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Molecular Mechanisms That Produce Secondary MDS/AML by *RUNX1/AML1* Point Mutations

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ABSTRACT

RUNX1/AML1 point mutations have been identified in myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) patients. A heterozygous germline mutation of the *RUNX1* gene causes a familial platelet disorder with a predisposition to AML. *RUNX1* mutations have also been detected with high frequency in minimally differentiated AML M0 subtypes and myelodysplastic/myeloproliferative neoplasms. Here we propose a new disease category of myelodysplastic neoplasms (MDN) consisting of MDS refractory anemia with excess blasts and AML with myelodysplasia-related changes, including therapy-related cases. *RUNX1* mutations have been detected in about 20% of patients with ''MDN''. Among the MDN cases, histories of radiation exposure, therapy-related myeloid neoplasms after successful treatment for acute promyelocytic leukemia, and leukemic transformation of myeloproliferative neoplasms have been reported to have a strong association with *RUNX1* mutations. The mutations occur in a normal, a receptive, or a disease-committed hematopoietic stem cell. It is suspected that the ''MDN'' phenotypes are defined by the *RUNX1* mutations in addition to some other abnormalities. J. Cell. Biochem. 112: 425–432, 2011. © 2010 Wiley-Liss, Inc.

KEY WORDS: RUNX1/AML1; MYELODYSPLASTIC NEOPLASMS; MOLECULAR MECHANISM; THERAPY-RELATED MYELOID NEOPLASMS

RUNX1/AML1 point mutations have been identified in myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) since the first report in 1999 [Osato et al., 1999]. Most of the mutants lose *trans*-activation potential leading to a loss of normal function, indicating that RUNX1 dysfunction is one of the major pathogenic mechanisms of MDS and AML [Harada et al., 2003, 2004]. Some types of RUNX1 mutants show a dominant-negative effect on the *trans*-activation activity, suggesting that they may have some oncogenic potential in addition to the loss of normal function. Biological analysis using a mouse bone marrow transplantation model and human $CD34^+$ cells transduced with RUNX1 mutants has confirmed the oncogenic ability of RUNX1 mutants [Watanabe-Okochi et al., 2008; Harada and Harada, 2009]. These data suggest that RUNX1 mutants are factors that initiate MDS-genesis by inhibiting differentiation of hematopoietic stem cells (HSC). One type of RUNX1 mutants requires that cells acquire the ability to proliferate, while another type may induce proliferation directly. Thus, RUNX1 mutants play a central role in the pathogenesis of MDS and AML.

To what disease category do *RUNX1* mutations contribute? In this prospect, we focus on *RUNX1* mutations in patients with ''secondary'' (i.e., radiation-induced, therapy-related, and blastic crisis from chronic phase [CP]) MDS and AML, in which we can assume the onset of the mutations, and we attempt to describe the relationship between *RUNX1* mutations and secondary MDS and AML.

MYELOID NEOPLASMS CAUSED BY *RUNX1/AML1* POINT MUTATIONS

RUNX1/AML1 point mutations have been reported in various myeloid neoplasms (Fig. 1). A heterozygous germline mutation of the *RUNX1* gene is known to cause familial platelet disorder with a predisposition to AML (FPD/AML) [Song et al., 1999; Osato, 2004], an autosomal dominant disorder characterized by congenital qualitative and quantitative platelet defects and the propensity to develop MDS or AML at a high incidence (20–50%). The affected

