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ONLINE METHODS

Human subjects, mice and cells. The study involving human samples was approved by the Ethics Committee of Hirosaki University Graduate School of Medicine, and all clinical samples were obtained with informed consent. C57BL/6 mice were purchased from CREA Japan. NGF-FKBP-Fas transgenic mice[10] (Jackson Laboratories), *Csf1r*-deficient mice[12] (provided by E.R.S.), PU.1-null (*Sfpi1*$^{-/-}$) and PU.1 conditionally deficient (*Sfpi1* floxed) mice[29] (provided by D.G.T.), CreERT2 knock-in mice (TaconicArtemis GmbH)[30] and MOZ-deficient mice[5] were backcrossed to C57BL/6 mice at least five times. Mouse experiments were performed in a specific pathogen–free environment at the Japan National Cancer Center animal facility according to institutional guidelines and with approval of the Japan National Cancer Center Animal Ethics Committee. PUER cells[20] were provided by H. Singh.

Generation of acute myeloid leukemia mouse models. MSCV-MOZ-TIF2-IRES-EGFP, MSCV-N-Myc-IRES-EGFP, MSCV-CSF1R-pgk-pac and MSCV-PU.1-pgk-pac constructs were generated by inserting cDNAs encoding MOZ-TIF2, N-Myc, CSF1R or PU.1 into the appropriate vector. The constructs were transfected into Plat-E cells[31] cells using the FuGENE 6 reagent (Roche Diagnostics) and supernatants containing retrovirus were collected 48 h after transfection. c-Kit$^+$ cells (1×10^5 cells) were selected from bone marrow or fetal liver cells using CD117-specific MicroBeads (Miltenyi Biotec); the cells were then incubated with retroviruses using RetroNectin (Takara Bio) for 24 h in StemPro-34 serum-free medium (Invitrogen) containing cytokines (20 ng ml^{-1} stem cell factor (PeproTech), 10 ng ml^{-1} interleukin-6 (PeproTech), 10 ng ml^{-1} interleukin-3 (a gift from Kirin Pharmaceuticals)). The infected cells were then transplanted together with bone marrow cells (2×10^5) into lethally irradiated (9 Gy) 6- to 8-week-old C57BL/6 mice by intravenous injection. Secondary transplants were performed by intravenous injection of bone marrow cells from primary AML mice into sublethally irradiated (6 Gy) C57BL/6 mice.

Administration of AP20187, imatinib or Ki20227. AP20187 (a gift from Ariad Pharmaceuticals; 10 mg per kg body weight) was administered daily by intravenous injection for 5 d, and then 1 mg per kg body weight AP20187 was administered every 3 d thereafter as described previously[10]. Mice were orally administered imatinib mesylate (Novartis Pharmaceuticals; 100 mg per kg body weight), Ki20227 (ref. 13) (a gift from Kirin Pharmaceuticals; 20 mg per kg body weight) or solvent twice daily from 7 d after transplantation.

Immunofluorescent staining, detection of side population cells, flow cytometric analysis and cell sorting. Bone marrow cells from mice with AML were preincubated with rat IgG and then incubated on ice with the following staining reagents: antibody to CD115 (AFS98) conjugated to phycoerythrin (PE) (eBioscience), antibody to Mac-1 (M1/70) conjugated to PE-Cy7 (eBioscience), antibody to Gr-1(RB6-8C5) conjugated to allophycocyanin (APC) (BD Pharmingen) and antibody to c-Kit (2B8) conjugated to APC (BD Pharmingen). For the detection of side population cells, bone marrow cells were stained with 5 μg ml^{-1} Hoechst 33342 in the presence or absence of 50 μM verapamil at 37 °C for 60 min. Flow cytometric analysis and cell sorting were performed using the JSAN cell sorter (Baybioscience) and the results were analyzed with FlowJo software (Tree Star).

Reporter analysis. *CSF1R*-luciferase constructs were generated by insertion of *CSF1R* promoter constructs, either wild type or lacking the PU.1-binding site[32], into pGL4.10 (luc2) (Promega). SaOS2 cells (a gift from T. Taya) were transfected with *CSF1R*-luciferase constructs and pGL4.75 (hRL-CMV) (Promega) together with various expression constructs (pLNCX-AML1 (ref. 18), pLNCX-PU.1 (ref. 33), pLNCX-MOZ[18], pLNCX-MOZ-TIF2 (ref. 18) and pLNCX-MOZ-CBP[18]) in 24-well plates, and luciferase activity was assayed 24 h after transfection using the microplate luminometer GLOMAX (Promega). The results shown for the reporter assays represent average values for relative luciferase activity generated from at least three independent experiments; relative values were obtained by normalizing to the luciferase activity of phRL-CMV, which served as an internal control.

Immunoprecipitation and immunoblotting. For Flag tag immunoprecipitation experiments, cells were lysed in a lysis buffer containing 250 mM NaCl, 20 mM sodium phosphate (pH 7.0), 30 mM sodium pyrophosphate, 10 mM NaF, 0.1% NP-40, 5 mM dithiothreitol, 1 mM phenylmethanesulfonylfluoride and Complete protease inhibitor (Roche). Cell lysates were incubated with Flag-specific antibody–conjugated agarose beads (Sigma) and rotated at 10 r.p.m. (TAITEC RT-50) at 4 °C overnight. The adsorbed beads were washed three times with lysis buffer. Precipitated proteins were eluted from the beads by Flag peptide and dissolved with the same volume of 2× SDS sample buffer. When immunoprecipitation was not performed, total protein lysates were prepared in 2× SDS sample buffer. Antibodies were detected by chemiluminescence with ECL plus Detection Reagents (Amersham Biosciences). The primary antibodies used in this study were Flag-specific antibody (M2) (Sigma), hemagglutinin-specific antibody (3F10) (Roche) and MOZ-specific antibody[18], which was generated by immunizing rabbit with peptides corresponding residue 441–460 of human MOZ.

