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LETTER TO THE EDITOR

Helicobacter pylori-induced thrombocytosis clinically indistinguishable from essential thrombocythemia

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Eradication of *Helicobacter pylori* is becoming a first-line therapy in patients with idiopathic thrombocytopenic purpura (ITP). The relationship between the immune destruction of platelets and *H. pylori* infection has been extensively discussed, but still remains unclear. On the other hand, a high frequency of gastrointestinal lesions and higher susceptibility to *H. pylori* have been reported in patients with myeloproliferative neoplasms (MPNs) including essential thrombocythemia (ET) [1,2], while the clinical outcome of patients with ET after eradication of *H. pylori* is totally unknown. Here we report, with bibliographic consideration, a 66-year-old female with putative ET, which was dramatically ameliorated by *H. pylori* eradication therapy alone.

A 66-year-old female patient with Hashimoto disease and Sjögren syndrome had been undergoing levothyroxine replacement therapy for over 25 years. At the age of 60, her peripheral platelet count had increased to $87.5 \times 10^4/\mu L$. A bone marrow study showed normocellular marrow with an increased number of megakaryocytes, but either JAK2 V617F or c-MPL W515L mutation tested negative. Soon after this, her peripheral platelet count increased to over $100 \times 10^4/\mu L$. She was suffering from autoimmune disorders which could have caused chronic inflammation, but the level of her platelet count could not be explained by reactive thrombocytosis to inflammation. Further diagnostic procedures confirmed that there was no evidence of a tumor possibly producing thrombopoietin and interleukin-6, and so on. She was tentatively diagnosed as having ET and began to receive 1000 mg/day hydroxycarbamide (HU) and 100 mg/day acetylsalicylic acid for anti-platelet therapy. The platelet count was maintained at around $80 \times 10^4/\mu L$ (Figure 1). At the age of 65, she developed an intractable skin ulcer at the left external malleolus which was suspected to be an adverse effect of HU, and hence intravenous injection of ranimustine (MCNU) was substituted for HU, but only once.

Just after the ranimustine admnistration, she was referred to our hospital and then continued on only acetylsalicylic acid. During this period she complained of dyspepsia, and for the first time underwent a gastrointestinal endoscopic study, which indicated that she had chronic active gastritis. Since she was highly positive for anti-*H. pylori* immunoglobulin G (IgG), 1-week eradication therapy consisting of lansoprazole (60 mg/day), clarithromycin (400 mg/day) and amoxicillin (1500 mg/day) was performed, and eradication of *H. pylori* was confirmed by the rapid urease test. The platelet count at the start of therapy was $164 \times 10^4/\mu L$, and 4 weeks later it had decreased to $115.6 \times 10^4/\mu L$. Thereafter, the platelet count continued to decrease gradually, and 1 year after the eradication therapy the platelet count was constantly below $40 \times 10^4/\mu L$, almost within the normal range, without any chemotherapeutic drugs. The present clinical course strongly suggests reactive thrombocytosis induced by *H. pylori* infection, instead of ET.

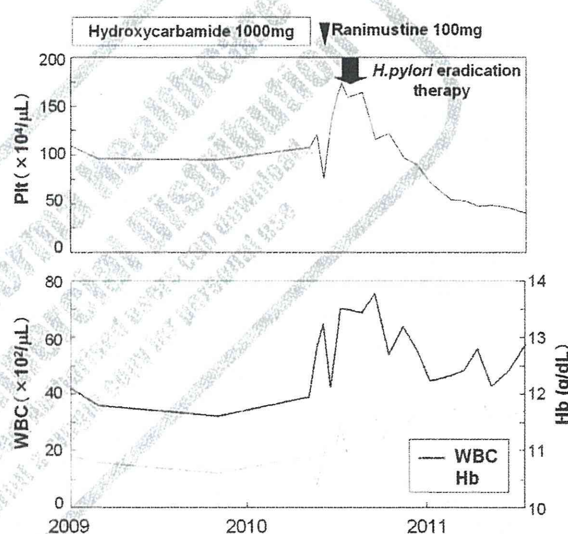

Figure 1. Changes in platelet count before and after *H. pylori* eradication therapy. The platelet count increased after discontinuation of hydroxycarbamide (HU), but has been gradually decreasing to the normal range after *H. pylori* eradication therapy in September 2010.

ITP results from the accelerated destruction of peripheral platelets by a platelet-specific autoimmune reaction and

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additional impairment of platelet production. The efficacy of *H. pylori* eradication therapy in patients with ITP was first reported in 1998 [3], and it is now becoming a first-line therapy. With regard to the pathogenetic relationship between *H. pylori* infection and ITP development, several explanations have been proposed according to the following clinical data: (1) a subset of anti-platelet autoantibodies, which might cross-react with the *H. pylori*-derived cytotoxin-associated gene A (CagA) protein, disappeared after eradication therapy [4]; (2) monoclonal antibodies against *H. pylori* urease B cross-reacted with the platelet glycoprotein IIIa [5]; and (3) monocytes from *H. pylori*-infected patients showed enhanced phagocytic activity and reduced expression of the inhibitory Fcγ receptor IIB (FcγRIIB) [6]. Nevertheless, the true pathogenesis remains to be elucidated.

