic stem and progenitor cell survival (Fig.

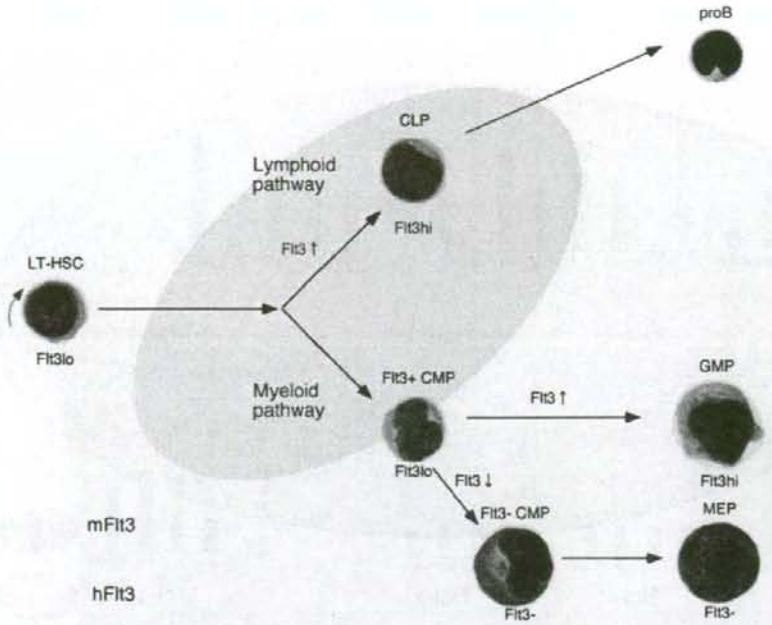

FIGURE 7. Proposed differential expression of human and mouse Flt3 in steady-state hematopoiesis. Cellular morphology of directly sorted each progenitors (May-Giemsa ×1000) is shown here. In human, the most primitive LT-HSC expressed hFlt3 at a low level and its expression is up-regulated at the early GM and the lymphoid progenitor stages, while it is down-regulated in MEPs. In contrast, the mouse LT-HSC lacks mFlt3 expression, and mFlt3 is expressed in cells primed to the lymphoid pathway, including CLPs and a fraction of CMPs.

5*B*). These data strongly suggest that Flt3 signaling does not instruct hematopoietic lineage commitment in hFlt3-expressing myeloid progenitors, but it does promote their survival.

SCF, the ligand for c-Kit, has also been shown to play a critical role in the maintenance of survival in early hematopoiesis. Both c-Kit and Flt3 belong to the class III receptor tyrosine kinase (RTK) family, sharing their major signaling cascade (44). Human HSCs, CMPs, and GMPs expressed both c-Kit and Flt3 at the single cell level (Fig. 1). Thus, we tested the anti-apoptotic effect of SCF in this system. As shown in Fig. 5*C*, in all HSC, CMP, and GMP populations, SCF also displayed anti-apoptotic effects whose impact on cell survival is similar to that of FL. Furthermore, in HSCs and CMPs, the combination of FL and SCF further increased percentages of live cells as compared with those in the presence of either FL or SCF alone, suggesting that SCF and FL signals collaborate to maintain cell survival of HSCs and CMPs.

Flt3 signaling up-regulates Mcl-1, but not Bcl-2 or Bcl-x_L, expression in human hematopoietic stem and progenitor cells

The question: is the mechanism of cell survival enhancement by signaling of RTKs, such as Flt3 and c-Kit? We have shown that in murine hematopoiesis, Mcl-1, a Bcl-2 homologue, is indispensable for hematopoietic stem and progenitor cell survival, and that c-Kit signaling is one of the most critical inducers for Mcl-1 expression in mHSCs (45). We therefore hypothesized that Flt3, as well as c-Kit, signaling may up-regulate Mcl-1 to maintain cell survival in human hematopoiesis as well.

Fig. 6*A* shows the distribution of the transcripts of Bcl-2 family molecules including Mcl-1, Bcl-2, and Bcl-x_L in human stem and progenitor cells. Mcl-1 is expressed at the highest level in HSCs. CMPs and CLPs expressed similar levels of Mcl-1, and MEPs expressed Mcl-1 at the lowest level. This expression pattern of Mcl-1 transcript in human hematopoiesis is consistent with that in murine hematopoiesis (45). In contrast, Bcl-2 was highly expressed in GMPs and CLPs, whereas Bcl-x_L was expressed in MEPs at the highest level.