GST pull-down assay. The HindIII-ClaI fragment corresponding to the N-terminal region (1–664) of MOZ was cloned into the pSP64polyA vector. [^{35}S]-MOZ (1–664) was produced by incubating pSP64polyA-MOZ with [^{35}S]-methionine using the TNT Coupled Rabbit Reticulocyte Lysate System (Promega). pGEX-6P-PU.1 and pGEX-6P-AML1 were generated by subcloning full-length human PU.1 and AML1 cDNAs into pGEX-6P (GE Healthcare). GST, GST-PU.1 and GST-AML1 were produced in *Escherichia coli* BL21 containing pGEX-6P, pGEX-6P-PU.1 and pGEX-6P-AML1, respectively. The [^{35}S]-MOZ (1-664) protein was incubated with GST-, GST-PU.1– or GST-AML1–conjugated glutathione-agarose at 4 °C for 60 min in lysis buffer, washed three times with lysis buffer, analyzed by SDS-PAGE and detected by autoradiography.

Statistical analyses. We performed unpaired two-tailed Student's *t* tests for comparisons and a log-rank test for survival data with JMP8 software (SAS Institute).

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ONCOGENOMICS

Array-based genomic resequencing of human leukemia

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To identify oncogenes in leukemias, we performed large-scale resequencing of the leukemia genome using DNA sequence arrays that determine ~9 Mbp of sequence corresponding to the exons or exon–intron boundaries of 5648 protein-coding genes. Hybridization of genomic DNA from CD34-positive blasts of acute myeloid leukemia ($n = 19$) or myeloproliferative disorder ($n = 1$) with the arrays identified 9148 nonsynonymous nucleotide changes. Subsequent analysis showed that most of these changes were also present in the genomic DNA of the paired controls, with 11 somatic changes identified only in the leukemic blasts. One of these latter changes results in a Met-to-Ile substitution at amino-acid position 511 of Janus kinase 3 (JAK3), and the JAK3(M511I) protein exhibited transforming potential both *in vitro* and *in vivo*. Further screening for *JAK3* mutations showed novel and known transforming changes in a total of 9 out of 286 cases of leukemia. Our experiments also showed a somatic change responsible for an Arg-to-His substitution at amino-acid position 882 of DNA methyltransferase 3A, which resulted in a loss of DNA methylation activity of >50%. Our data have thus shown a unique profile of gene mutations in human leukemia.

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Keywords: resequencing; AML; JAK3; DNMT3A

Introduction

Leukemias are clonal disorders of hematopoietic stem cells or immature progenitors. Several subtypes of leukemia are associated with disease-specific karyotype anomalies in the malignant blasts. Most cases of acute promyelocytic leukemia a subtype of acute myeloid leukemia (AML), for instance, are associated with a t(15;17) chromosomal rearrangement that results in the production of the PML-RARA fusion-type oncoprotein (Tallman and Altman, 2008). Similarly, another subtype of AML is associated with a t(8;21) rearrangement, resulting in the production of the oncogenic RUNX1-CBFA2T1 protein (Nimer and Moore, 2004).

The karyotype of leukemic blasts is an important determinant of the long-term prognosis of affected individuals. AML with t(15;17), t(8;21) or inv(16) rearrangements thus constitutes a subgroup of leukemias with a 'favorable' karyotype, with a 5-year survival rate of >60%, whereas AML with an 'adverse' karyotype (monosomy 7, monosomy 5 or complex anomalies) has a 5-year survival rate of only <15% (Grimwade *et al.*, 1998). The prognosis of AML with a normal karyotype (constituting ~50% of all AML cases) is substantially worse than that with a favorable karyotype, with a 5-year survival rate of 24% (Byrd *et al.*, 2002), indicating that blasts with a normal karyotype may contain transforming genes generated as a result of (1) sequence alterations, (2) epigenetic abnormalities or (3) small chromosomal rearrangements not detectable by the G-banding technique. Indeed, several genes, including *NPM1* and *KIT*, have been found to be mutated and activated in AML blasts with a normal karyotype (Schlenk *et al.*, 2008).

The identification of transforming genes in AML will require large-scale resequencing of the blast genome. Although a new generation of sequencing technologies is now available, whole-genome resequencing of many samples remains a demanding task (Bentley *et al.*, 2008; Wheeler *et al.*, 2008). Although DNA microarray-based sequencing is suitable for analysis of multiple samples, currently available platforms are limited in the number of nucleotides that each array is able to probe. To overcome such limitations, we have now applied the extra-large arrays ('wafers') manufactured by Perlegen Sciences (Mountain View, CA, USA) (originally developed for typing of single-nucleotide polymorphisms) (Patil *et al.*, 2001) to

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resequencing of the human genome. Our two-step analysis of human leukemia specimens ($n=20$) has identified a novel transforming mutation in the gene for Janus kinase 3 (JAK3) and a hypomorphic mutation in that for DNA methyltransferase 3A (DNMT3A).