There are paradoxical reports that infection with *H. pylori* increases the number of peripheral platelets in patients without ITP, which can be reversed by eradication therapy [7,8]. This observation is supported by experimental data that *H. pylori*-induced chronic inflammation can up-regulate the synthesis of interleukin-6 by local macrophages [9], which in turn stimulates platelet production directly or indirectly through up-regulation of thrombopoietin [10]. However, platelet counts are generally much less than $100 \times 10^4/\mu L$ in reactive thrombocytosis, except for cases bearing certain tumors that constitutively produce megakaryocyte-stimulating factors such as thrombopoietin and interleukin-6, and our patient is not such a case. The revised World Health Organization criteria for diagnosis of ET includes the demonstration of JAK2 V617F or other clonal marker, or no evidence of reactive thrombocytosis in the absence of JAK2 V617F. Recent advances in genomic analysis of MPN have identified many mutations in additional genes including CBL, LNK, SOCS, TET2, ASXL1, EZH2 and IDH, which may contribute to the pathogenesis of MPN and offer novel clonal markers [11]. Although evidence of clonality was not demonstrated in this case, the initial diagnosis of ET was probable according to the sustained platelet count over $100 \times 10^4/\mu L$ in the absence of documented inflammation or tumor. In this case, the titer of serum anti-*H. pylori* IgG was extremely high (199 U/mL; upper limit 9.9 U/mL) at the start of therapy, then decreased over time, but still remains moderate (34 U/mL) 1 year after therapy. Thus, one possible explanation for the pathogenesis of *H. pylori*-induced thrombocytosis is that a subset of anti-*H. pylori* IgG might work as a thrombopoietin-mimetic molecule to stimulate its downstream signal.

In conclusion, to our knowledge, this is the first reported case of *H. pylori*-induced thrombocytosis closely resembling ET. Now, we plan to conduct a clinical study of screening for *H. pylori* infection in patients with apparent thrombocytosis that lacks defined molecular abnormalities to confirm a correlation between *H. pylori* infection and thrombocytosis. It is worthwhile trying *H. pylori* eradication therapy in patients with putative ET who have no clonal markers such as JAK2 V617F mutation and have definite signs for *H. pylori* infection.

Potential conflict of interest: Disclosure forms provided by the authors are available with the full text of this article at www.informahealthcare.com/lal.

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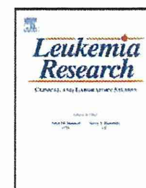

Unrelated cord blood transplantation after myeloablative conditioning regimen in adolescent and young adult patients with hematologic malignancies: A single institute analysis

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ABSTRACT

We report the results of unrelated cord blood transplantation (CBT) after myeloablative conditioning regimen in 16 patients with hematologic malignancies from 15 to 20 years old. The median times of myeloid and platelet engraftment were 21 and 38 days, respectively. The cumulative incidences of acute graft-*vs*-host disease (GVHD) was 62.0%, all of which were grade I or II, and that of extensive-type chronic GVHD was 12.5%. The probabilities of overall and disease-free survival at 3 years were 68.2% and 48.6%, respectively, comparable to adult or childhood cases. Adolescents and young adult patients with hematologic malignancies who have no HLA-matched adult donors could be considered as candidates for CBT.

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1. Introduction

Recently the medical needs of adolescents and young adults with hematologic malignancies have become better defined. In comparison of outcome of patients with 16–21 years of age with acute lymphoblastic leukemia (ALL) treated with pediatric *vs* adult clinical trials, pediatric trials yielded better outcome than adult trials [1]. In patients with acute myelocytic leukemia (AML), outcome of children younger than age 15 years has significantly improved for the last several decades, but that of patients with 15–19 years remains poor [2]. Thus, adolescents and young adults with hematologic malignancies are distinct in terms of their therapeutic requirements compared to adults or children. However, there have been no data defined adolescent and young adult patients for cord blood transplantation (CBT) after conventional myeloablative conditioning regimen. We here first report the clinical results for a group of 16 adolescent and young adult patients with hematologic malignancies treated with CBT in our institute, showing the safety and efficacy comparable to those for adults and children.

2. Patients and methods

This is a retrospective single-center analysis. Between September 1999 and July 2009, 16 patients at adolescent and young adult ages from 15 to 20 years old were treated with CBT as the first allogeneic stem cell transplantation at The Research Hospital, Institute of Medical Science, University of Tokyo. One patient received an autologous bone marrow transplantation before he had come to our hospital. Written informed consent for treatment was obtained from all patients with the Declaration of Helsinki. Patients were qualified as being standard risk and high risk according to the criteria in the previous reports [3,4].

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2.1. HLA typing and donor selection

HLA-A and HLA-B antigens were identified by serological typing. HLA-DRB1 alleles were determined by high-resolution molecular typing using polymerase chain reaction sequence-specific primers. Patients who did not have HLA-matched family or unrelated adult donors were considered to be eligible for CBT. In the selection of cord blood unit for transplantation, all cord blood grafts were evaluated by HLA-A, HLA-B and HLA-DRB1 typing, and nucleated cell counts. Preferred cord blood units matched 4 of 6 to 6 of 6 HLA loci and contained a minimal cell count of 1.5×10^7 nucleated cells/kg body weight before freezing according to the criteria of our institution as shown in the previous reports [3,4]. All cord blood units were obtained from cord blood banks belonging to the Japan Cord Blood Bank Network.

2.2. Conditioning regimen, GVHD prophylaxis, and supportive care

All patients received fractionated 12 Gy total body irradiation and chemotherapy as a myeloablative conditioning regimen (Table 1). Fifteen patients received standard cyclosporin (CsA) and methotrexate (MTX), and one patient received CsA alone for a graft-*vs*-host disease (GVHD) prophylaxis [3,4]. Fifteen mg/m^2 of MTX was given intravenously on day 1, and 10 mg/m^2 on days 3 and 6 as previously reported [3,4]. Both acute and chronic GVHD (aGVHD and cGVHD, respectively) were graded according to the previously published criteria [5,6]. The criterion to stop immunosuppression depended on patients' disease status. All patients received recombinant human granulocyte colony-stimulating factor starting on day 1 until durable granulocyte recovery was achieved.