Purified stem and progenitor populations were incubated with FL and/or SCF in serum-free media. Both FL and SCF dramatically up-regulated the expression of Mcl-1 in a dose-dependent manner, and it reached its peak 30 min after initiation of culture at a concentration of 5 ng/ml (data not shown). Fig. 6*B* shows the relative expression level of Mcl-1, Bcl-2, and Bcl-x_L in the presence of 20 ng/ml FL and/or SCF. We found that both FL and SCF significantly up-regulated the expression of Mcl-1, but not of Bcl-2 or Bcl-x_L, in HSCs, CMPs, and GMPs. These data collectively suggest that one of the important functions of these class III RTKs is to specifically activate Mcl-1 expression. Interestingly, in HSCs, FL and SCF displayed an additive effect on the up-regulation of Mcl-1. Therefore, Flt3 and c-Kit signaling collaborate to protect $Flt3^+$ HSCs and early myeloid progenitors from apoptotic cell death, presumably through activating anti-apoptotic Mcl-1 transcription. In CLPs, however, FL activated not only Mcl-1 but also Bcl-2 transcription.

Discussion

In this study, by using a multicolor FACS and a highly efficient xenograft system, we provide evidence that the distribution of Flt3 RTK is quite different in human and mouse hematopoiesis. First, although mouse LT-HSCs do not express mFlt3, the HSC-enriched $hCD34^+hCD38^-hLin^-$ population, that can reconstitute human hematopoiesis for a long-term in our xenogenic mouse model, uniformly expresses hFlt3 in both BM and CB. It is still unclear whether SCID-repopulating cells directly correspond to hLT-HSCs. However, because the $hCD34^+hCD38^+hLin^-$ cells never

reconstituted in xenogenic hosts for a long-term in our and others' experiments (42), it is highly likely that $hCD34^+hCD38^-hLin^-$ population is highly enriched for hLT-HSCs. Therefore, it is suggested that the negative expression of hFlt3 does not mark LT-HSCs in human, while mFlt3 does in mouse (16, 17). Second, in contrast to mouse hematopoiesis, where mFlt3 expression is restricted within progenitor populations of lymphoid potential including CLPs and a minority of CMPs that can differentiate into B cells (20), hFlt3 is expressed in human CMPs and GMPs, as well as in CLPs. The Flt3 expression is suppressed after cells are committed into the MegE lineage in both human and mouse. The distribution of Flt3 in mouse and human hematopoiesis is schematized in Fig. 7. The significant difference of Flt3 distribution in human and mouse hematopoiesis suggests that the critical role of Flt3 signaling in hematopoietic development could also be different between these species.

We further found that the important function of hFlt3 should include the maintenance of cell survival via the up-regulation of anti-apoptotic Mcl-1 in early hematopoiesis. Previous studies have demonstrated that FL can support in vitro survival of human long-term culture-initiating cells (24, 46, 47). MCL-1 is a non-redundant anti-apoptotic protein, at least in mouse hematopoiesis, because the removal of Mcl-1 from hematopoietic cells in a conditional knockout system caused fatal hematopoietic failure, and because in vitro disruption of *Mcl-1* in mouse HSCs, CMPs, or CLPs rapidly induced their apoptotic cell death (45). The expression level of Mcl-1 was the highest at the HSC stage and gradually declined as HSCs differentiate into myeloid and lymphoid progenitors in mouse hematopoiesis (45). The pattern of Mcl-1 distribution is well preserved in human hematopoiesis (Fig. 6*A*), suggesting that Mcl-1 might also be essential for hHSC survival. In mouse HSCs, Mcl-1 is up-regulated by signals from cytokines including SCF, IL-6, and IL-11, and SCF exerts the most potent effect on the up-regulation of Mcl-1(45). In contrast to mouse LT-HSCs that express c-Kit but not Flt3, functional hLT-HSCs coexpress c-Kit and Flt3 (Fig. 1), and importantly, FL as well as SCF are potent inducers for Mcl-1 transcription (Fig. 6). The fact that FL and SCF activated only Mcl-1, but not Bcl-2 or Bcl-x_L, in turn suggests that Mcl-1 might be the most critical survival factor controlled by exogenous cytokine signals at the HSC stage. Although it remains unclear whether hFlt3 and/or c-Kit signaling is absolutely required for hHSC survival, our data suggest that, to maintain the Mcl-1 level in hHSCs, the Flt3/FL system could work as an alternative to the SCF/c-Kit system. This is of interest because the SCF/c-Kit system is non-redundant in mouse hematopoiesis (48), where mouse LT-HSCs express only c-Kit, but not Flt3.