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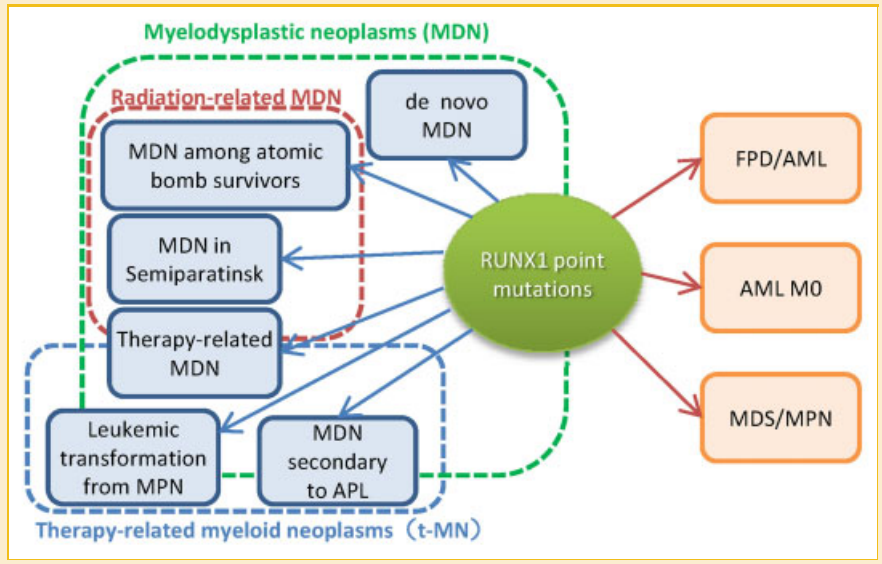

Fig. 1. *RUNX1* mutations in MDN.

individuals may develop leukemia at various times throughout their life span, suggesting that the acquisition of additional mutations is needed to cause leukemia during this long latency period.

It has been demonstrated that *RUNX1* mutations occur at low frequency in de novo AML without myelodysplastic features, but they have been detected in 15–35% of cases of minimally differentiated AML M0 subtypes [Osato, 2004]. Moreover, half of the *RUNX1*-mutated AML M0 cases lost the wild-type *RUNX1*, and de novo AML with *RUNX1* mutation is associated with acquired but not congenital trisomy 21 [Preudhomme et al., 2000]. A significant association between *RUNX1* mutations and activating *FLT3* mutations [Matsuno et al., 2003] and trisomy 13 with increasing *FLT3* expression levels [Dicker et al., 2007] was detected. Furthermore, *RUNX1*-mutated AML M0 is a distinct entity, with expression of both myeloid and B-lymphoid genes [Silva et al., 2009]. Recently, *RUNX1* mutations were identified in 13.2% of de novo non-M3 AML cases [Tang et al., 2009], however, no information was given about their myelodysplasia.

RUNX1 mutations were also reported in 37% of chronic myelomonocytic leukemia (CMML) [Kuo et al., 2009] and 14% of myelodysplastic/myeloproliferative neoplasms (MDS/MPN) [Ernst et al., 2010]. MDS/MPN, including CMML, atypical chronic myeloid leukemia (aCML) and MDS/MPN unclassifiable, are clonal myeloid neoplasms characterized by the simultaneous presence of both myelodysplastic and myeloproliferative features at the time of their initial presentation [Orazi and Germing, 2008]. The molecular pathogenesis of MDS/MPN is only partially understood in the "myeloproliferative" side, but *RUNX1* mutations may explain the molecular mechanism of the "myelodysplastic" side of MDS/MPN. However, the discrimination criteria between MDS/MPN and MDS refractory anemia with excess blasts (RAEB) is unclear.

On the other hand, the frequency of *RUNX1* point mutations in MDS was initially reported to be low [Osato et al., 1999; Song et al., 1999; Imai et al., 2000; Preudhomme et al., 2000], mainly because the MDS patients in those reports comprised many cases with a low blast percentage (<5%). However, subsequent analyses of *RUNX1* gene mutations have indicated that they occur in about 10–20% of patients classified as MDS-RAEB and AML following MDS, and their frequency is substantially higher among radiation-associated (including atomic-bomb survivors) and therapy-related cases [Harada et al., 2003, 2004; Christiansen et al., 2004; Zharlyganova et al., 2008].

THE CONCEPT OF "MYELODYSPLASTIC NEOPLASMS"

MDS is distinguished from AML by the blast threshold, defined as 20% blasts in the blood or bone marrow, according to the World Health Organization (WHO) classification system that takes advantage of morphological, genetic, immunophenotypic, biological, and clinical features to define specific disease entities [Swerdlow et al., 2008]. Unlike the classification for AML, which is based on cytogenetic and genetic abnormalities, the classification for MDS still relies on morphological findings alone, due to unsatisfactory insights into the molecular pathogenesis. Only one category of 5q− syndrome in MDS is well defined, and its molecular mechanisms and appropriate therapies have been investigated in recent years [Boultwood et al., 2010], however, other types of MDS are not established as definite disease categories based on molecular mechanisms. It is necessary to clarify the molecular mechanisms of MDS in order to establish a new classification scheme that would include a characteristic constellation of clinical, genetic, and pathologic findings, similar to AML.