Results

Sequencing strategy

Oligonucleotide probes on the sequencing wafer for the first phase of our study were designed to detect nonsynonymous nucleotide changes in the coding exons of the genome. Intronic sequences (GT in the splicing donor sequence AG-GT and AG in the splicing acceptor sequence AG-G) adjacent to coding exons were also interrogated with the wafer to capture splicing anomalies. Genes examined by the wafer included those known to be mutated in cancer and reported in the catalog of somatic mutations in cancer (COSMIC, http://www.sanger.ac.uk/genetics/cgp/cosmic) as of September 2006 ($n=338$) and those related to the regulation of DNA repair ($n=419$), chromatin structure ($n=299$), redox regulation ($n=102$), epigenetic regulation ($n=44$), cell signaling ($n=2490$), protein kinases ($n=314$), gene transcription ($n=797$), cell cycle ($n=297$), apoptosis ($n=312$), DNA replication ($n=144$) or other functions ($n=92$) (Figure 1a). A total of 5648 genes were thus analyzed with the wafer.

To efficiently isolate oncogenes generated by point mutation using our sequencing array, we selected leukemic blasts with a karyotype characterized by few chromosome anomalies and by few copy number variations of chromosomes, as determined by comparative genomic hybridization with single-nucleotide polymorphism-typing arrays (Supplementary Figure S1). We isolated 15 cases of *de novo* AML, 4 cases of AML that developed from myelodysplastic syndrome, and 1 case of myeloproliferative disorder negative for the JAK2(V617F) and MPL(W515L) mutations (Kralovics *et al.*, 2005; Pikman *et al.*, 2006) (Supplementary Table S1).

From each of these 20 individuals enrolled in the study, we purified immature blasts positive for the surface expression of CD34 (leukemic fraction) as well as a paired control fraction of mature T cells positive for the surface expression of CD4. Although monocytes-macrophages may also express a low level of CD4 at the cell surface, our magnetic bead-based purification system preferentially enriched mature T cells with a high level of CD4 expression; contamination of the mature T-cell fraction with monocytes-macrophages was judged to be $<9\%$ by flow cytometry (Supplementary Figure S2).

Given the potential presence of substantial numbers of unreported single-nucleotide polymorphisms in the human genome, we adopted a two-step analysis to select somatic changes (Figure 1b). In phase I, genomic DNA was isolated from the CD34$^+$ fraction, subjected to mid-range PCR amplification and hybridized with the wafer to examine $\sim$9 Mbp of nucleotide sequence. In phase II, we constructed a smaller wafer to investigate only the

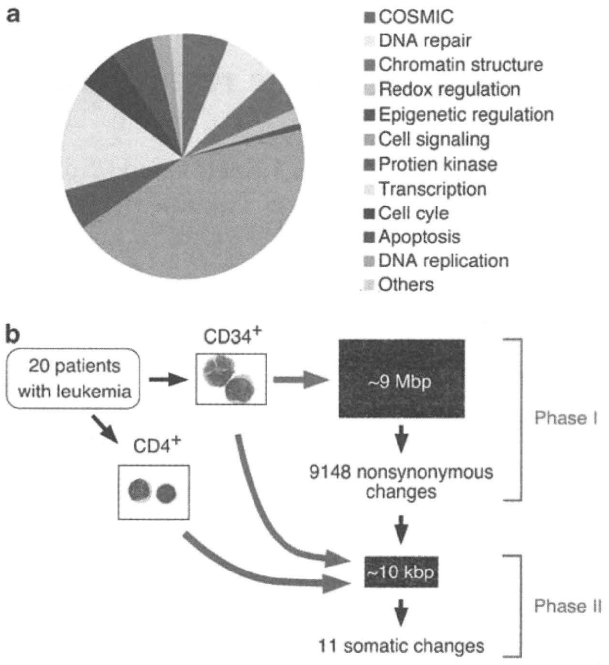

Figure 1 Resequencing of the leukemia genome with wafers. (**a**) Genes interrogated by the phase I wafer ($n=5648$) included those listed in the COSMIC database and those categorized on the basis of function of the encoded protein as indicated. (**b**) CD34$^+$ and CD4$^+$ cell fractions were purified from individuals with leukemia ($n=20$). Genomic DNA of the former fractions was assayed with the phase I wafer including $\sim$9 Mbp of sequence, resulting in the isolation of 9148 nonsynonymous nucleotide changes in 3403 independent genes. The phase II wafer was then constructed to analyze these 9148 changes and was hybridized with genomic DNA from both CD34$^+$ and CD4$^+$ fractions separately. Only 11 mutations were found to be present in the former fraction but not in the latter.

nucleotides shown to be changed in phase I relative to the human reference sequence. Genomic DNA isolated from leukemic blasts and paired control fractions was then analyzed individually with the phase II wafer. We assumed that a nucleotide change was a germline polymorphism if it was observed in both leukemic and control fractions of the same individual, and that it was a somatic mutation if it was observed in the former fraction but not in the latter.

Identification of the JAK3(M511I) mutation

Screening of the leukemic blasts of the 20 individuals for point mutations in phase I yielded 9148 nonsynonymous changes among 3403 independent genes, a frequency similar to that observed in other large-scale resequencing studies performed with capillary sequencers (Sjoblom *et al.*, 2006; Greenman *et al.*, 2007). However, analysis of CD4$^+$ fractions showed that most of these sequence changes were also present in the paired control genome, leaving only 11 nonsynonymous somatic mutations in 11 genes (Supplementary Table S2). Such small number of somatic mutations is in a good agreement with the eight somatic mutations found in AML through whole-genome resequencing using the

Illumina Genome Analyser (Illumina, San Diego, CA, USA) (Ley *et al.*, 2008). All of our 11 somatic changes were confirmed by analysis of both genomic DNA and cDNA of the corresponding specimens with a capillary sequencer (data not shown). These data thus support the necessity of examining paired noncancerous specimens to pinpoint somatic changes in the cancer genome.