The anti-apoptotic effect of hFlt3 signaling was also seen in hFlt3-expressing myeloid progenitor populations. The incubation of CMPs and GMPs with FL significantly prevented their apoptotic cell death in vitro, and FL, as well as SCF, rapidly activated the Mcl-1 transcription in these progenitors. Interestingly, in CLPs, FL activated not only Mcl-1 but also Bcl-2. In lymphopoiesis, Bcl-2 (49, 50), as well as Mcl-1 (51), is critical. FL may collaborate with IL-7 to maintain lymphoid cell survival by up-regulating both Bcl-2 and Mcl-1. Collectively, in humans, Flt3 signaling might support cell survival in early hematopoietic stages with only the exception of the MegE lineage developmental pathway.

Our data also provides an important insight into pathogenesis of AML with *FLT3* mutations. A total of 15–35% of AML patients have either internal tandem duplications (ITDs) in the juxtamembrane domain or mutations in the activating loop of *FLT3* (28, 29), resulting in ligand-independent constitutive signal activation. The *FLT3* mutations are rarely found in acute lymphoblastic leukemia (28, 29). The etiologic link of *FLT3* mutations with AML does not fit the lymphoid-only expression pattern of Flt3 in mouse hematopoiesis. In mouse models, however, the ectopic expression of *FLT3*-ITDs in the bone marrow promotes development of myeloproliferative disorders, but these mutations themselves do not cause leukemia (52). We have found that AML cells with *FLT3*-ITD mutations possess extremely high levels of Mcl-1, and transduction of *FLT3*-ITD into normal HSCs induces rapid up-regulation of Mcl-1 of up to >10-fold higher levels (G. Yoshimoto and K. Akashi, manuscript in preparation). Because the expression of *FLT3* mutations should occur in concert with that of normal Flt3, our data suggest that once *FLT3* mutations are acquired in human hematopoiesis, abnormal survival-promoting signals of Mcl-1 should be expressed in LT-HSCs, and is progressively up-regulated in GMPs. It has been shown that both LT-HSCs and GMPs are the critical cellular target for leukemic transformation. The reinforced survival of CMPs/GMPs by blocking two independent apoptotic pathways (53), or the enforced expression of bcr-abl together with survival-promoting Bcl-2 at the GMP stage (54), results in AML development in mouse models. In human bcr-abl-positive chronic myelogeneous leukemia, GMPs could be the target for blastic transformation by acquisition of β-catenin signaling (55). GMPs can also be converted into leukemic stem cells simply by transducing leukemia fusion genes, such as MLL-ENL (56) or MOZ-TIF2 (57). Thus, these data collectively suggest that the acquisition of *FLT3* mutations in human hematopoiesis might induce the reinforced survival of cells at the HSC and myeloid progenitor stages, where *FLT3* mutations might collaborate with other genetic abnormalities to achieve full AML transformation.

In conclusion, our data show that the distribution of Flt3 is quite different in mouse and human hematopoeisis. hFlt3 targets LT-HSCs and myeloid progenitors except for MEPs. Flt3 signaling might support cell survival in early hematopoiesis including the HSC and the myeloid progenitor stages through up-regulation of Mcl-1. This is a striking example that the expression pattern of key molecules could be significantly different between human and mouse. Accordingly, special considerations are required in using mouse models to understand the role of Flt3 and *FLT3* mutations in human hematopoiesis.

Disclosures

The authors have no financial conflict of interest.

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CASE REPORT

Successful treatment of parainfluenza virus 3 pneumonia with oral ribavirin and methylprednisolone in a bone marrow transplant recipient

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Abstract We report a case of severe parainfluenza (PIV) 3 pneumonia in a hematopoietic stem cell transplant recipient that was successfully treated with oral ribavirin and methylprednisolone. A 42-year-old woman diagnosed with acute myelogenous leukemia (FAB M5a) in first complete remission underwent allogeneic bone marrow transplantation from an HLA-matched unrelated donor in May 2006. In July 2007, she developed PIV3 pneumonia. Her respiratory status progressively worsened and she required O_2 inhalation at 6 L/min. After an informed consent was obtained, oral ribavirin was initiated (16 mg/kg per day) for 1 week on July 31. By day 3 of treatment, the high-grade fever had disappeared. However, it recurred after ribavirin was discontinued. In addition, the patient's hypoxia continued to worsen, requiring O_2 inhalation at 9 L/min. To suppress the inflammatory reaction in the lung caused by PIV3 pneumonia, intravenous methylprednisolone (1,000 mg once a day for 3 days) was started along with high-dose oral ribavirin (16 mg/kg per day) on August 11. The patient showed dramatic clinical improvement, and oxygen inhalation was discontinued on September 3. Our case suggests that with concomitant effective anti-viral treatment, corticosteroids may suppress host inflammatory or immune reactions that lead to respiratory failure.