In Japan, most people have a medical examination including blood cell counts every year, as required by their employers, and it is easy for everybody to undergo a blood test because of the full-cover obligatory health insurance system of Japan. Thus, many patients with hematological diseases are diagnosed before they develop apparent subjective symptoms. Patients whose disorders are

diagnosed early usually have fewer blast cells, compared with symptomatic patients, and therefore they are frequently diagnosed as MDS. Because most patients with *RUNX1*-mutated MDS progress to AML, it is suspected that we may analyze a patient in the MDS phase and others may analyze the AML phase of the same myeloid neoplasm.

Among the disease categories of AML according to the WHO classification, a category of ''AML with myelodysplasia-related changes (AML/MLC)'' calls our attention to the biological and clinical importance of MDS-related AML, which is associated with multilineage dysplasia, poor-risk cytogenetic findings, age-dependent increased incidence, and a poor response to therapy [Nimer, 2008]. In contrast to de novo AML without significant myelodysplastic features, MDS-related AML is generally considered similar to MDS-RAEB, which develops as a result of accumulated genetic abnormalities in HSC [Nolte and Hofmann, 2008]. It is suspected that MDS-related AML and MDS-RAEB probably, at least in part, develop via identical molecular mechanisms. Furthermore, a category of ''therapy-related myeloid neoplasms (t-MN)'' is also classified independently. However, gene abnormalities found in therapy-related MDS and AML are also found in sporadic MDS and AML. The frequency of some abnormalities that are sensitive to chemicals is higher in therapy-related cases than in sporadic cases. It seems that the difference between therapy-related cases and spontaneous cases is only that therapy-related cases progress a few steps ahead of spontaneous cases during the stepwise mechanism of myeloid neoplasms. Thus, we think that it is not necessary to consider the molecular mechanisms of these two categories separately, and the molecular mechanism of the therapy-related cases can be applicable to spontaneous cases.

On the basis of these genetic findings, we propose a disease category of ''MDN'' consisting of MDS-RAEB and AML/MLC, including therapy-related cases. *RUNX1* mutations have been detected in about 20% of patients with ''MDN'' in our analysis.

RUNX1 MUTATIONS IN RADIATION-EXPOSED PATIENTS WITH MDN

Hematological diseases among the atomic-bomb survivors in Hiroshima and Nagasaki have been well analyzed [Preston et al., 1994]. Acute and chronic leukemias among atomic-bomb survivors appeared after a minimum latency period of 2–3 years, reached a maximum after 6–7 years, decreased slowly with time and then returned to the background level after 30 years [Kato and Shimizu, 1995]. However, MDS incidence increased after long minimum latency periods of 10 or more years, then continued to increase with time, and is still high, even now, more than 60 years after exposure, having similar kinetics to cancers.

The *RUNX1* gene was reported as a target of gene alteration by ionizing radiation and anticancer drugs in experimental systems [Stanulla et al., 1997; Deininger et al., 1998]. Moreover, human leukemias associated with *RUNX1* gene translocations after anticancer therapy or low-dose radiation have been reported [Roulston et al., 1998; Hromas et al., 2000]. These data prompted us to test the frequency of point mutations in the *RUNX1* gene in patients with hematological malignancies, including atomic-bomb survivors in Hiroshima. We found that the *RUNX1* gene was frequently mutated in MDN patients among atomic-bomb survivors and radiation therapy-related MDN patients [Pedersen-Bjergaard et al., 2002; Harada et al., 2003, 2004]. These studies indicate that exposure to radiation may have an effect on the development of MDN through mutations of the *RUNX1* gene.