2.3. Endpoints and statistical analysis

The chimerism status after CBT, engraftment, graft failure, treatment-related mortality (TRM), and disease-free survival (DFS) were defined as described in the previous reports [3,4].

Data analysis was performed on 1 December 2010. The probability of overall survival (OS) and DFS were estimated using the Kaplan–Meier method.

3. Results and discussion

The characteristics of the 16 patients and the cord blood units are shown in Table 1. Six patients were classified as standard risk while 10 patients as high risk. Six patients (2 ALL, 3 myelodysplastic syndrome (MDS) and 1 chronic myelocytic leukemia (CML)) were initially treated by Pediatric units. All patients received a single and HLA-mismatched cord blood unit. The median numbers of cryopreserved nucleated cells and CD34$^+$ cells were 2.50×10^7/kg (range 2.05 to 3.73×10^7/kg) and 0.94×10^5/kg (range 0.46 to 1.33×10^5/kg), respectively. The median numbers of infused nucleated and CD34$^+$ cells were 2.11×10^7/kg (n = 11; range 1.36 to 2.38×10^7/kg) and 0.76×10^5/kg (n = 11; range 0.25 to 2.55×10^5/kg), respectively.

Fourteen patients (87.5%) successfully achieved myeloid reconstitution and 2 patients went into graft failure regardless of above of median number of total nucleated cells (2.71 and 2.45×10^7/kg, respectively) and CD34$^+$ cells (1.09 and 1.13×10^5/kg, respectively) transplanted. One had full recovery with 100% of host chimerism by day 52, and the other took a second cord blood graft on day 30. All patients with myeloid reconstitution showed full donor chimerism at the first bone marrow examination after CBT. The median time to an absolute neutrophil count $>0.5 \times 10^9$/L among the patients with engraftment was 21 days (range 19–32 days). The cumulative

Table 1
Characteristics of patients, cord blood units, and outcomes.

Characteristics	
Patients, *n*	16
Male/female, *n*	9/7
Median age, years (range)	17 (15–20)
Median weight (kg) (range)	52 (45–71)
Median number of cryopreserved nucleated cells	
$\times 10^7$/kg (range)	2.50 (2.05–3.73)
Median number of cryopreserved CD34$^+$ cells	
$\times 10^5$/kg (range)	0.94 (0.46–1.33)
Median number of infused nucleated cells, *n*	11
$\times 10^7$/kg (range)	2.11 (1.36–2.38)
Median number of infused CD34$^+$ cells, *n*	11
$\times 10^5$/kg (range)	0.76 (0.25–2.55)
Median time from diagnosis to transplantation	
Days (range)	429 (65–1898)
Recipient CMV status, positive/negative, *n*	13/3
Diagnosis	
De novo AML [*n* (%)]	3 (19)
CR1, *n*	1
CR2, *n*	1
Not in remission, *n*	1
ALL [*n* (%)]	7 (44)
CR1, *n*	2
CR2, *n*	4
CR3, *n*	1
CML BC [*n* (%)]	1 (6)
MDS [*n* (%)]	4 (25)
RA, *n*	1
RCMD, *n*	1
Advanced (*n*)	2
MDS/MPD [*n* (%)]	1 (6)
Disease status at transplant*	
Standard risk [*n* (%)]	6 (37)
High risk [*n* (%)]	10 (63)
Conditioning regimen	
TBI + CY + AraC, *n*	1
TBI + CY + AraC/G-CSF, *n*	9
TBI + CY, *n*	4
TBI + CY + Tepa, *n*	1
TBI + Flu + Mel, *n*	1
GVHD prophylaxis	
CSP + sMTX, *n*	15
CSP, *n*	1
Number of HLA-A, B, DRB1 mismatches	
1, *n*	4
2, *n*	5
3, *n*	6
4, *n*	1
Engraftment [day (range)]	
Median time to neutrophil count $>0.5 \times 10^9$/L	21 (19–32)
Median time to platelet count $>50 \times 10^9$/L	38 (33–98)
Acute GVHD [*n* (%)]	
0	1 (7)
Grade I	6 (43)
Grade II	7 (50)
Grade III	0
Grade IV	0
Chronic GVHD [*n* (%)]	
None	0
Limited	10 (83)
Extensive	2 (17)
Immunosuppressant termination (*n* = 7)	
Median time [day (range)]	267 (83–952)
Cause of death [*n* (%)]	
Relapse	4 (80)
MOF	1 (20)

CMV, cytomegalovirus; AML, acute myelogenous leukemia; CR1, CR2, CR3: first, second, third complete remission, respectively; ALL, acute lymphoblastic leukemia; CML, chronic myelogenous leukemia; BC, blast crisis; MDS, myelodysplastic syndrome; RA, refractory anemia; RCMD, refractory cytopenia with multilineage dysplasia; Advanced, patients with MDS-related secondary AML; MPD, myeloproliferative disease; TBI, total body irradiation; Ara-C, cytosine arabinoside; G-CSF, granulocyte colony-stimulating factor; CY, cyclophosphamide; Tepa, thiotepa; Flu, fludarabine; Mel, melphalan; CsA, cyclosporine; sMTX, short-term methotrexate; MOF, multiple organ failure.