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Keywords Ribavirin · Parainfluenza virus · Pneumonia · Bone marrow transplantation

1 Introduction

Community-acquired respiratory virus infections are an important cause of morbidity and mortality after hematopoietic stem cell transplantation (HSCT) [1]. The best studied pathogens include respiratory syncytial virus (RSV), parainfluenza virus (PIV), and influenza virus [2]. PIV, an enveloped paramyxovirus containing single-stranded RNA, is classified into four serotypes. The latency period is 1–4 days [3, 4]. Only HSCT from an unrelated donor has been identified as a risk factor for infection with PIV [1]. In recipients of T-cell-depleted grafts, the degree of CD4 lymphopenia has been reported to increase the risk of all upper respiratory virus infections, including PIV [5]. Upper respiratory tract infection (URTI) is the predominant presentation. Progression to pneumonia seems to be less common than RSV. The most important risk factor for the progression from URTI to pneumonia is the use of corticosteroids and resulting lymphopenia [1, 2]. Thus, for PIV infection, host immunity seems to be a major determinant. Factors associated with a poor outcome after pneumonia include the presence of other infections and the use of ventilators. In these patients, PIV3 infections have been associated with a high mortality rate of up to 50% [1]. According to a few clinical studies, anti-viral treatment with ribavirin may improve the outcome of PIV infection in hematology patients. However, in the majority of the cases described, ribavirin has been administered in the aerosolized form, while only very few studies have addressed the use of ribavirin in the intravenous or oral form [1, 6–11]. Here, we report a case of severe PIV3

pneumonia in an HSCT recipient that was successfully treated with oral ribavirin and methylprednisolone.

2 Case report

A 42-year-old woman diagnosed with acute myelogenous leukemia (FAB M5a) in first complete remission underwent allogeneic bone marrow transplantation (BMT) from an HLA-matched unrelated donor in May 2006. The conditioning regimen comprised total body irradiation (12 Gy) and cyclophosphamide (120 mg/kg), and the graft-versus-host disease (GVHD) prophylaxis consisted of tacrolimus and short-term methotrexate. She developed grade 1 acute GVHD that was well controlled with prednisolone. She had no chronic GVHD.

In July 2007 (14 months after BMT), while taking 0.5 mg of tacrolimus every other day, she presented with fever, nasal discharge and cough. Chest examination revealed no significant findings. Despite treatment with oral levofloxacin (300 mg per day), her cough and fever worsened. On admission to our hospital on July 28, she was hypoxic with an SpO_2 of 93% on room air, necessitating O_2 inhalation. Infiltrations in the right middle and left lower lung fields were seen on her chest radiograph. Computed tomography (CT) scans demonstrated ground-glass opacification throughout the right middle and left lower lobes (Fig. 1). Despite the treatment with broad-spectrum antibiotics, her respiratory state deteriorated. Bronchoscopic examination was performed. The bronchoalveolar lavage fluid (BALF) aspirate contained many neutrophils and was negative for bacterial or fungal culture. PCR examination of BALF was positive for PIV3 and negative for other bacterial, fungal, or viral pathogens, including *Pneumocystis carinii*, *Mycobacterium tuberculosis*, adenovirus, RSV, varicella-zoster virus, herpes simplex virus, and cytomegalovirus.

The patient was diagnosed with PIV3 pneumonia. At the onset of PIV3 pneumonia, the number of CD4 + T cells and CD8 + cells were 74/mm^3, 391/mm^3, respectively, indicating the poor recovery of her cellular immunity. The patient's respiratory state worsened progressively, requiring O_2 inhalation at 6 L/min. After an informed consent was obtained, oral ribavirin was initiated (16 mg/kg per day) for 1 week on July 31. Her fever improved gradually. However, it recurred after ribavirin was discontinued and her hypoxia worsened, requiring O_2 inhalation at 9 L/min. CT scans demonstrated diffuse interstitial lesions (Fig. 2). To suppress the inflammatory reaction in the lung of PIV3 pneumonia, intravenous methylprednisolone (1,000 mg once a day for 3 days) was started with high-dose oral ribavirin (16 mg/kg per day) on August 11. The patient showed marked clinical improvement, and oxygen inhalation was discontinued on September 3.