One of the gene mutations found only in the $CD34^+$ fractions results in a Met-to-Ile change at amino-acid position 511 of JAK3. A heterozygous *JAK3* mutation responsible for the amino-acid change was confirmed in both genomic DNA and cDNA from the $CD34^+$ fraction, but not in those from the corresponding $CD4^+$ fraction of patient ID JM07 (Supplementary Figure S3), who had *de novo* AML (M1 subtype) and a normal karyotype (Supplementary Table S1). In contrast to JAK2, activating mutations in which are preferentially associated with myeloproliferative disorder, several gain-of-function mutations (such as I87T, P132T, Q501H, A572V, R657Q and V722I) of JAK3 have recently been associated with acute megakaryoblastic leukemia of children (Walters *et al.*, 2006; Sato *et al.*, 2008). Other JAK3 mutations (such as A573V and A593T) were also identified in the same disorder, and an M576L substitution was detected in an adult with acute megakaryocytic leukemia (AML, M7 subtype) (Kiyoi *et al.*, 2007), although the transforming potential of these changes remains unknown.

Given that the M511I mutant of JAK3 has not previously been described and that the relevance of JAK3 to the pathogenesis of adult AML has not been extensively investigated, we first focused on the function of JAK3(M511I). The M511 residue is located in the linker region between the Src homology 2 (SH2) domain and the pseudokinase domain of JAK3 (Figure 2a). The transforming mutation Q501H that is associated with juvenile acute megakaryoblastic leukemia (Sato *et al.*, 2008) is also located in this region. Given that JAK3 is abundant in and has an essential role in the development of lymphocytes (Russell *et al.*, 1995), we examined the expression level of *JAK3* in AML blasts. The gene was expressed at a high level in most AML specimens ($n = 52$), with its expression level being greater than that of *JAK2* in all but three cases (Supplementary Figure S3).

To examine the transforming potential of JAK3(M511I), we introduced the mutant or wild-type protein into the interleukin-3 (IL-3)-dependent mouse cell line 32D (Greenberger *et al.*, 1983). Although 32D cells forced to express wild-type JAK3 underwent rapid apoptosis after withdrawal of IL-3, those expressing JAK3(M511I) continued to grow even in the absence of IL-3, although at a reduced rate compared with that of cells expressing the artificially generated, highly transforming mutant JAK3(V674A) (Choi *et al.*, 2007) (Supplementary Figure S3). 32D cells differentiate into terminal granulocytes in the presence of granulocyte colony-stimulating factor. However, cells expressing the M511I or V674A mutant of JAK3 maintained an exponential rate of growth, without any sign of differentiation, in the presence of granulocyte colony-stimulating factor (Figure 2b, Supplementary Figure S3), supporting the notion that the M511I mutant has transforming potential.

To directly examine the leukemogenic activity of JAK3(M511I), we generated a recombinant retrovirus encoding this mutant and used it to infect murine hematopoietic stem cells. Reconstitution of the bone marrow of lethally irradiated mice with such infected cells resulted in marked lymphocytosis in peripheral blood and enlargement of the spleen in the recipient animals (Figure 2c). The cells in the peripheral blood, spleen and bone marrow of the recipients responsible for these phenotypes manifested a medium-sized, blastic morphology, and flow cytometric analysis revealed them to be $CD8^+$ T cells (Figure 2c). The clonal nature of these proliferating T cells was further confirmed by Southern blot analysis (Supplementary Figure S4), indicative of the development of T-cell acute lymphoblastic leukemia in the recipient mice.

To assess the prevalence of *JAK3* mutations in adult leukemia, we further examined the nucleotide sequence of the entire coding region of *JAK3* cDNA in an additional 266 specimens of leukemic blasts. The coding region of *JAK3* cDNA was successfully amplified by PCR from 83 specimens. We could further identify 4 distinct *JAK3* sequence changes in 8 of these 83 samples: 1 case with G62S, 4 cases with Q501H, 2 cases with R657Q and 1 case with R918C (Figure 2a). Taking into account the 20 cases evaluated in the phase I analysis, we thus identified a total of 9 cases with a mutant form of JAK3 (3.1%) among 286 cases of leukemia (Supplementary Table S3). Our identification of known transforming JAK3 mutants (Q501H and R657Q) originally associated with acute megakaryoblastic leukemia prompted us to determine the prevalence of these two changes in another cohort of AML ($n = 148$), revealing two cases with JAK3(Q501H) and one case with JAK3(R657Q). In addition, analysis of a hematopoietic cell line (KCL22) (Kubonishi and Miyoshi, 1983) established from a patient with chronic myeloid leukemia in BC revealed yet another mutation (L1017M) of JAK3 (Figure 2a).

To directly compare the transforming potential of these various JAK3 mutants, we introduced each protein into the IL-3-dependent mouse B-cell line BA/F3 and examined the growth properties of the resulting transfectants. Whereas all cells expressing the JAK3 mutants proliferated in a similar manner in the presence of IL-3 (data not shown), culture without IL-3 revealed marked differences in the transforming potential among the mutants. JAK3(M511I) was the most efficient oncokinase, with a transforming activity similar to that of JAK3(V674A). The frequent mutants JAK3(Q501H) and JAK3(R657Q) exhibited weaker but still pronounced transforming potential, whereas the remaining mutants (G62S, R918C and L1017M) showed an even lower potential (Figure 2d).

