Acquisition of loss of the wild-type *NRAS* locus with aggressive disease progression in a patient with juvenile myelomonocytic leukemia and a heterozygous *NRAS* mutation

In a patient with juvenile myelomonocytic leukemia, a *NRAS* mutation at codon 12 (GGT>TGT) was initially heterozygous, but became homozygous after blastic crisis (BC). According to microsatellite and FISH analyses, the post-BC homozygous mutation might result from the loss of the wild-type *NRAS* locus through mitotic recombination.

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Somatic point mutations of the RAS genes are found in approximately 25% of patients with juvenile myelomonocytic leukemia (JMML).[1] Although at present allogeneic hematopoietic stem cell transplantation is the only curative treatment for JMML, high relapse rate is a serious problem. Among variables analyzed, transformation to acute leukemia, often referred to as blastic crisis (BC), at the time of transplantation was associated with increased cumulative incidence of post-transplant relapse.[2] The genetic mechanism underlying BC, however, remains to be determined.

A 2-month old boy was diagnosed as having JMML and treated with 6-mercaptopurine. After 2 years, the laboratory findings revealed a marked leukocytosis, thrombocytopenia, and 43% blasts in bone marrow. The blasts were positive for myeloperoxidase, alpha-naphthyl butylate esterase, and CD13/CD33, indicating progression toward myeloid BC. Three months later, chromosomal analysis of the bone marrow cells showed the advent of monosomy 7. The details of the clinical course have been previously reported.[3]

A single nucleotide substitution at codon 12 of the NRAS gene from GGT to TGT was detected with the concomitant normal sequence in peripheral blood mononuclear cells (PB MNCs) obtained before treatment with 6-mercaptopurine, as shown in Figure 1A. There were no mutations at codons 12, 13, and 61 of the *KRAS2* gene or at exons 3, 8, and 13 of the *PTPN11* gene. However, PB MNCs obtained 5 months after BC showed the homozygous *NRAS* mutation (GGT>TGT). Mutant allele-specific amplification (MASA) analysis was then performed on PB-derived granulocyte-macrophage (GM) colony-constituent cells generated with GM colony-stimulating factor (CSF) and stem cell factor (SCF) in a methylcellulose culture.[4] As shown in Figure 1B, both the mutant PCR product and the wild-type product were detected in all of 11 GM colony-constituent cells before BC. By contrast, only the mutant product was amplified from all of 24 GM colony-constituent cells after BC. Thus, the patient's malignant myeloid lineage cells might initially possess the heterozygous *NRAS* mutation and might subsequently acquire the homozygous *NRAS* mutation. Using microsatellite analyses, we attempted to verify whether the homozygous *NRAS* mutation after BC was due to loss of heterozygosity (LOH) related to the *NRAS* gene according to the procedure previously described.[5] Since there were no polymorphic microsatel-