* Patients qualified as being standard risk or high risk according to the criteria described in previous reports [3,4].

ErbB receptor tyrosine kinase/NF-κB signaling controls mammosphere formation in human breast cancer

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Breast cancer is one of the most common cancers in humans. However, our understanding of the cellular and molecular mechanisms underlying tumorigenesis in breast tissues is limited. Here, we identified a molecular mechanism that controls the ability of breast cancer cells to form multicellular spheroids (mammospheres). We found that heregulin (HRG), a ligand for ErbB3, induced mammosphere formation of a breast cancer stem cell (BCSC)–enriched population as well as in breast cancer cell lines. HRG-induced mammosphere formation was reduced by treatment with inhibitors for phosphatidyl inositol 3-kinase (PI3K) or NF-κB and by expression of IκBα-Super Repressor (IκBαSR), a dominant-negative inhibitor for NF-κB. Moreover, the overexpression of IκBαSR in breast cancer cells inhibited tumorigenesis in NOD/SCID mice. Furthermore, we found that the expression of IL8, a regulator of self-renewal in BCSC-enriched populations, was induced by HRG through the activation of the PI3K/NF-κB pathway. These findings illustrate that HRG/ErbB3 signaling appears to maintain mammosphere formation through a PI3K/NF-κB pathway in human breast cancer.

EGF | HER | tumor sphere | cancer stem cells | inflammation

Cancer stem cells (CSCs), which make up only a small proportion of heterogeneous tumor cells, may possess a greater ability to maintain tumorigenesis than other tumor cell types (1, 2). CSCs can self-renew and simultaneously produce differentiated daughter cells; thus they can strongly proliferate until they reach their final differentiated state. With improvements in the isolation of CSCs, there is now a growing body of evidence that, in some cases of hematologic and solid tumors, a cancer stem cell population can be enriched based on phenotype (3–10). In human breast cancers, breast cancer stem cells (BCSCs) are enriched in the CD44^{high}/CD24^{low} cell population, whereas the CD44^{low}/CD24^{high} cells represent a more differentiated phenotype with limited stem cell–like potential (3). Because BCSCs withstand anoikis in culture, they expand under anchorage-independent conditions, giving rise to clonal spheroids (mammospheres), which can be serially passaged in vitro (11, 12). These processes can in part recapitulate the breast tumorigenesis process (13–16). To develop more effective cancer therapies, it would be reasonable to target molecules that have a critical role in the maintenance of mammospheres. However, the molecular mechanism by which mammospheres are maintained is still largely obscure.

NF-κB is a transcription factor complex that is typically a heterodimer of p50, p52, p65 (RelA), RelB, and c-Rel. It is usually inactive and bound to IκB, an inhibitory protein, in the cytoplasm. The primary mechanism of regulation of NF-κB activity is through activation of the IKK complex, including heterodimers of IKKα and IKKβ, as a result of various signaling pathways. The serine–threonine kinase Akt is one of the activators of IKKβ (17), and the activated IKK complex phosphorylates the IκBα protein, resulting in its ubiquitination, proteasome-mediated degradation, and subsequent release of NF-κB for nuclear translocation. Released NF-κB translocates to the nucleus and binds to the κB sequence, where it promotes the transcription of various genes, including inflammatory chemokines. Recently, we found activation of inflammatory signaling pathways in association with an increase in NF-κB activity in BCSC-enriched populations (18, 19). However, the role of NF-κB and the molecular mechanisms by which NF-κB is activated during mammosphere formation remain unknown.

Heregulin (HRG, also called neuregulin) is a ligand for ErbB3, which is one of the four members of the EGF receptor ErbB family (20). Expression of HRG in the mammary gland induces adenocarcinomas in animal models (21) and favors metastatic spread of breast cancer cells (22). HRG is expressed in 30% of human breast cancer patients (23) and correlates with poor histological grade (24). Recently, it was reported that ErbB2 overexpression increases the stem/progenitor cell population of both normal and malignant mammary cells (25); however the role of HRG and ErbB3 in regulating the properties of BCSC-enriched populations remains largely unknown.

In the present study, we showed that HRG induced mammosphere formation of cancer cells from a BCSC-enriched population. Moreover, our findings suggest that the activity of phosphatidyl inositol 3-kinase (PI3K)/NF-κB is essential for the HRG-induced mammosphere formation.

Results

HRG Induces Mammosphere Formation of a BCSC-Enriched Population. To test the mammosphere-forming ability of a BCSC-enriched population, we initially isolated CD44^{high}/CD24^{low}/lineage^{-} BCSC-enriched population and CD44^{low}/CD24^{high}/lineage^{-} nonstem cells from human breast cancer tissue. When these cells were cultured with conventional mammosphere culture media containing EGF, bFGF, and B27 supplement (13, 26), the CD44^{high}/CD24^{low}/lineage^{-} BCSC-enriched population generated mammospheres, whereas the CD44^{low}/CD24^{high}/lineage^{-} nonstem cells did not form mammospheres (Fig. 1 *A* and *B*). These observations suggest that cells with the capacity to

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The authors declare no conflict of interest.