We also analyzed gene mutations in MDS patients among nuclear victims around the world. The former Soviet Union's first nuclear bomb test was conducted at the Semipalatinsk Nuclear Test Site (SNTS) in the Republic of Kazakhstan, on August 29, 1949. During the following 40 years, there were 456 nuclear explosions including atmospheric and surface events between 1949 and 1962 [Mikhailov, 1996]. As a result, it is suspected that several hundreds of thousands of residents near the SNTS in Kazakhstan were exposed to radiation due to extensive radioactive contamination from the test site. Considerable efforts have been made to assess the radiation doses and the effect of ionizing radiation on populations residing around the SNTS [Gordeev et al., 2002; Stepanenko et al., 2006], and it is well known that solid cancers and leukemias occur more frequently among residents near the SNTS than in the general population [Abylkassimova et al., 2000; Bauer et al., 2005].

The number of patients with MDN in this area is increasing. Morphology of the bone marrow cells from patients with leukemia in the radiation-affected area is quite strange with strong myelodysplasia. The frequency of *RUNX1* mutations in radiation-exposed patients with MDN among the residents near the SNTS was significantly higher compared with unexposed patients. Furthermore, a significant association between *RUNX1* mutations in MDN patients and individual radiation doses was detected [Zharlyganova et al., 2008]. These results suggested that radiation might contribute to the development of MDS/AML through *RUNX1* mutations among the residents near the SNTS. Considering these results, *RUNX1* point mutations might be a specific biomarker that differentiates radio-induced MDN from spontaneous MDN.

In general, the apparent difference in the pattern of onset between leukemia and MDS may be explained by different molecular mechanisms. Chromosomal translocations caused by double-strand DNA breaks resulting from high-dose radiation are likely to contribute to the development of leukemia after a short latency time, whereas point mutations of genes, especially *RUNX1*, induced by low-dose radiation may contribute to the development of MDS decades later. The reason why atomic-bomb survivors have increased risks of various cancers even 60 years after a single radiation exposure is because radiation-induced mutations required for the initiation of carcinogenesis were presumably recorded in long-lived stem cells in various organs with self-renewal capacity [Langlois et al., 1987; Kyoizumi et al., 1996]. Cytogenetic and molecular findings provide evidence that a model of stepwise genetic progression may explain the development and evolution of MDN [Rosenfeld and List, 2000]. In this model, a primary genetic event incites the initial DNA damage and subsequently increases its susceptibility to further damage. Secondary genetic events promote acquisition of the cytogenetic or molecular-genetic abnormalities common to MDN and precipitate additional abnormalities. Thus, one HSC that acquired a *RUNX1* gene mutation due to radiation

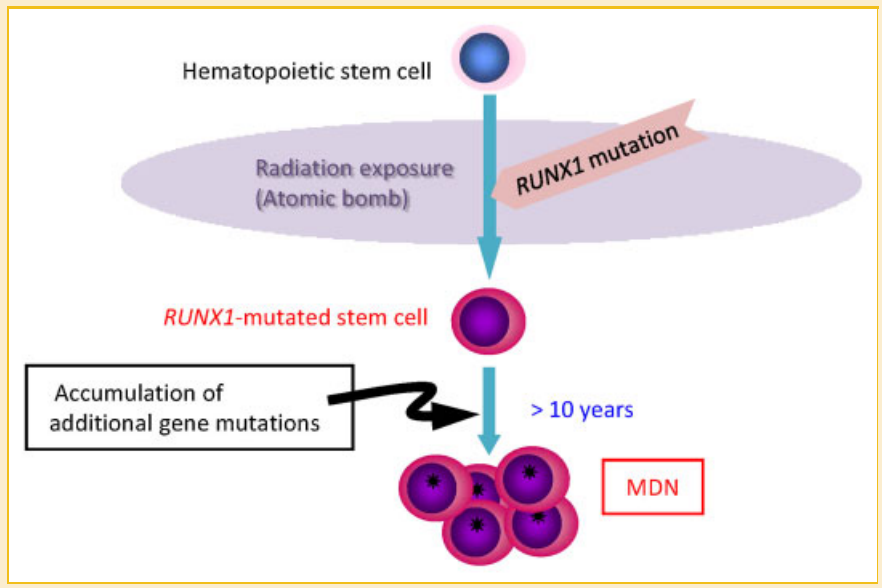

Fig. 2. Molecular mechanisms of radiation-associated MDN.

exposure took decades to be transformed by the accumulation of additional gene alterations, which then led to the development of MDN (Fig. 2).

