Brief report

**AML1/RUNXI point mutation possibly promotes leukemic transformation in myeloproliferative neoplasms**

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Myeloproliferative neoplasms (MPNs), including polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF), are clonal hematopoietic stem cell disorders characterized by proliferation of one or more myeloid cell lineages. Some patients exhibit leukemic transformation (LT) by unknown mechanisms, and chemotherapy may increase the risk of LT. To clarify the molecular mechanisms of LT, gene alterations involved in LT from patients in the chronic phase (CP) of MPNs were identified. Among 18 patients who progressed to leukemia, AML1/RUNXI mutations were detected in 5 patients at the LT but in none at the CP. Therefore, we attempted to identify gene alterations involved in LT from patients in the chronic phase (CP) of MPNs. The D171N point mutation resulted in proliferation of immature myeloid cells, enhanced self-renewal capacity, and proliferation of primitive progenitors. Taken together, these results indicate that AML1/RUNXI point mutations may have a leukemogenic potential in MPN stem cells, and they may promote leukemic transformation in MPN. (Blood. 2009;114:5201-5205)

Introduction

Myeloproliferative neoplasms (MPNs), including polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF), are clonal hematopoietic stem cell disorders characterized by proliferation of one or more myeloid cell lineages, and they are associated with the JAK2V617F mutation,1,2 whose detection is used in the differential diagnosis of MPN.3,4 Some patients with MPN exhibit leukemic transformation (LT) after several years of disease,5-7 but the mechanism of LT has been a matter of some controversy because of insufficient insight into the underlying molecular pathogenesis. LT may be a natural sequela of these diseases, whereas treatment with alkylating agents, hydroxycarbamide, or their combination may increase the risk of LT.8-10 Previous studies have reported that chromosomal abnormalities, including −5/5q− and −7/7q−, were frequently detected in patients with MPN at the time of LT,11 suggesting that the development of cytogenetic abnormalities may be associated with the LT of patients with MPN. However, the genes involved in LT still remain obscure. Therefore, we attempted to identify gene alterations involved in LT from patients in the chronic phase (CP) of MPN.

Methods

Patients

Patients with MPN were diagnosed and classified according to the World Health Organization criteria12 at Hiroshima University Hospital between 1985 and 2007. Patients with PV, ET, PMF, MPN-unclassified, and LT of these diseases were examined as approved by the Institutional Review Board at Hiroshima University. Patients gave written informed consent, in accordance with the Declaration of Helsinki.

Genomic DNA and total RNA were prepared as described previously.13 The JAK2V617F mutation was screened using the amplification refractory mutation system assay.3 Identification of AML1, FLT3, N-RAS, c-KIT, PTPN11, and TP53 mutations was performed as described previously.13,14 For CEBA mutation screening by PCR-single strand conformation polymorphism analysis, the following forward/reverse (F/R) oligonucleotides were used: 1F/R, 5′-CATGCCCCGGAGAAGCTTAACTGCTGTGAAG-3′; 2F/R, 5′-ATCGACATCAGCGCCTACATGAGAAG-3′; 3F/R, 5′-GCTGTTGATCAGCAAGCAGGACG-3′; 4F/R, 5′-CCCATGCGGAGTCCAGATCG-3′; 5F/R, 5′-TAGACACGGATACTCGAG-3′.

Retrovirus production

AML1D171N cDNA was subcloned into the pMXs-IRES-GFP (pMXs) retroviral expression vector15 (see Figure 1A). The PLAT-GP packaging cell line was transfected with pMXs.IG and pCMV.DR-IG using FuGene6 (Roche Diagnostics) and cultured in Dulbecco modified eagle medium containing 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine, and 100 units/mL penicillin/streptomycin. Virus-containing culture supernatants were collected after 3 days. CD34+ cells were purified from MPN patients using a CD34 MicroBead Kit (Miltenyi Biotec) and autoMACS (Miltenyi Biotec) according to the manufacturer’s instructions. The cells were precultured for 3 days in MethoCult H4034 medium (StemCell Technologies) containing stem cell factor (SCF), granulocyte/macrophage-colony stimulating factor (GM-CSF), interleukin-3 (IL-3), and erythropoietin. The cells were resuspended in Iscove modified Dulbecco medium containing 20% FBS, 100 ng/mL Fms-related tyrosine kinase 3 (FLT-3) ligand, 20 ng/mL GM-CSF, 100 ng/mL SCF, 100 ng/mL thrombopoietin (all cytokines were from PeproTech), 2 mM L-glutamine, and 100 units/mL penicillin/streptomycin (complete cytokine medium) and added to plates coated with Cin (complete cytokine medium) and added to plates coated with
<table>
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<th>Sex</th>
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MPN indicates myeloproliferative neoplasm; PV, polycythemia vera; ET, essential thrombocytosis; MF, myelofibrosis; PMF, primary myelofibrosis; BUS, busulfan; HU, hydroxyurea; 6-MP, 6-mercaptopurine; IFN-α, interferon-α; VP16, etoposide; MCNU, ranimustine; VD 3, vitamin D 3; PSL, prednisolone; MNL, metenolone; CP, chronic phase; LT, leukemic transformation; ND, not done; +, positive; and -, negative.