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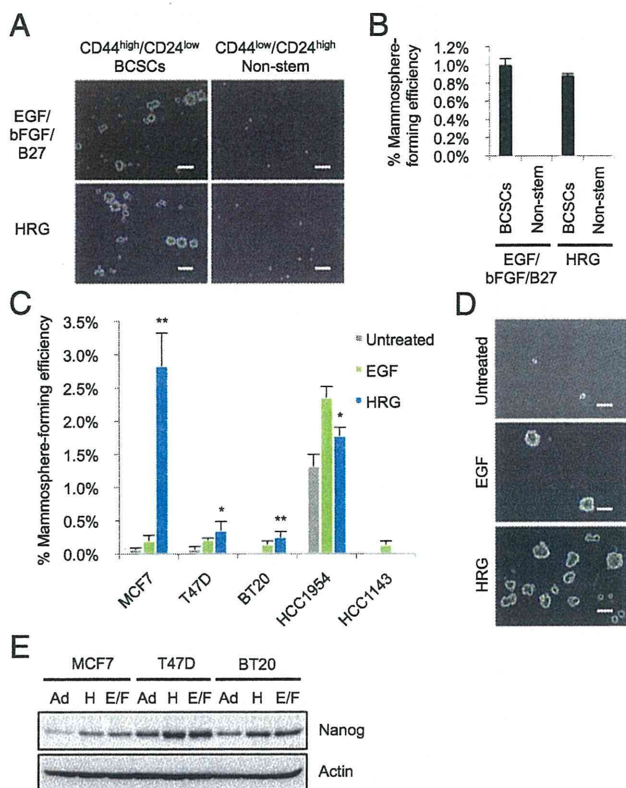

Fig. 1. Effect of HRG on mammosphere formation of a BCSC-enriched population. (*A*) Representative images of primary cultures of mammospheres formed from the sorted $CD44^{high}/CD24^{low}/lineage^{-}$ BCSC-enriched population (*Left*) and the $CD44^{low}/CD24^{high}/lineage^{-}$ nonstem cell population (*Right*) obtained from a specimen of invasive ductal carcinoma (IDC1, Table S1). The cells from IDC1 were incubated with EGF/bFGF/B27 (*Top*) or with 20 ng/mL HRG (*Bottom*). Scale bar = 100 μm. (*B*) The spheres were counted and the percentage of mammosphere-forming cells were determined in each group (data are mean ± SD; $n = 4$). (*C*) Mammosphere assay in MCF7, T47D, BT20, HCC1954, and HCC1143 breast cancer cell lines.treated with 20 ng/mL EGF or 20 ng/mL HRG (data are mean ± SD; $n = 4$, $^{**}P < 0.01$, $^{*}P < 0.05$, relative to the values in the respective untreated controls). (*D*) Images showing mammosphere formation in MCF7 cells treated as indicated in (*C*). Scale bar = 100 μm. (*E*) Nanog protein expression levels in the parental cells growing in 2D adherent (Ad) culture, sphere cells cultured with 20 ng/mL HRG (H) and sphere cells cultured with EGF/bFGF/B27 (E/F).

form mammospheres are enriched in $CD44^{high}/CD24^{low}/lineage^{-}$ cells, confirming that mammospheres can be derived from BCSC-enriched populations as described previously (26, 27).

To examine the effects of HRG on the mammosphere formation, we cultured these cells with HRG in the absence of EGF, bFGF, or B27 supplement and then counted the number of mammospheres that formed. $CD44^{high}/CD24^{low}/lineage^{-}$ BCSC-enriched population cultured with HRG generated mammospheres at similar frequencies as those cultured with EGF/bFGF/B27, whereas $CD44^{low}/CD24^{high}/lineage^{-}$ nonstem cells did not generate mammospheres (Fig. 1 *A* and *B*). These findings suggest that HRG has the ability to induce mammosphere formation of BCSC-enriched population.

To further investigate the effect of HRG, we examined mammosphere formation in five breast cancer cell lines treated with HRG. Similar to the effects of HRG on primary human breast cancer cells, HRG increased mammosphere formation in four of the five breast cancer cell lines (Fig. 1 *C* and *D*) with an efficiency comparable to that of EGF. The HRG-induced mammospheres expressed the stem cell marker Nanog, comparable to mammospheres cultured with EGF/bFGF/B27 (Fig. 1*E*). Together, these results suggest that HRG plays an important role in enhancing the mammosphere formation of BCSC-enriched populations.

HRG Up-Regulates NF-κB Through PI3K/Akt Activation. To examine whether HRG treatment activates the ErbB2/ErbB3 pathway, we investigated the effect of HRG on the phosphorylation levels of ErbB2, ErbB3, ERK, and Akt in three breast cancer cell lines. HRG markedly induced the phosphorylation of ErbB2, ErbB3, ERK, and Akt (Fig. 2*A*) in all three cell lines, suggesting that HRG strongly activates ErbB2 and ErbB3, which leads to the activation of ERK and the PI3K/Akt pathway. To confirm that HRG promotes the interaction between ErbB2 and ErbB3, we performed an immunoprecipitation analysis after treatment with HRG. The analysis revealed that treatment with HRG led to increased interactions between ErbB3 and ErbB2 (Fig. 2*B*).