The chest radiographs and CT scans demonstrated resolving infiltrates (Figs. 3, 4). High-dose ribavirin therapy resulted in the complication of myelosuppression requiring red blood cell and platelet transfusion, and administration of granulocyte-colony stimulating factor (G-CSF). The serology showed an increase in the PIV3 antibody titer (tenfold

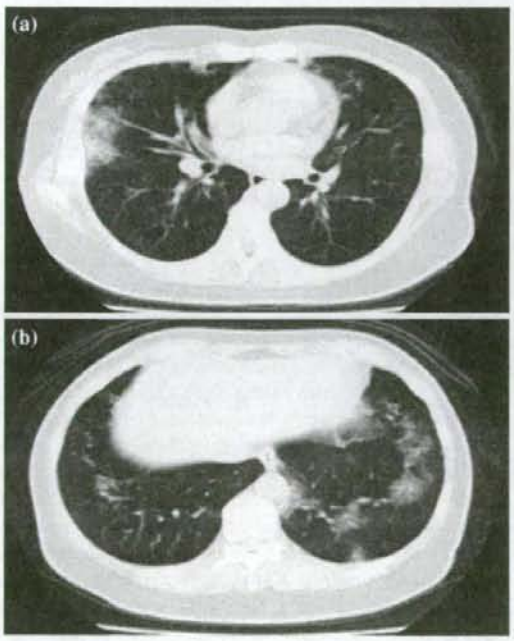

Fig. 1 Chest CT scans on admission demonstrating ground-glass opacification throughout the right middle (**a**) and left lower (**b**) lobes

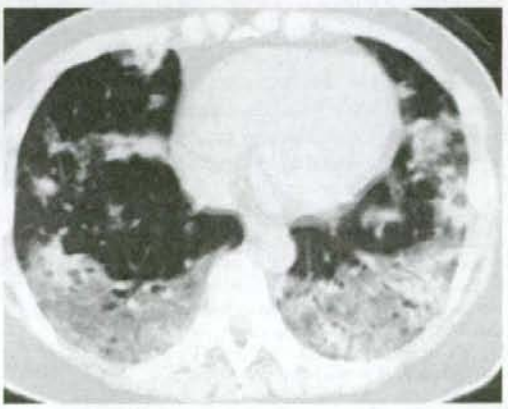

Fig. 2 Chest CT scan before initiating methylprednisolone demonstrating diffuse interstitial lesions

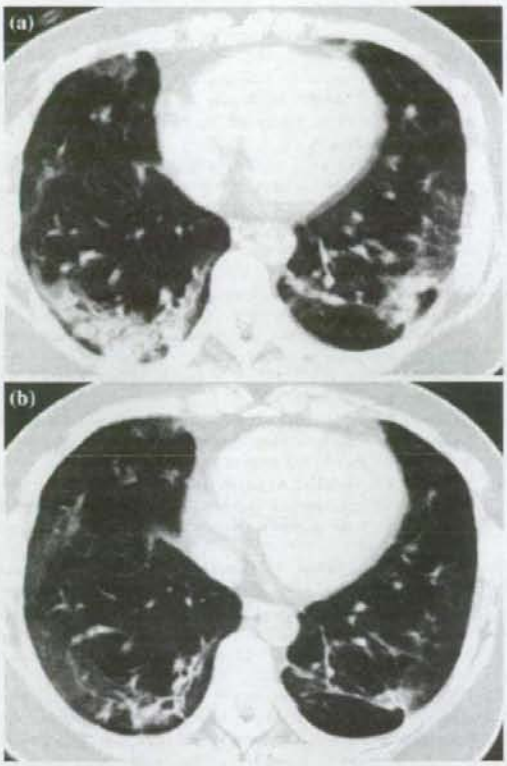

Fig. 3 Chest CT scans 2 (**a**) and 4 (**b**) weeks after initiating methylprednisolone demonstrating amelioration of interstitial lesions

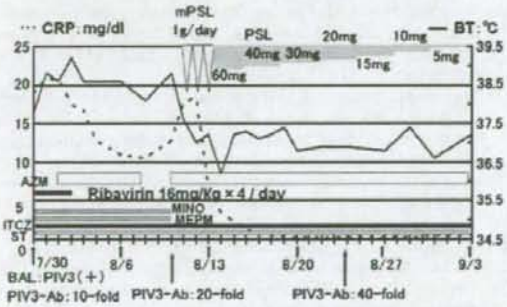

Fig. 4 Clinical course of treatment with oral ribavirin and methylprednisolone. *mPSL* methylprednisolone, *AZM* azithromycin, *MINO* minocycline, *MEPM* meropenem, *ITCZ* itraconazole, *ST* sulfamethoxazole/trimethoprim, *BAL* bronchoalveolar lavage, *PIV3* parainfluenza virus 3, *PIV3-Ab* parainfluenza virus 3 antibody titer

on admission, 20-fold 2 weeks after admission, and 40-fold 4 weeks after admission), and no evidence of any other agent. The patient was discharged on October 16, and she remained well 4 months after discharge.