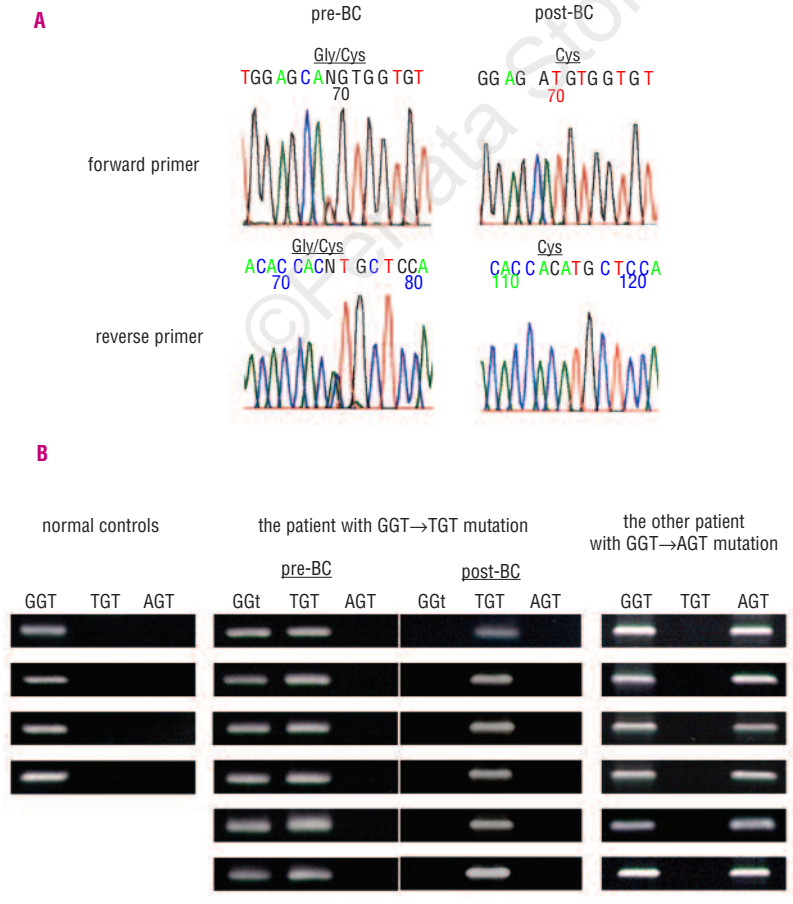


Figure 1. Mutation analyses of the *NRAS* gene (A). The direct sequencing traces showing codon 12 of the *NRAS* gene in PB cells before and after blastic crisis (BC) of the patient. The heterozygous missense mutation was observed before BC, whereas the homozygous mutation was observed after BC. (B) Data show PCR products of the mutated and/or wild-type *NRAS* alleles obtained from PB-MNCs of 4 normal controls, 6 pre-BC GM colony-constituent cells and 6 post-BC GM colony-constituent cells of the present case with codon 12 GGT→TGT mutation, and 6 GM colony-constituent cells of another case with codon 12 GGT→AGT mutation, using the MASA method. This latter case was used to show the reliability of our mutant allele-specific amplification technique. Primers 5'-CTGGTGGTGGTTGGAGCAT-3' and *NRAS-R* (5'-TCCGACAAGTGAGAGACAGG-3'), were used to detect the mutant *NRAS* sequence (GGT→TGT), whereas the primers 5'-TGGTGGTGGTTGGAGCAG-3' and NRAS-R, were used to detect the wild-type *NRAS* sequence. For the other mutant *NRAS* sequence (GGT→AGT), we used the primers 5'-CTGGTGGTGGTTGGAGCAA-3' and NRAS-R. GGT, wild-type allele; TGT, GGT→TGT mutant allele; AGT, GGT→AGT mutant allele.