Somatic mutations of DNMT3A

Another somatic mutation identified in the phase II data set was a heterozygous change in *DNMT3A* that results

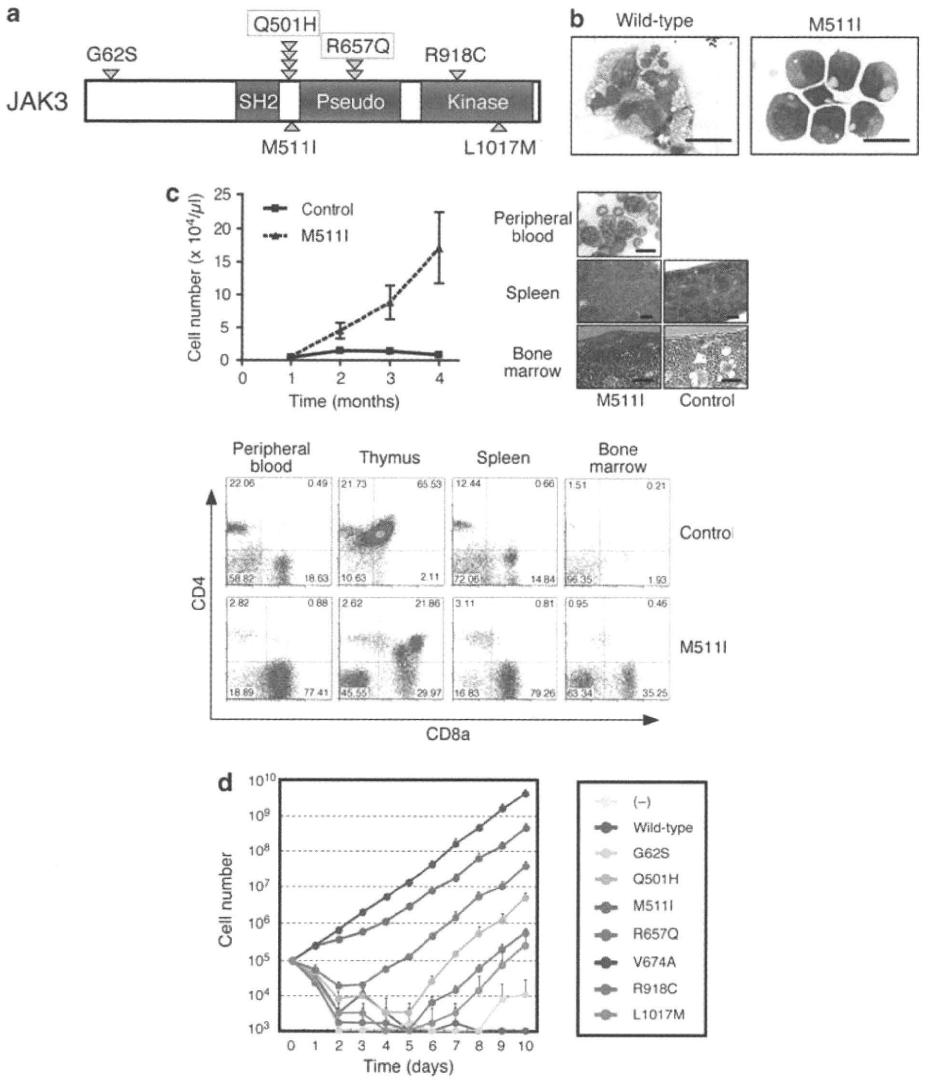

Figure 2 Identification of JAK3 mutants in leukemia. (**a**) Amino-acid substitutions detected in this study are shown relative to the domain organization of JAK3. The mutations M511I (one case) and Q501H (four cases) are located in the linker region between the SH2 and pseudokinase domains of JAK3, whereas G62S (one case), R657Q (two cases) and R918C (one case) are located in the amino-terminal region, the pseudokinase domain and the kinase domain, respectively. The KCL22 cell line also harbors an L1017M mutation within the kinase domain of JAK3. Previously known activating mutations of JAK3 (Q501H and R657Q) are indicated by red rectangles. (**b**) Mouse 32D cells expressing wild-type human JAK3 or the JAK3(M511I) mutant were incubated with G-CSF (0.5 ng/ml) for 14 days, stained with Wright–Giemsa solution and examined by light microscopy. Scale bars, 20 μm. (**c**) C57BL/6 mice were irradiated and then injected intravenously with syngeneic CD34$^-$KSL hematopoietic stem cells infected with a retrovirus encoding JAK3(M511I) or the corresponding empty virus (control). The number of white blood cells in peripheral blood was counted at the indicated times thereafter; data are means ± s.d. for 10 mice in each group (upper left panel). Peripheral blood, spleen and bone marrow isolated from recipient mice 3 months after cell injection were stained with the Wright–Giemsa solution (peripheral blood) or hematoxylin–eosin (spleen and bone marrow) and were then examined by light microscopy (upper right panel); scale bars represent 10, 200 and 50 μm, respectively. Mononuclear cells isolated from peripheral blood, thymus, spleen and bone marrow of recipient mice 3 months after cell injection were subjected to flow cytometric analysis of surface expression of CD4 and CD8a (lower panel). (**d**) Control BA/F3 cells (–) or those expressing the indicated JAK3 mutants were cultured without IL-3 for the indicated times, after which the cell number was determined. Data are means + s.d. of triplicates from a representative experiment.

in an R882H substitution in the encoded protein (Figure 3a, Supplementary Figure S5). DNMT3A, together with DNMT3B, has an essential role in *de novo* methylation of the human genome (Okano *et al.*, 1999), and an aberrant methylation profile (hypermethylation of CpG islands and hypomethylation of other regions) is a hallmark of cancer cells (El-Osta, 2004). Despite a direct linkage between such methylation changes and silencing of tumor-suppressor genes in cancer, the molecular mechanism responsible for such abnormal methylation remains unknown. Our data thus provide the first evidence of somatic mutation of a DNA methyltransferase gene in cancer cells. Mutations in the catalytic domain of DNMT3B have been shown to be