3 Discussion

This is the first case report to document the successful treatment of PIV3 lower respiratory tract infection with combined high-dose oral ribavirin and corticosteroid therapy in an HSCT recipient. PIV3 infection in an immunocompromised patient can be fatal, and the prognosis is poor especially in HSCT recipients with lower respiratory tract infections. In this specific patient group, the mortality rate has varied between 30 and 50% [1]. In a retrospective analysis, neither aerosolized ribavirin nor immunoglobulin led to improved outcomes for PIV3 pneumonia or a reduction of viral shedding following PIV3 pneumonia [1, 7]. The use of systemic ribavirin has only been reported in case observations. The association of high-dose steroid treatment and lymphopenia with progression to disease suggests host immunity to be a major determinant. In this context, reduction of immunosuppressive therapy is desired when possible [1, 2, 12, 13].

Compared with aerosolized ribavirin, intravenous administration of the drug is easy, although potentially inferior in clinical efficacy [1, 2, 8, 14]. However, there are reports showing that the intravenous form of ribavirin is effective in other respiratory virus infections, such as adenovirus or RSV pneumonia and pneumonitis [2, 15–18].

In Japan, the intravenous form of ribavirin is not commercially available, but the oral form of the drug has been approved for the treatment of hepatitis C virus infection.

In the current report, oral ribavirin in combination with corticosteroids was successful in treating PIV3 pneumonia [19]. The dose and duration of oral ribavirin for the treatment of PIV3 pneumonia after HSCT have not been established. It is reported that the bioavailability of oral ribavirin is approximately 50% [20–23].

Corticosteroids have proven to be clinically useful in the treatment of *P. carinii* pneumonia with hypoxemia and the treatment of children with croup [24–27]. However, in HSCT patients with PIV URTI, corticosteroids are a risk factor for progression to lower respiratory tract infection [1]. On the other hand, some researches of antiviral therapy combined with steroid on viral pneumonia except PIV pneumonia in immunocompetent adults have been reported [28]. According to the study, the only combination of acyclovir and steroid against varicella zoster virus pneumonia was associated with a better outcome; however, the dosage and duration of steroid therapy for all of the viral pneumonia were heterogeneous in the study. Therefore the effectiveness of the combination of antiviral and steroid is not certain, and the authors suggested that future randomized clinical studies were necessary. The mechanism of corticosteroids for the treatment of viral pneumonia is not clarified. The mechanism of lung injury is considered not specifically a virally mediated lung injury, but rather an

abandoned host immune response to the virus. For viral pneumonia, the role of steroids is supposed to be similar to that in *P. carinii* pneumonia and miliary tuberculosis; both infections cause T-cell mediated responses. Corticosteroids directly inhibit both T cell function and neutrophil adherence to epithelial cells [29]. In our case, the use of oral ribavirin had limited efficacy, and a dramatic clinical improvement was observed following the introduction of corticosteroids with oral ribavirin.

Our case suggests that with concomitant effective antiviral treatment, corticosteroids may suppress host inflammatory or immune reactions that lead to respiratory failure.

In conclusion, we describe a case of severe PIV 3 pneumonia in an HSCT recipient successfully treated with oral ribavirin and methylprednisolone. The optimal use of oral ribavirin for the treatment of PIV pneumonia remains to be determined.

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HapMap scanning of novel human minor histocompatibility antigens

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HapMap scanning of novel human minor histocompatibility antigens

Running head: Minor antigen mapping with HapMap resources

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Abstract

Minor histocompatibility antigens (mHags) are molecular targets of allo-immunity, associated with hematopoietic stem cell transplantation (HSCT) and involved in graft-versus-host disease, but they also have beneficial anti-tumor activity. mHags are typically defined by host SNPs that are not shared by the donor and are immunologically recognized by cytotoxic T-cells isolated from post-HSCT patients. However, the number of molecularly identified mHags is still too small to allow prospective studies of their clinical importance in transplantation medicine, mostly due to the lack of an efficient method for isolation. Here we show that when combined with conventional immunological assays, the large data set from the International HapMap Project can be directly used for genetic mapping of novel mHags. Based on the immunologically determined mHag status in HapMap panels, a target mHag locus can be uniquely mapped through whole genome association scanning taking advantage of the unprecedented resolution and power obtained with >3,000,000 markers. The feasibility of our approach could be supported by extensive simulations and further confirmed by actually isolating two novel mHags as well as one previously identified example. The HapMap data set represents an invaluable resource to investigate human variation, with obvious applications in genetic mapping of clinically relevant human traits.