RUNX1 MUTATIONS IN THERAPY-RELATED MYELOID NEOPLASMS AFTER SUCCESSFUL TREATMENTS FOR ACUTE PROMYELOCYTIC LEUKEMIA

Acute promyelocytic leukemia (APL) is a distinct subtype of AML characterized by a t(15;17) translocation leading to a *PML-RARA* fusion gene. APL is a highly curable disease with excellent complete remission (CR) and long-term survival rates. All-*trans* retinoic acid combined with anthracycline-based chemotherapy yields a CR rate of approximately 90% for newly diagnosed APLs. The relapse rate is approximately 20%, and with the development of new molecular target therapies such as arsenic trioxide, a cure can now be expected even for relapsed patients. However, the development of t-MN is being reported with an increasing frequency of 0.97–6.5% in patients successfully treated for APL [Latagliata et al., 2002; Lobe et al., 2003] and may be more popular than AML other than APL. The t-MN secondary to APL is usually difficult to treat, and it is one of the prognosis-limiting factors for the curable APL disease.

We clarified the different clinical features and hematological findings between t-MN and relapsed APL cases and found that *RUNX1* gene alterations were associated with t-MN [Imagawa et al., 2010]. Among 108 patients during their first CR from APL, 10 patients (9.3%) relapsed and 11 patients (10.2%) developed t-MN after a median follow-up of 8.6 years. It seems that inclusion of VP16 in chemotherapy and the accumulation of chemotherapeutic agents in the maintenance phase may increase the risk of t-MN [Lobe et al., 2003; Asou et al., 2007]. All of the relapse patients had the *PML-RARA* gene, whereas none of the patients with t-MN had *PML-RARA*. Instead, translocations involving 21q22 of *RUNX1* (*RUNX1-MTG16)* or 11q23 of *MLL* (*MLL-FOXO3* and *MLL-CBP*), four *RUNX1* mutations and one *CEBPA* mutation were detected. These abnormalities were not detected at the primary APL diagnosis or in the relapsed patients with APL.

It is assumed that *RUNX1* or other abnormalities may be induced in $CD34^{+}$ cells during chemotherapy resulting in t-MN after successful treatment of APL. *PML-RARA*-negative t-MN may develop from a "receptive" HSC or from a normal HSC, which is a myeloid committed progenitor, by the accumulation of chemotherapy-induced gene abnormalities, including *RUNX1* mutations (Fig. 3).

RUNX1 MUTATIONS IN LEUKEMIC TRANSFORMATION OF MYELOPROLIFERATIVE NEOPLASMS

The mechanisms that produce MDN from MPN are more complicated, as they involve *JAK2*V617F mutations. MPN including polycythemia vera, essential thrombocythemia, and primary myelofibrosis, are clonal HSC disorders characterized by proliferation of one or more myeloid cell lineages, and they are associated with the *JAK2*V617F mutation [James et al., 2005; Kralovics et al., 2005], whose detection is used in the differential diagnosis of MPN [Jones et al., 2005]. Some patients with MPN exhibit leukemic transformation (LT) after several years of disease, and treatment with alkylating agents, hydroxycarbamide, or their combination may increase the risk of LT [Kiladjian et al., 2006]. Recently, gene alterations involved in LT from patients in the CP of MPN have been identified [Ding et al., 2009; Beer et al., 2010]. Among these gene alterations, including translocations and mutations, a high frequency of *RUNX1* mutations was detected in patients at the LT, whereas no mutation was detected in patients at CP.