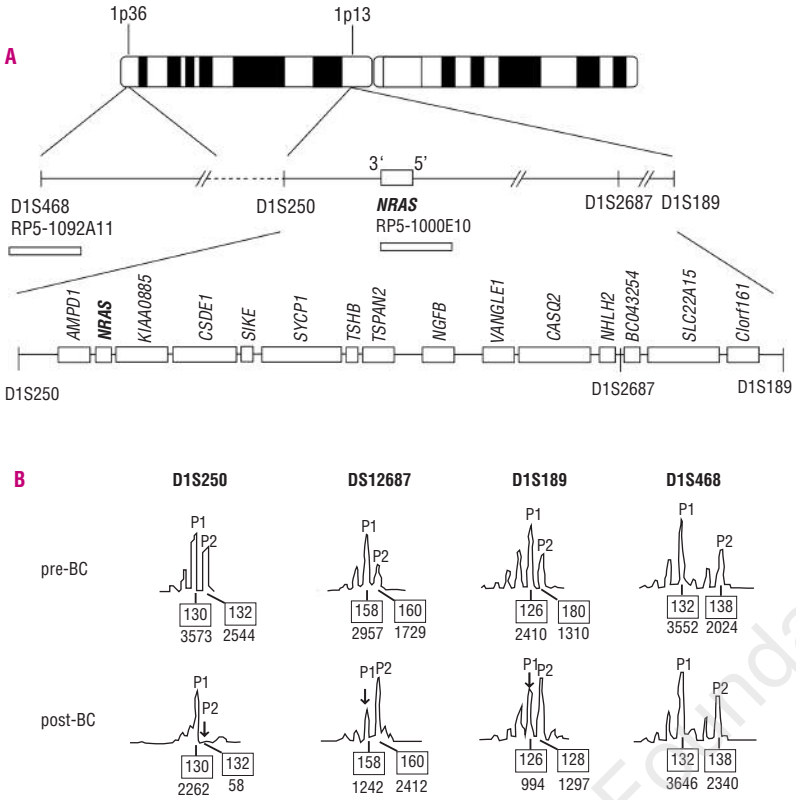
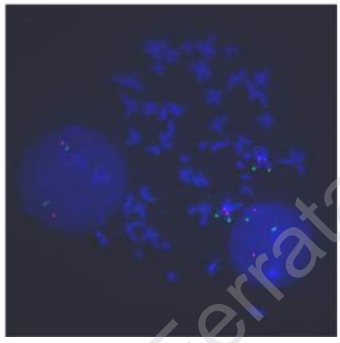


Figure 2. LOH analyses of the NRAS gene. (A) Chromosome 1p showing the markers used for microsatellite PCR analysis and FISH. (B) Analyses of polymorphic microsatellite marker DNAs on the short arm of chromosome 1 We made 9 pairs of pre-blastic crisis (BC) and post-BC GM colony samples (sequences were all determined by the MASA method), and then compared numbers of the dinucleotide repeats in 4 marker DNAs: D1S250, 40kb downstream from the *NRAS* gene; D1S2687 and D1S189, 1 Mb and 1.5 Mb upstream to the gene, respectively; D1S468, 112 Mb downstream from the *NRAS* gene. The forward primers were labeled with a fluorescence dye marker (5-6-carboxyfluorescein). Alleles were defined as the two highest peaks (P1 and P2) within the expected size range. Numbers within the rectangle indicate the size of the short tandem repeat (STR), and numbers below the rectangles indicate the height of the STR peaks. According to the maufacurer's instructions, when the ratio of P1:P2 in the post-BC sample/P1:P2 in the pre-BC sample was either less than 0.67 or greater than 1.35, a LOH was confirmed.



C. FISH analysis using probes for the region containing the NRAS gene on chromosome 1p13 and for the region containing D1S468 on chromosome 1p36 was performed on post-BC GM colony-constituent cells generated with GM-CSF and SCF. Orange signals, RP5-1000E10 encompassing the NRAS gene; green signals, RP5-1092A11 encompassing D1S468. On a metaphase cell, the orange signals were recognized on both chromosome 1 homologs.

lite markers inside the *NRAS* gene, we used three microsatellite markers (D1S250, D1S2687 and D1S189) near the gene on the short arm of chromosome 1p13 (Figure 2A). D1S468, 112 Mb downstream from the *NRAS* gene, was used as a control. We made 9 pairs of pre-BC and post-BC GM colony samples whose sequences were all determined by the MASA method, and then compared numbers of the dinucleotide repeats in the marker DNAs. As shown in Figure 2B, the ratio of P1:P2 in post-BC/P1:P2 in pre-BC of D1S250 was higher than 1.35 (27.25±1.57, mean±SD). Ratios of post-P1:P2/pre-P1:P2 in D1S2687 and D1S189 were less than 0.67 (0.26±0.05 and 0.37±0.04, respectively). By contrast, the ratio of post-P1:P2/pre-P1:P2 in D1S468 was 0.85±0.05. Thus, these results suggest that LOH related to the *NRAS* locus joined the heterozygous *NRAS* mutation after BC. Finally, we examined the mechanism of the LOH. FISH analysis (using probes for the region containing the *NRAS* gene on chromosome 1p13 and for the region containing D1S468 on chromosome 1p36)[6,7] and MASA assay were simultaneously performed on post-BC GM colony-constituent cells generated with GM-CSF and SCF. Thirteen out of 30 GM colonies with the homozygous mutation contained 1-2 metaphase cells per colony. In each of the 23 metaphase cells examined, the signals for RP5-1000E10 encompassing the NRAS gene were found on both chromosome 1 homologs (Figure 2C). These results suggest that the LOH resulted from duplication of the mutated allele through mitotic recombination, rather than from deletion of the NRAS part of wild-type chromosome 1p.