Introduction

The antitumor activity of allogeneic hematopoietic stem cell transplantation (HSCT), which is a curative treatment for many patients with hematological malignancies, is mediated in part by immune responses that are elicited as a consequence of incompatibility in genetic polymorphisms between the donor and the recipient.[1,2] Analysis of patients treated for post transplant relapse with donor lymphocytes has shown tumor regression to be correlated with expansion of cytotoxic T lymphocytes (CTLs) specific for hematopoiesis-restricted minor histocompatibility antigens (mHags).[3,4] mHags are peptides, presented by major histocompatibility complex (MHC) molecules, derived from intracellular proteins that differ between donor and recipient due mostly to single nucleotide polymorphisms (SNPs) or copy number variations (CNVs).[1,2,5] Identification and characterization of mHags that are specifically expressed in hematopoietic but not in other normal tissues, could contribute to graft versus leukemia/lymphoma (GVL) effects, while minimizing unfavorable graft versus host disease, one of the most serious complications of allo-HSCT.[1,2] Unfortunately, however, efforts to prospectively target mHags to invoke T cell mediated selective GVL effects have been hampered by the scarcity of eligible mHags, largely due to the lack of efficient methods for mapping the relevant genetic loci. Several methods have been developed to identify mHags, including peptide elution from MHC,[6,7] cDNA expression cloning,[8,9] and linkage analysis,[3,10] We have recently reported a novel genetic method that combines whole genome association scanning with conventional chromium release cytotoxicity assays (CRAs). With this approach, the genetic loci of the mHag gene recognized by a given CTL clone can be precisely identified using SNP array analysis of pooled-DNA generated from immortalized lymphoblastoid cell lines (LCLs) that are immunophenotyped into $mHag^+$ and $mHag^-$ groups by CRA.[11] The mapping resolution has now been improved from several Mb for conventional linkage analysis to an average haplotype block size of less than 100kb,[12] usually

containing a handful of candidate genes. Nevertheless, it still requires laborious DNA pooling and scanning of SNP arrays with professional expertise for individual CTLs.[11] To circumvent these drawbacks, we have sought to take advantage of publicly available HapMap resources. Here, we describe a powerful approach for rapidly identifying mHAg loci using a large genotyping data set and LCLs from the International HapMap Project for genome wide association analysis.[13-15]

Methods

Cell lines and CTL clones

The HapMap LCL samples were purchased from the Coriell Institute (Camden, NJ). All LCLs were maintained in RPMI1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine and 1mM sodium pyruvate. Because the recognition of a mHag requires presentation on a particular type of HLA molecule, the LCLs were stably transduced with a retroviral vector encoding the restriction HLA cDNA for a given CTL clone when necessary.[16]

CTL lines were generated from recipient peripheral blood mononuclear cells obtained posttransplant by stimulation with those harvested before HSCT after irradiation (33 Gy), and thereafter stimulated weekly in RPMI 1640 supplemented with 10% pooled human serum and 2 mM L-glutamine. Recombinant human inerleukin-2 was added on days 1 and 5 after the second and third stimulations. CTL clones were isolated by standard limiting dilution and expanded as previously described.[10,17] HLA restriction was determined by conventional CRAs against a panel of LCLs sharing HLA alleles with the CTLs. All clinical samples were obtained after written informed consent based on a protocol approved by the Institutional Review Board Committee at Aichi Cancer Center and the University of Tokyo according to the Declaration of Helsinki.

Immunophenotyping of HapMap LCLs and high-density genome wide scanning of mHag loci

Case ($mHag^+$) - control ($mHag^-$) LCL panels were generated by screening corresponding restriction HLA-transduced CHB and JPT HapMap LCL panels with each CTL clone using CRAs. Briefly, target cells were labeled with 0.1 mCi of ^{51}Cr for 2 h, and 1×10^3 target cells/well were mixed with CTL at a predetermined E:T ratio in a standard 4-h CRA. All

assays were performed at least in duplicate. The percent specific lysis was calculated by ((Experimental cpm - Spontaneous cpm) / (Maximum cpm - Spontaneous cpm)) x100. After normalization by dividing their percent specific lysis values by that of positive control LCL (typically recipient-derived LCL corresponding to individual CTL clones), the mHag status of each HapMap LCL was defined as positive, negative or undetermined.

To identify mHag loci, we performed association tests for all the Phase II HapMap SNPs, by calculating χ^2 test statistics based on 2 x 2 contingency tables with regard to the mHag status as measured by CRA and the HapMap genotypes (presence or absence of a particular allele) at each locus. χ^2 were calculated for the two possible mHag alleles at each locus and the larger value was adopted for each SNP. While different test statistics may be used showing different performance, the χ^2 statistic is most convenient for the purpose of power estimation as described below. The maximum value of the χ^2 statistics was evaluated against the thresholds empirically calculated from 100,000 random permutations within a given LCL set. The program was written in C++ and will run on a unix clone. It will be freely distributed on request. Computation of the statistics was performed within several seconds on a Macintosh equipped with 2 x quadcore 3.2 GHz Zeon processors (Apple, Cupertino, CA), although 100,000 permutations took several hours on average.