*Other mutations include the mutations of AML1, C/EBPα, N-RAS, SHP2, FLR-3, C-KIT, and TP53.
†Missense/deletion mutations in the runt homology domain.
‡Frame-shift mutations in the runt homology domain resulting in truncated forms of AML1.
RetroNectin (Takara) preloaded with virus. Three days after transduction, GFP+ cells were sorted using a FACS Aria (BD Biosciences).

**CFC replating assays**

A total of 10 000 GFP+ sorted cells were resuspended in MethoCult H4034 medium and plated into a 35-mm dish. After 14 days, colonies were counted. Cells were then suspended in Iscove modified Dulbecco medium, and 10 × 10^6 cells were plated again for a colony-forming cell (CFC) replating assay until no colony was observed.

**Long-term cultures on stroma cells**

Aliquots of 10 × 10^5 cells were added to 12-well plates precoated with MS5 stroma cells, which were used as a feeder layer after mitomycin C treatment. Cells were cultured in complete cytokine medium for 5 weeks with weekly one-half medium changes.

**LTC-IC assay**

A total of 10 000 cells were suspended in Myelocult H5100 (StemCell Technologies) containing 12.5% horse serum, 12.5% FBS, 0.2mM inositol, 16μM folic acid, 10−M 2-mercaptoethanol, and 2mM L-glutamine in α-minimum essential medium with 1μM hydrocortisone (StemCell Technologies). The cells were divided into two 35-mm dishes precoated with MS5 stroma cells and cultured for 5 weeks with weekly one-half medium changes. Long-term culture-initiating cell (LTC-IC) cultures were harvested, and clonogenic progenitors were assayed in Methocult GF+ GF435 (StemCell Technologies) methylcellulose medium containing SCF, GM-CSF, IL-3, IL-6, granulocyte-colony stimulating factor, and erythropoietin. After 20 days, LTC-IC–derived CFCs were counted.

**Results and discussion**

A total of 417 patients with MPN (152 PV, 212 ET, 29 PMF, and 24 MPN-unclassified) were evaluated between 1985 and 2007. Eighteen of these patients (4.3%) progressed to leukemia within a minimal follow-up duration of 12 months at our hospital (Table 1). All 14 patients with PV or ET received advanced cytoreductive therapies, including busulfan and/or hydroxycarbamide. The patients with myelofibrosis had been treated with steroids, vitamin D3, and/or interferon-α, and one was treated with etoposide. At LT, 13 of the 18 patients showed additional cytogenetic abnormalities, including −7/7q−, i(17)(q10) related to the TP53 gene, 11q23 rearrangements of the MLL gene, and inv(3)(q21q26) related to the EVI-1 gene. These abnormalities were detected at LT in both JAK2V617F+ and JAK2V617F− MPN patients, raising the possibility that the HSCs may have been transformed into leukemic blasts as a result of gene abnormalities. Furthermore, most of the patients had undergone chemotherapy, suggesting that the leukemic transformation in patients with MPN may be caused in part by gene abnormalities acquired as a result of chemotherapy. Among these gene abnormalities in the MPN patients, we focused on AML1 mutations. The AML1 mutations in the runt homology domain resulted in a loss of the corresponding protein’s ability to bind to DNA.