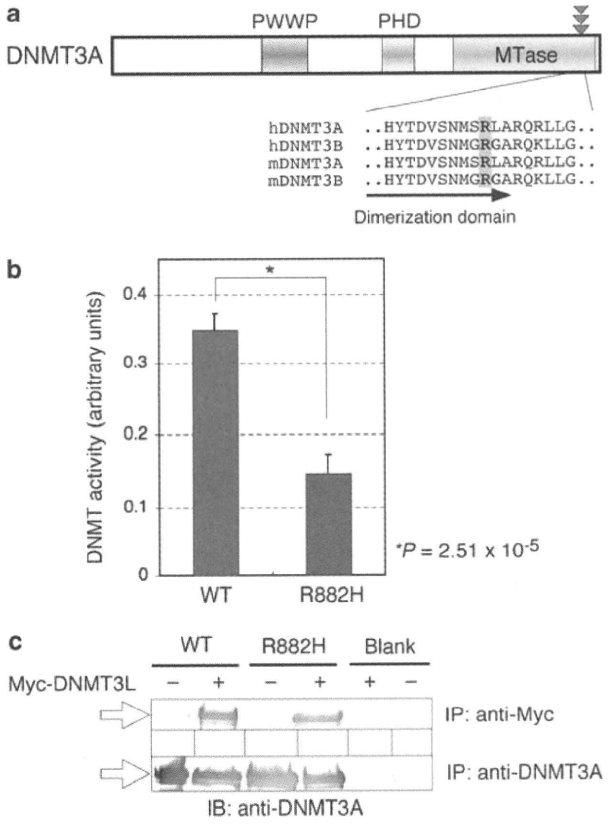

Figure 3 Identification of a DNMT3A mutant in leukemia. (**a**) Domain organization of human DNMT3A showing that the R882 residue found to be mutated in leukemia is conserved among human (h) and mouse (m) members of the DNMT3 family. DNMT3A contains a tetrapeptide PWWP domain, polybromo homology domain (PHD) and methyltransferase (MTase) domain. The R882 residue is located in the homodimerization region present within the MTase domain. (**b**) Wild-type (WT) and R882H forms of DNMT3A were expressed in and purified from insect cells and then subjected to an *in vitro* assay of methyltransferase activity. Data are means + s.d. of triplicates from a representative experiment. The *P*-value was determined by Student's *t*-test. (**c**) Lysates of HEK293 cells expressing Myc epitope-tagged DNMT3L and wild-type or R882H forms of DNMT3A, as indicated, were subjected to immunoprecipitation (IP) with antibodies to Myc or to DNMT3A, and the resulting precipitates were subjected to immunoblot analysis (IB) with antibodies to DNMT3A. The position of DNMT3A (wild-type or mutant) is indicated by an open arrow.

responsible for a hereditary syndrome characterized by ICF (immunodeficiency, instability of the centromeric region of chromosomes and facial anomalies) in humans (Ehrlich, 2003). One of the mutation sites of DNMT3B (R823) associated with the ICF syndrome corresponds to the residue of DNMT3A (R882) shown to be mutated in this study.

The R882 residue of DNMT3A is considered to participate in the homodimerization and activation of the protein (Jia *et al*., 2007) (Figure 3a). To determine whether the R882H mutation affects the catalytic activity of DNMT3A, we expressed mutant and wild-type proteins separately in insect cells, purified them to near homogeneity and subjected them to an *in vitro* assay of methyltransferase activity with a synthetic substrate (Suetake *et al*., 2003). The catalytic activity of DNMT3A(R882H) was $<50\%$ of that of the wild-type protein (Figure 3b). DNMT3L acts as a coactivator for the methyltransferase activity of DNMT3A or DNMT3B through its association with the latter proteins (Jia *et al*., 2007). The R882H mutation did not affect the interaction of DNMT3A with DNMT3L in transfected mammalian cells (Figure 3c) or its sensitivity to DNMT3L as examined by the *in vitro* assay of methyltransferase activity (data not shown). These data thus suggested that the R882H mutation directly inhibits the enzymatic activity of DNMT3A.

Screening of another cohort of leukemia cases ($n = 54$) for mutant forms of *DNMT3A* revealed another two patients with a mutation of the same amino acid (R882H in one patient and R882C in the other) (Supplementary Table S4). Therefore, we identified a total of 3 cases with an R882 mutation (4.1%) among 74 cases of leukemia. Screening for mutations of *DNMT3B* failed to detect any somatic changes in the same individuals (data not shown), suggesting that *DNMT3A* is a preferential target in leukemia.

Multistep transformation in leukemia

Although >99% of nucleotide changes in the phase I data were also observed in the paired $CD4^+$ cells, it is unlikely that all of these changes are actually germline polymorphisms because they include established oncogenic mutations. They thus include 190 nucleotide changes previously described in cancer cells (Supplementary Table S5), such as those giving rise to NRAS(Q61H) in patient ID JM17 and to FLT3(D835Y) in patient ID JM08 (Figure 4a). Given that both NRAS(Q61H) and FLT3(D835Y) are well-characterized oncoproteins (Yamamoto *et al*., 2001), it is unlikely that these individuals harbored such nucleotide changes in the germ line. There are at least two possible explanations for these findings. First, it is possible that purification of the $CD4^+$ fraction was not efficient, with the result that this fraction was contaminated by $CD34^+$ cells. However, the *CD4* expression ratio for the $CD4^+$ and $CD34^+$ fractions of each individual was $\geqslant 17.1$ (median = 40.1) (Figure 4b), and contamination of the $CD4^+$ fraction with $CD34^+$ cells at such a level would not likely produce detectable changes in Sanger sequencing outputs (compare, for instance, the signal intensities of the normal and mutant alleles in Figure 4a).