Evaluation of the power of association tests using HapMap samples

The genotyping data of the Phase II HapMap[14] were obtained from the International HapMap Project web site (http://www.hapmap.org/genotypes/latest_ncbi_build35), among which we used the non-redundant data sets (excluding SNPs on the Y chromosome) from 60 CEU (Utah residents with ancestry from northern and western Europe) parents, 60 YRI (Yoruba in Ibadan, Nigeria) parents, and the combined set of 45 JPT (Japanese in Tokyo, Japan) and 45 CHB (Han Chinese in Beijing, China) unrelated individuals. They contained 3,901,416 (2,624,947

polymorphic), 3,843,537, (2,952,93 polymorphic), and 3,933,720 (2,516,310 polymorphic) SNPs for CEU, YRI, and JPT+CHB, respectively.

To evaluate the power, we first assumed that the Phase II HapMap SNP set contains the target SNP of the relevant mHag or its complete proxies, and that the immunological assays can completely discriminate i mHag$^+$ and j mHag$^-$ HapMap LCLs. Under this ideal condition, the test statistic, or χ^2, for these SNPs takes a definite value, $f(i,j)=i+j$, which was compared with the maximum χ^2 value, or its distribution, under the null hypothesis, i.e., no SNPs within the Phase II HapMap set should be associated with the mHag locus. Unfortunately, the latter distribution cannot be calculated in an explicit analytical form but needs to be empirically determined based on HapMap data, since Phase II HapMap SNPs are mutually interdependent due to extensive linkage disequilibrium within human populations. For this purpose, we simulated 10,000 case-control panels by randomly choosing i mHag$^+$ and j mHag$^-$ HapMap LCLs for various combinations of (i,j) and calculated the maximum χ^2 values (χ^2_{max}) for each panel to identify those (i,j) combinations, in which $f(i,j)$ exceeds the upper one percentile point of the simulated 10,000 maximum values, $g(i,j)^{P=0.01}$.

When proxies are not complete (i.e., $r^2<1$), the expected values will be decayed by the factor of r^2, and further reduced due to the probabilities of false positive (f_P) and negative (f_N) assays, and expressed as $\hat{f}(i,j)=(i+j)\times\hat{r}^2$ through an apparent r^2 ($\hat{r}^2$) as provided in formula (1). Under given probabilities of assay errors and maximum LD strength between markers and the mHag allele, we can expect to identify target mHag loci for those (i,j) sets that satisfy $\hat{f}(i,j)_{>}g(i,j)^{P=0.01}$.

Empirical estimation of distributions of r^2

The maximum r^2 value (r^2_{max}) between a given mHag allele and one or more Phase II HapMap SNPs was estimated based on the observed HapMap data set. Each Phase II HapMap SNP was assumed to represent a target mHag allele, and the r^2_{max} was calculated, taking into account all the Phase II HapMap SNPs less than 500kb apart from the target SNP.

Confirmatory genotyping

Genotyping was carried out either by TaqMan MGB technology with primers and probes for HA-1 mHag according to the manufacturer's protocol using an ABI 7900HT with the aid of SDS 2.2 software (Applied Biosystems, Foster City, CA) or by direct sequencing of amplified cDNA for the *SLC1A5* gene. cDNA was reverse transcribed from total RNA extracted from LCLs, and PCR was conducted with cDNA with the corresponding primers. Amplified DNA samples were sequenced using BigDye Terminator 3.1 (Applied Biosystems). The presence or absence (deletion) of the *UGT2B17* gene was confirmed by genomic PCR with two primer sets for exons 1 and 6 as described previously[18] using DNA isolated from LCLs of interest.

Epitope mapping

A series of deletion mutant cDNAs were designed and cloned into pcDNA3.1/V5-His TOPO plasmid (Invitrogen, Carlsbad, CA). Thereafter, 293T cells that had been transduced with restricting HLA class I cDNA for individual CTL clones were transfected with each of the deletion mutants and co-cultured with the CTL clone overnight to induce interferon-γ release, which was then evaluated by enzyme-linked immunosorbent assay (ELISA) as previously described.[9]

For *SLC1A5*, expression plasmids encoding full-length cDNA and the exon 1 of recipient and donor origin were first constructed because only the SNP in the exon 1 was