Braun *et al.*[8] reported that somatic activation of a latent $KRAS^{G12D}$ allele rapidly induces a fatal myeloproliferative disorder in mice. However, our recent study revealed hematologic improvement despite no chemotherapy during a 2- to 4-year follow-up in JMML patients with a NRAS or KRAS2 glycine to serine substitution,[9] implying that all types of *RAS* mutations are not always sufficient to confer the disease progression. Given the results of a 7,12-dimethylbenz[a]anthracene-induced rat leukemia model,[10] the two stages of genetic change in our case suggest that oncogenic heterozygous *RAS* mutations are an

early event of leukemogenesis, but in JMML the aggressive development requires other genetic lesions.

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References

1. Lauchle JO, Braun BS, Loh ML, Shannon K. Inherited predispositions and hyperactive Ras in myeloid leukemogenesis. Pediatr Blood Cancer 2006;46:579-85.
2. Locatelli F, Nöllke P, Zecca M, Korthof E, Lanino E, Peters C, et al. Hematopoietic stem cell transplantation (HSCT) in children with juvenile myelomonocytic leukemia (JMML): results of the EWOG-MDS/EBMT trial. Blood 2005;105: 410-9.
3. Nakazawa T, Koike K, Agematsu K, Itoh S, Hagimoto R, Kitazawa Y, et al. Cytogenetic clonality analysis in monosomy 7 associated with juvenile myelomonocytic leukemia: clonality in B and NK cells, but not in T cells. Leuk Res 1998;22:887-92.
4. Sakashita K, Koike K, Kinoshita T, Shiohara M, Kamijo T, Taniguchi S, et al. Dynamic DNA methylation change in the CpG island region of p15 during human myeloid development. J Clin Invest 2001;108:1195-204.
5. Matsuda K, Yamauchi K, Tozuka M, Suzuki T, Sugano M, Hidaka E, et al. Monitoring of hematopoietic chimerism using short tandem repeat, and effect of CD selection upon its sensitivity. Clin Chem 2003;50:2411-4.
6. Kobayashi N, Matsuda K, Sakashita K, Matsuzaki S, Iwasaki R, Koike K. Bilineage acute leukemia of T-lymphoid and myeloid lineages. Haematologica 2004;89:1139-41.
7. Baxter EJ, Scott LM, Campbell PJ, East C, Fourouclas N, Swanton S et al. Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. Lancet 2005; 365:1054-61.
8. Braun BS, Tuveson DA, Kong N, Le DT, Kogan SC, Rozmus J, et al. Somatic activation of oncogenic Kras in hematopoietic cells initiates a rapidly fatal myeloproliferative disorder. Proc Natl Acad Sci USA 2004;101:597-602.
9. Matsuda K, Shimada A, Yoshida N, Ogawa A, Watanabe A, Yajima S, et al. Spontaneous improvement of hematologic abnormalities in patients having juvenile myelomonocytic leukemia with specific RAS mutations. Blood 2007;109:5477-80.
10. Osaka M, Matsuo S, Koh T, Sugiyama T. Loss of heterozygosity at the N-ras locus in 7,12-dimethylbenz[a] anthracene-induced rat leukemia. Mol Carcinog 1997;18: 206-12.