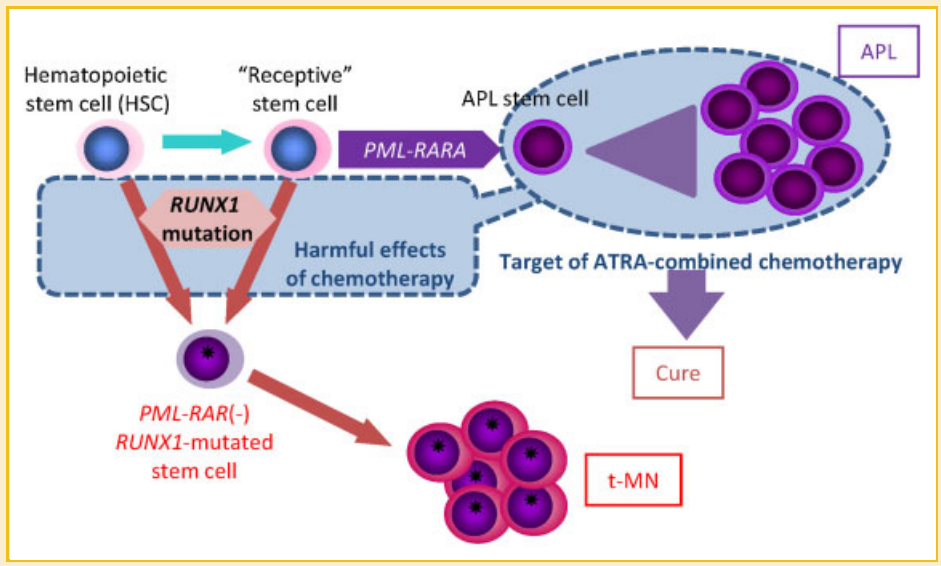

Fig. 3. Molecular mechanisms of t-MN after successful treatment for APL.

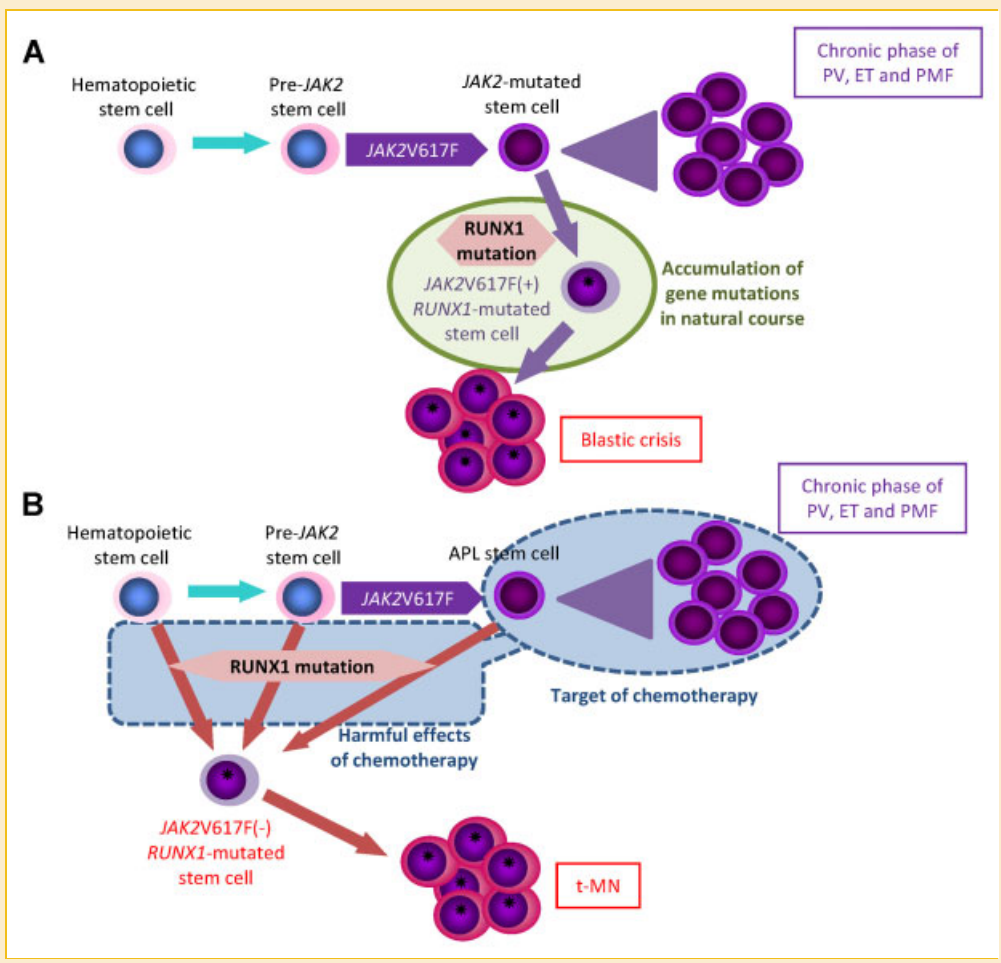

Fig. 4. Molecular mechanisms of LT of myeloproliferative neoplasms (A) blastic crisis-like pathway (B) t-MN pathway.