Furthermore, although CD4 expression has been occasionally observed in AML blasts (Schwonzen *et al*., 2007), quantitation of *CD4* and *CD34* mRNA within our purified $CD34^+$ fractions failed to detect a significant level of the former message in the blasts (Supplementary Figure S6). Therefore, it is unlikely that contamination of $CD4^+$ leukemic blasts within the purified, control $CD4^+$ fraction substantially affected the sequencing results in our phase II experiment.

Rather, it is more likely that leukemia may develop in a stepwise manner with a substantial time interval

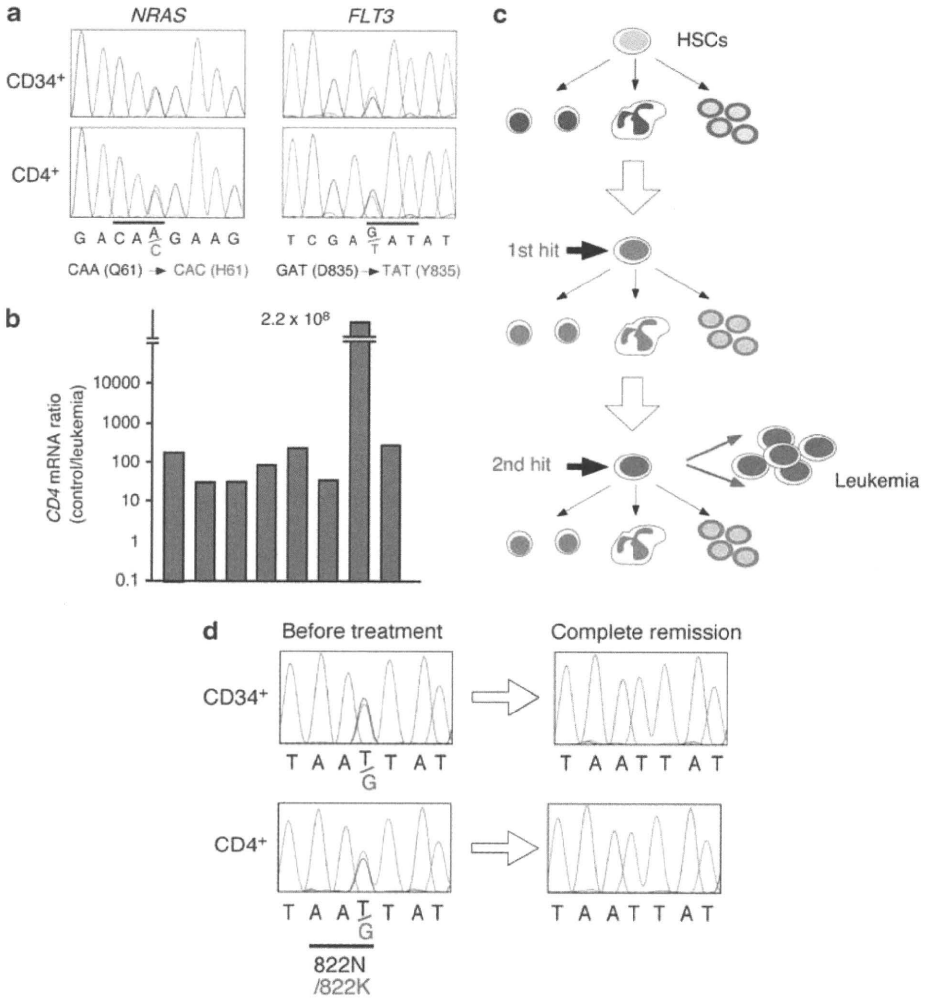

Figure 4 Proposed stepwise nature of leukemogenesis. (**a**) Sequencing electrophoretograms for the regions surrounding codon 61 of *NRAS* or codon 835 of *FLT3* in genomic DNA from the CD34$^+$ and CD4$^+$ fractions of patient IDs JM17 and JM08, respectively. Heterozygous nucleotide changes that give rise to NRAS(Q61H) or FLT3(D835Y) were detected in both fractions of the corresponding patients. (**b**) The amount of *CD4* mRNA in the CD4$^+$ (control) and CD34$^+$ (leukemia) fractions of leukemia patients (with a substantial amount of control *GAPDH* mRNA) was quantitated by reverse transcription and real-time PCR analysis and expressed as the control/leukemia ratio. (**c**) Hematopoietic stem cells (HSCs) give rise to a wide range of mature blood cells. Even after the first hit (mutation) of the genome, HSCs retain their full differentiation capacity, and therefore produce differentiated cells harboring this first hit. After the second hit, the affected cell fraction undergoes full transformation to leukemia. (**d**) Sequencing electrophoretograms for the genome of CD34$^+$ and CD4$^+$ fractions from patient ID JM03 showing a heterozygous mutation for KIT(N822K) before chemotherapy but not after.

between steps (Figure 4c). If a first hit occurs in the genome of hematopoietic stem (or progenitor) cells and if such a somatic change does not result directly in the generation of full-blown leukemia, the preleukemic clones may give rise to terminally differentiated blood cells (including CD4$^+$ cells). After a certain period, a second (or possibly a third) hit occurs in the immature cells and triggers the rapid growth of leukemic clones without differentiation. In such a scenario, terminally differentiated 'normal' cells may still harbor the first hit in their genome.