Because NF-κB is a downstream target of Akt, we investigated whether the NF-κB signaling pathway was also altered by HRG treatment. IKKα/β are the upstream kinases involved in the phosphorylation of IκBα, which leads to the nuclear translocation of NF-κB. Treatment with HRG markedly induced the phosphorylation of Akt and IKKα/β within 10 min and the phosphorylation of IκBα and the NF-κB subunit RELA after 30 min (Fig. 2*C*). To examine the DNA-binding activity of RELA after HRG stimulation, we quantified the intensity of the RELA/DNA complex by ELISA at various time intervals. Treatment with HRG induced a marked increase in the binding activity of RELA after 1 h, and then this activation gradually decreased until 4 h (Fig. 2*D*). To test whether the activation of RELA by HRG was dependent on the PI3K/Akt pathway, we pretreated cells with LY294002, an inhibitor of PI3K before the addition of HRG. As anticipated, the HRG-induced activation of NF-κB was completely inhibited by LY294002 in a manner similar to the inhibition after treatment with DHMEQ, a specific inhibitor of NF-κB (28) (Fig. 2*E* and Fig. S1). These results showed that NF-κB was activated by HRG through the PI3K/Akt pathway. Because our previous observations suggested that the NF-κB pathway is enriched in BCSCs (19), we speculated that the HRG/PI3K/Akt/NF-κB axis may have a role in regulating mammosphere formation.

HRG/PI3K/NF-κB Axis Controls Mammosphere Formation. To elucidate whether NF-κB or PI3K influences HRG-induced mammosphere formation, we treated MCF7 cells with HRG, together with DHMEQ or LY294002. Treatment with DHMEQ or LY294002 decreased the frequency of mammosphere formation in a dose-dependent manner (Fig. 3*A*); however, the sizes of the mammospheres were not significantly changed, suggesting that the activities of NF-κB or PI3K affect mammosphere initiation but do not primarily influence cell proliferation during mammosphere growth. To test secondary mammosphere formation, primary mammospheres generated in the presence of DHMEQ or LY294002 were dissociated into single cells and incubated with HRG in the absence of the inhibitors (Fig. 3*B*). We found that the cells derived from primary mammospheres formed in the presence of DHMEQ or LY294002 did not form secondary mammospheres as efficiently as cells from untreated mammospheres (Fig. 3 *C* and *D*). These findings suggest that the activities of NF-κB and PI3K are required to maintain mammosphere cells with the ability to initiate HRG-induced mammosphere formation. In agreement with these findings, we found that lapatinib, an inhibitor of EGF receptor and ErbB2 tyrosine kinases, decreased NF-κB activity and mammosphere formation (Fig. S2 and *SI Results*). To determine whether DHMEQ or LY294002 induces apoptosis, we stained mammosphere cells with propidium iodide (PI) following inhibitor treatments and then analyzed the cell-cycle status by flow cytometry (Fig. S3). There was no apparent sub-G1 cell population, indicating that

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ORIGINAL ARTICLE

Efficacy and safety of nilotinib in Japanese patients with imatinib-resistant or -intolerant Ph+ CML or relapsed/refractory Ph+ ALL: a 36-month analysis of a phase I and II study

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Abstract Although the tyrosine kinase inhibitor (TKI) imatinib is often used as first-line therapy for newly diagnosed chronic myelogenous leukemia (CML), some patients fail to respond, or become intolerant to imatinib. Nilotinib is a potent and selective second-generation TKI, with confirmed efficacy and tolerability in patients with imatinib-resistant or -intolerant CML. A phase I/II study was conducted in Japanese patients with imatinib-resistant or -intolerant CML or relapsed/refractory Ph+ acute lymphoblastic leukemia. Thirty-four patients were treated with nilotinib for up to 36 months. Major cytogenetic response was achieved in 15/16 patients (93.8%) with chronic-phase CML within a median of approximately 3 months. Major molecular response was achieved in 13/16 patients (81.3%). These responses were sustained at the time of the most recent evaluation in 13 patients and 11 patients, respectively. Hematologic and cytogenetic responses were also observed in patients with advanced CML. The BCR-ABL mutation associated with the most resistance to available TKIs, T315I, was observed in three patients. Common adverse events included rash, nasopharyngitis, leukopenia, neutropenia, thrombocytopenia, nausea, headache and vomiting. Most adverse events resolved following nilotinib dose interruptions/reductions. These results support the favorable long-term efficacy and tolerability of nilotinib in Japanese patients with imatinib-resistant or -intolerant chronic-phase chronic myeloid leukemia.

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This trial is registered at http://www.clinicaltrials.gov, number NCT01279473.

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Keywords Chronic myeloid leukemia · Acute lymphoblastic leukemia · Tyrosine kinase inhibitors · Nilotinib

Introduction

The tyrosine kinase inhibitor (TKI) imatinib (ST1571, Glivec™; Novartis) has been shown to induce durable responses in a high proportion of patients with chronic-phase chronic myeloid leukemia (CML-CP) [1–5]. However, disease progression caused by resistance to imatinib occurs in some CML patients treated with this drug [6].

CML patients in the accelerated phase (CML-AP) or in blast crisis (CML-BC) also show a complete cytogenetic response (CCyR) following treatment with imatinib, but the proportion of such patients achieving CCyR is considerably lower than that of CML-CP patients [7, 8]. Moreover, imatinib resistance and relapse are also common in CML-AP and -BC patients [6, 9]. Imatinib is also used to treat patients with Philadelphia chromosome-positive (Ph+) acute lymphoblastic leukemia (ALL), and many of these patients also achieve CCyR. However, the CCyRs in these patients are not sustained for as long as they are in CML-CP patients, both in Japan [10] and in other countries [11].

Approximately half of the cases of imatinib resistance are now known to result from mutations in *BCR-ABL* [12–16], which make particular leukemic cells resistant to BCR-ABL tyrosine kinase inhibition by imatinib.