In contrast to the fact that the *BCR-ABL* fusion gene is retained in all cases of chronic myeloid leukemia (CML) in blast crisis (BC), half of the patients with *JAK2*V617F-positive MPN were reported to transform to *JAK2*V617F-negative AML, suggesting that leukemia developed from a *JAK2*V617F-negative (pre-*JAK2*) HSC [Campbell et al., 2006; Levine and Gilliland, 2008] or normal HSC [Beer et al., 2010]. However, other *JAK2*V617F-positive patients did not lose their *JAK2*V617F mutation during CP to LT, indicating that their leukemia probably arose in *JAK2*V617F-positive HSCs [Ding et al., 2009; Beer et al., 2010]. This hypothesis about origins of LT cells was supported by the recent report of chromosomal abnormalities analyzed by high-resolution single nucleotide polymorphism array [Thoennissen et al., 2010]. Patients with *JAK2*V617F-positive AML showed the same chromosomal alterations, with some additional changes, as those in CP, suggesting that the origin of the LT cells was *JAK2*V617F-positive HSCs. Patients with *JAK2*V617F-negative AML from *JAK2*V617F-positive MPN revealed quite different patterns from their CP, indicating their different origin, but a few common genetic abnormalities were detected between CP and LT, supporting the origin of the pre-*JAK2* HSCs.

RUNX1 mutations were detected at LT in both *JAK2*V617F-positive and -negative MPN patients, raising the possibility that the HSCs may have been transformed into leukemic blasts as a result of *RUNX1* mutations. Furthermore, most of the patients had undergone chemotherapy, suggesting that the LT in patients with MPN may be caused in part by gene abnormalities acquired due to chemotherapy. However, a few patients with *JAK2*V617F-positive MPN who were not treated with chemotherapeutic reagents also transformed to *JAK2*V617F-positive leukemia with *RUNX1* mutation. Thus, there may be another LT pathway that acquires a *RUNX1* mutation in the natural course of MPN. This mechanism is similar to the BC of CML (CML-BC). *RUNX1* rearrangements such as t(3;21) are frequently seen in CML-BC, and a *RUNX1* mutation in CML-BC was also reported [Osato et al., 1999].

To clarify the leukemogenic effect of RUNX1 mutants, the RUNX1 D171N mutant was transduced into $CD34^+$ cells from patients in the CP of MPN [Ding et al., 2009]. The effect of this mutant on cell differentiation/proliferation was assessed by colony-forming cells re-plating assays. The D171N-transduced cells formed fewer erythroid colonies and more myeloid colonies, retained more $CD34^+$ cells, proliferated more strongly than the control, and formed colonies after a third plating. Furthermore, long-term culture-initiating cells, a small minority of more primitive progenitors/stem cells among the $CD34^+$ cells, that have capability of self-renewal and clonogenic capacity after prolonged in vitro culture, increased significantly in the cells transduced with D171N. Thus, the RUNX1 mutant transduced into $CD34^+$ cells from MPN patients promoted proliferation of primitive progenitors, i.e., leukemic stem cells. These results indicate that *RUNX1* mutations may have a leukemogenic potential in a *JAK2*V617-positive HSC, in a pre-*JAK2* HSC, or in a normal HSC, and they may promote LT in MPN (Fig. 4).

CONCLUSION

Our recent study showed that *RUNX1* mutations define the molecular mechanisms of MDN. Once a *RUNX1* mutation occurs in a normal HSC, spontaneous additional gene abnormalities, which may be induced by the *RUNX1* mutation in part, are accumulated in the cell during a long latency period, MDN may then develop. Meanwhile, if a *RUNX1* mutation occurs in a ''receptive'' HSC that has already accumulated other gene abnormalities, the cell develops MDN over a short period. Thus, the *RUNX1* mutation is considered to be one of the disease-deciding factors of MDN, and we strongly propose that *RUNX1* mutations could be one of the genetic classification categories of MDS and AML.

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