To clarify the leukemogenic effect of AML1 mutants, the AML1 D171N mutant was transduced into CD34+ cells from patients in the CP of MPN (Figure 1A-C). The effect of this mutant on cell differentiation/proliferation was assessed by CFC replating assays (Figure 1D). Although total colony numbers were comparable, the D171N plates contained fewer erythroid colonies and more myeloid colonies than the control in most experiments, similar to a previous report. More CD34+ cells were retained in the D171N-transduced cells compared with the control (Figure 1E). To evaluate self-renewal capacity, cells from the first colonies were replated. New colonies were detected on all D171N plates but on only 3 control plates (Figure 1D). Moreover, colonies were seen after a third plating of D171N plate no. 1 but not in the controls. During the CFC replating assay, the D171N-transduced cells proliferated more strongly than the controls. These results indicate that the D171N mutant has the potential to increase the myeloid immature cells and to enhance their self-renewal capacity.

The transduced CD34+ cells were next cultured on MS5 stroma cells because it was very difficult to culture the CD34+ cells in liquid media without stroma cells. After 5 weeks, the D171N-transduced cells retained more CD34+ cells than the controls (Figure 1F). Among the CD34+ cells, a small minority of more primitive progenitors/stem cells, known as LTC-ICs, is capable of self-renewal and remains clonogenic after prolonged in vitro culture. The cells transduced with D171N showed significantly more colonies in all 3 LTC-IC experiments (Figure 1G).

A recent study showed that a mouse BMT model transduced with the D171N mutant developed leukemia in concert with EVI-1 overexpression. In the present study, AML1 point mutations were detected with high frequency in patients at the LT from both JAK2V617F+ and JAK2V617F− MPN. Furthermore, the AML1 D171N mutant transduced into CD34+ cells from MPN patients promoted proliferation of primitive progenitors, that is, leukemic stem cells. These results indicate that AML1 point mutations may have a leukemogenic potential in JAK2V617+ stem cells or in pre-JAK2 stem cells, and they may promote leukemic transformation in MPN.
Figure 1. Retroviral transduction of CD34+ cells from patients with MPN. (A) pMXs.IG retroviral construct to express AML1 D171N. LTR indicates long terminal repeat. The FLAG-tagged D171N mutant was inserted into a retrovirus vector plasmid that encoded IRES-GFP. (B) Transduction of the AML1 D171N mutant into CD34+ cells obtained from patients with MPN. CD34+ cells were isolated from 8 patients with PV (nos. 1 and 2), ET (no. 3), PMF (no. 4), MPN-unclassified (nos. 5 and 6), post-PV MF (no. 7), and post-ET MF (no. 8). The cells were transduced either with a control vector (pMXs.IG) or with the AML1 mutant (D171N). A typical flow cytometric profile of cells transduced with either pMXs.IG or D171N retrovirus. The efficiency of retrovirus transduction was 30% to 50%. The GFP+ cells shown within the gate were collected. (C) Anti-FLAG immunoblotting of sorted GFP+ cells to confirm the expression of FLAG-tagged AML1 D171N protein. (D) Colony number and cell proliferation from CFC replating assays. The experiment was performed twice for patient nos. 6 and 7 and once for other patients. Shown are representative data for 8 of 13 patients. After 14 days of culture in methylcellulose, plates of cells from the patients with PV, ET, and PMF (nos. 1-4) contained more than 100 colonies, whereas those from patients with MPN-unclassified and post-PV/ET MF (nos. 5-8) contained only 1 to 80 colonies. Note that the y-axis scales vary. BFU-E indicates burst-forming unit-erythroid; CFU-GM, colony-forming unit-granulocyte, macrophage; GEMM, colony-forming unit-granulocyte, erythroid, macrophage, megakaryocyte. (E) Percentage of CD34+ cells in total cells recovered from the first CFC plates 14 days later. (F) Percentage of CD34+ cells in the cells cultured on MS5 stroma cells for 5 weeks. MS5 cells were excluded by flow cytometry gating. (G) The retrovirus-transduced and sorted cells were cultured on MS5 cells for 5 weeks and subjected to subsequent CFC assays. LTC-IC assays were carried out in triplicate, and the average numbers of LTC-ICs per 10 000 original input cells and SDs are indicated.
Acknowledgments

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Authorship

Contribution: Y.D. collected data and wrote the paper; Y.H. assembled and analyzed the data and revised the manuscript; J.I. collected data; A.K. provided patients’ samples; and H.H. designed the research, interpreted the data, and revised the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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References

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