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Nilotinib (AMN107, Tasigna®; Novartis) is a second-generation TKI that inhibits BCR-ABL-dependent cell proliferation and induces cell death in BCR-ABL phenotypic cells [17, 18]. Nilotinib was originally approved as second-line treatment for imatinib-resistant or -intolerant CML-CP and -AP patients [19–22]. More recently, it was approved as first-line therapy for CML-CP and -AP patients [23, 24] in Japan. Several studies have reported hematologic response (HR) and cytogenetic response (CyR) with nilotinib in patients with imatinib-resistant or -intolerant CML-BC and those with relapsed/refractory Ph+ ALL [25, 26].

We recently reported the results of a phase I and II study of nilotinib in which Japanese patients with imatinib-resistant or -intolerant Ph+ CML, or relapsed/refractory Ph+ ALL were treated for up to 12 months [22]. Here, we report the effects of treatment with nilotinib for up to 36 months in these patients, as well as the results of mutation analysis and the response by BCR-ABL mutation status.

Materials and methods

Study design and objectives

This was an open-label, multicenter, continuous-dose, 36-month extension of a phase I and II clinical study. The study protocol and documentation were approved by the institutional review boards of each participating center. The observation period was defined to be 36 months, including the entire 3 months of the Ph I/II clinical study. The study was conducted in accordance with the ethical principles established by the Declaration of Helsinki and in compliance with institutional guidelines.

The primary objective of this extension study was to evaluate the long-term safety of nilotinib, including chronic toxicity. Secondary objectives included the long-term efficacy of nilotinib, the relationship between BCR-ABL mutations or BCR-ABL transcript levels determined by quantitative RT-PCR, and the clinical efficacy of nilotinib. The time of last evaluation in this study was the time at which patients had received treatment for more than 3 years or the time at which the drug became commercially available at each of the study institutions, whichever was the later.

Patients

The inclusion and exclusion criteria are described in the original study report [22]. Briefly, Japanese patients were eligible if they had imatinib-resistant or -intolerant CML-CP, CML-AP, CML-BC or relapsed/refractory Ph+ ALL, were at least 20 years of age, had a World Health Organization (WHO) performance status (PS) ≤ 2, and had normal hepatic, renal and cardiac function.

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ORIGINAL ARTICLE

Allogeneic hematopoietic stem cell transplantation for acute myeloid leukemia with t(6;9)(p23;q34) dramatically improves the patient prognosis: a matched-pair analysis

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Acute myeloid leukemia (AML) with t(6;9)(p23;q34) is well known to have a poor prognosis treated with chemotherapy and autotransplantation. The presence of this karyotype is an indicator for allogeneic hematopoietic stem cell transplantation (HSCT); however, the impact of t(6;9)(p23;q34) on the HSCT outcome remains unclear. We conducted a matched-pair analysis of *de novo* AML patients with and without t(6;9)(p23;q34) using data obtained from the Japanese HSCT data registry. A total of 57 patients with t(6;9)(p23;q34) received transplants between 1996 and 2007, and 171 of 2056 normal karyotype patients matched for age, disease status at HSCT and graft source were selected. The overall survival, disease-free survival, cumulative incidence of relapse and the non-relapse mortality in t(6;9)(p23;q34) patients were comparable to those for normal karyotype patients. A univariate analysis showed that t(6;9)(p23;q34) had no significant impact on the overall survival. These findings suggest that allogeneic HSCT may overcome the unfavorable impact of t(6;9)(p23;q34) as an independent prognostic factor.
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Keywords: allogeneic hematopoietic stem cell transplantation; acute myeloid leukemia; unfavorable cytogenetic risk; t(6;9)(p23;q34)

Introduction

Acute myeloid leukemia (AML) is a hematological malignancy resulting from the proliferation of leukemic stem cells. Because of the resistance of leukemic stem cells to chemotherapy,[1] long-term survival is generally seen in only 50% of patients treated with chemotherapy alone. Therefore, allogeneic stem cell transplantation (HSCT) is often considered as a curative treatment option.[2] AML is the most common indication for HSCT in North America and in Japan, but fatal transplant-related adverse events are difficult to avoid, despite the improvements in supportive treatment in recent years. Therefore, treatment of AML is hard to standardize, and the attending physician must make a decision on a case-by-case basis, weighing the advantages and disadvantages of HSCT.

The results of previous large clinical trials have indicated that abnormalities of the chromosomal karyotype are considered to be one of the most powerful factors to predict the patient prognosis.[3,4] AML with the unfavorable cytogenetic risk group, such as a partial deletion of the long arm of chromosome 7 (del (7q)), monosomy of chromosome 7 (−7) or with a complex karyotype is considered to be a good indication for HSCT, even during the first remission, because of the high cytogenetic risk associated with chemotherapy and the beneficial outcome that can be achieved by HSCT.[5–8]

The translocation of chromosome (6;9)(p23;q34) forming the *DEK/NUP214* fusion mRNA is observed in ∼1% of AML cases.[9] The characteristics of AML with t(6;9)(p23;q34) are known to include development at a younger age,[10] resistance to chemotherapy and a very poor prognosis.[9] Therefore, the presence of this karyotype in AML patients is an indication for HSCT; however, the impact of t(6;9)(p23;q34) on the outcome of HSCT remains unclear because of the rarity of this entity. We conducted a retrospective study to examine the outcomes of HSCT in AML patients with t(6;9)(p23;q34) using the data from the Japan Society for Hematopoietic Cell Transplantation Data Registry.

Materials and methods

Study population

Clinical data were collected from the databases of the Japan Society for Hematopoietic Cell Transplantation and the Japan Cord Blood Bank Network using a standardized report form. Follow-up reports were submitted at 100 days, 1 year and annually after HSCT. Patients with *de novo* AML aged 15 years or older at the time of first HSCT and who received the transplant between January 1996 and December 2007 were extracted from the databases. We compared the clinical features and the outcomes among the patients with t(6;9)(p23;q34) and the patients with a normal karyotype in G-band staining. Cytogenetic data were analyzed according to the Southwestern Oncology Group criteria in each institution[7] instead of by central review. We selected patient pairs with t(6;9)(p23;q34)

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and the normal karyotype using an optimal matching method with the following three matching factors: recipient age, disease status at HSCT and graft source. This study was approved by the Committee for Nationwide Survey Data Management of the Japan Society for Hematopoietic Cell Transplantation. Written informed consent was obtained in accordance with the Declaration of Helsinki.

Statistical analysis

The overall survival (OS) was defined as the number of days from HSCT until death from any cause. Disease relapse was defined as the number of days from HSCT to relapse of the underlying disease. Non-relapse mortality was defined as death without relapse. Any patient who was alive at the last-follow-up date was censored. All statistical analyses were performed using the R version 2.13.0 software program (R Foundation for Statistical Computing; http://www.r-project.org). Probabilities and times-to-events were compared between the two groups using the Mantel–Haenszel method and stratified Cox's proportional hazard modeling, respectively. The cumulative incidences of non-relapse mortality and relapse were calculated considering each other event as a competing risk, and were compared using the stratified Grey test.[11] *P* values were two sided, and outcomes were considered to be significant when $P \leqslant 0.05$.

Results

Patients' characteristics

A total of 2577 AML cases met the inclusion criteria. The number of cases with t(6;9)(p23;q34) and a normal karyotype was 57 and 2056, respectively; and 171 patients with the normal karyotype were selected for matched-pair analysis by a 1:3 matching ratio. The characteristics of the patients are shown in Table 1; there were no statistically significant differences between the t(6;9)(p23;q34) patients and the normal karyotype patients except the use of total body irradiation as a preconditioning regimen.

Survival, relapse and non-relapse mortality

The probability of OS in the patients with t(6;9)(p23;q34) was as good as that for patients with a normal karyotype (the probability of 5-year OS in t(6;9)(p23;q34) and normal karyotype patients was 45 and 40%, respectively; Figure 1a). When the t(6;9)(p23;q34) patients and the normal karyotype patients were further categorized according to the disease status at HSCT, the OS of the t(6;9)(p23;q34) patients and the normal karyotype patients were comparable in both the complete remission (CR) at HSCT patients and the non-CR at HSCT patients (Figure 1b). The probability of disease-free survival in these patients was also not significantly different (the probability of 5-year disease-free survival in patients with t(6;9)(p23;q34) and the normal karyotype was 42 and 33%, respectively; Figure 1c). The cumulative incidence of relapse (Figure 2a) and the non-relapse mortality (Figure 2b) in t(6;9)(p23;q34) patients were also comparable to those for normal karyotype patients (the 5-year cumulative incidence was 42% in t(6;9)(p23;q34) patients and 45% in normal karyotype patients for relapse ($P=0.34$) and 16 and 22% ($P=0.85$) for non-relapse mortality). The prognostic factors affecting OS revealed that there were no significant differences related to karyotype, gender, gender mismatch between donor and recipient, human leukocyte antigen disparity, recipient cytomegalovirus serostatus and use of total body irradiation for the preconditioning regimen by the univariate analyses (Table 2).

Table 1 Patient characteristics

	t(6;9)(p23;q34)	*Normal karyotype*	*P-value*
Age			
15–24	14	42	0.999
25–34	14	45	
35–44	20	58	
45–54	7	20	
55–64	2	6	
Gender			
Male	34	97	0.758
Female	23	74	
Disease status at HSCT			
CR1 or CR2	29	87	1.0
Not in remission	28	84	
Preconditioning regimen, TBI			
No	21	33	0.0102
Yes	33	131	
Donor			
Related	26	78	1.0
Unrelated bone marrow	18	54	
Unrelated cord blood	13	39	
Number of HLA mismatch			
0	24	47	0.379
1	5	23	
2	10	27	
3	0	2	

Abbreviations: CR, complete remission; HLA, human leukocyte antigen; HSCT, hematopoietic stem cell transplantation; TBI, total body irradiation.

Discussion

Previous reports have confirmed the negative impact of t(6;9)(p23;q34) on the outcome after standard-dose chemotherapy and high-dose therapy with autologous stem cell transplantation in patients with AML.[9,10] The current matched-pair analysis of the nationwide survey demonstrated that the OS and the non-relapse mortality, as well as the relapse rate, were independent of the presence of t(6;9)(p23;q34) in allogeneic HSCT recipients, thus suggesting that allogeneic HSCT may be able to overcome the unfavorable effect of t(6;9)(p23;q34) in AML patients.

However, it is difficult to draw any firm conclusions regarding the results of the present analysis owing to the small number of patients in the matched-pairs subsets. These findings require confirmation in larger studies specifically in examining the impact of t(6;9)(p23;q34) status. Nevertheless, the suggestion that allogeneic HSCT appears to overcome the adverse survival impact of t(6;9)(p23;q34) is supported by other studies.[12,13] In a EBMT study of AML patients with t(6;9)(p23;q34), allogeneic HSCT produced responses that were independent of t(6;9)(p23;q34), and the 3-year OS of patients with t(6;9)(p23;q34) was as high as $51 \pm 7\%$, comparable to AML patients with the normal karyotype.[13] Also, the incidence of relapse following allogeneic HSCT appeared to be similar in patients with t(6;9)(p23;q34) compared with those without