Support for this latter possibility was provided by patient ID JM03, who had AML (M2 subtype) with a t(8;21) chromosome anomaly. Before chemotherapy, the genomic DNA of both CD34$^+$ and CD4$^+$ fractions from this patient harbored a heterozygous mutation of *KIT* that results in the production of a constitutively activated mutant protein, KIT(N822K) (Shimada *et al.*, 2006) (Figure 4d). The same change was also detected in cDNA prepared from the CD34$^+$ fraction (data not shown). Leukemic blasts in this patient were sensitive to standard chemotherapeutic regimens, and the patient underwent complete remission. Examination of CD34$^+$ and CD4$^+$ fractions obtained during the remission period revealed that the N822K codon change was no longer detectable not only in the CD34$^+$ fraction but also in the CD4$^+$ fraction (Figure 4d). These data thus support the scenario shown in Figure 4c: The N822K

change represents the first hit and was present in differentiated blood cells, and the corresponding pre-leukemic clones were simultaneously eradicated together with the leukemic clones by chemotherapy.

On the other hand, as shown in Supplementary Tables S1 and S2, a heterozygous mutation for NRAS(G12S) was found only in the $CD34^+$ fraction, but not in the $CD4^+$ fraction of the patient ID JM16. Conventional chemotherapy for this patient eradicated the leukemic blasts carrying the mutation (Supplementary Figure S7), also confirming that a successful treatment results in the disappearance of cells with a (possible) 'second hit'.

Our hypothesis of the stepwise leukemogenesis is also consistent with the previous detection of the *RUNX1-CBFA2T1* oncogene in differentiated blood cells (Kwong *et al.*, 1996; Miyamoto *et al.*, 1996, 2000).

Discussion

Our large-scale genomic resequencing of human leukemia specimens with DNA microarrays has identified recurrent nucleotide changes responsible for the generation of JAK3 and DNMT3A mutants. Whereas JAK3 mutants were unexpectedly found in adult AML, their transforming ability, and possibly their contribution to leukemogenesis, varied substantially. However, our bone marrow transplantation experiments showed that at least one of these JAK3 mutants (M511I) directly participates in the development of leukemia. Identification of the M511I mutation of JAK3 in the leukemic fraction but not in the control fraction of patient ID JM07 suggests that this mutation may be the second hit triggering AML. Given that the blasts of this patient had a normal karyotype, it is likely that the first hit is present in the genome of both fractions. Karyotyping of other patients with *JAK3* mutations showed a total of three cases with a normal karyotype, one case with t(8;21), and one case with a numerical anomaly of several chromosomes (Supplementary Table S3), suggesting that *JAK3* mutations may be preferentially associated with leukemia with a normal karyotype.

Although JAK3(M511I) was identified in AML, our bone marrow transplantation experiments with hematopoietic stem cells expressing this mutant yielded T-cell acute lymphoblastic leukemia. In contrast to human leukemia, in which JAK3 changes may constitute a second hit (probably in progenitor cells), JAK3(M511I) may have been expressed in all hematopoietic cells of the recipient mice. JAK3(M511I) thus likely triggered leukemia within a T-cell fraction the intracellular context of which is optimized for JAK3 signaling.

It has been frequently observed that transgenic mouse or bone marrow transplantation experiments for leukemic oncogenes do not accurately recapitulate the original leukemia subtypes (Wong and Witte, 2001). Transgenic mice expressing $p210^{BCR\text{-}ABL1}$, for instance, usually develop T-cell lymphoma or acute lymphoblastic leukemia, not chronic myeloid leukemia. Furthermore, bone marrow transplantation with hematopoietic progenitor cells expressing $p210^{BCR\text{-}ABL1}$ often leads to development of lymphoma, AML, acute lymphoblastic leukemia or macrophage tumors. Generation of malignancy in such systems may, thus, be elaborately influenced by mouse strains, promoter fragments for artificial expression and/or cell types to be used for gene transduction.

Our detection of recurrent DNMT3A hypomorphic mutations in leukemia clones may indicate the presence of an abnormal methylation profile in the genome of such blasts. However, given the limited amount of the specimens available, we were able to investigate microsatellite stability only at certain loci (Koinuma *et al.*, 2005), revealing no apparent microsatellite instability (data not shown). We also generated BA/F3 cells expressing wild-type or R882H forms of DNMT3A to compare the methylation status of some CpG islands in the genome; again, we detected no discernable differences between the two cell preparations (data not shown). However, given that BA/F3 cells contained two copies of wild-type *Dnmt3a* in addition to multiple copies of mutant *DNMT3A*, whereas the leukemic blasts likely harbor one copy each of the wild-type and mutant *DNMT3A* alleles, the clinical relevance of the R882 mutant requires further examination under the latter condition. Cell proliferation/differentiation is indeed influenced substantially by the copy number of *DNMT3* genes (Okano *et al.*, 1999; Ehrlich, 2003).

Our observations indicate the importance of preparing paired normal fractions in large-scale resequencing projects, but they also reveal a difficulty in the preparation of *bona fide* 'normal' fractions in the case of leukemic disorders. Our data thus indicate that nonleukemic blood cells may harbor early genomic hits, rendering them inappropriate as controls. Furthermore, a substantial proportion of fingernail DNA was recently shown to be derived from donor cells among recipients of allogeneic stem cell transplants (Imanishi *et al.*, 2007), indicating that nonblood cells may contain DNA derived from transplanted cells. Therefore, it is possible that buccal, fingernail or even hair cells may not be suitable as normal cell controls. In contrast to solid tumors, for which blood cells are appropriate as paired normal fractions, leukemic disorders require that caution be taken to discriminate somatic nucleotide changes from germline polymorphisms.

Materials and methods

Wafer sequencing

$CD34^+$ and $CD4^+$ fractions were isolated from leukemic individuals using CD34microbeads and CD4microbeads, respectively, and a MidiMACS separator (Miltenyi Biotec, Gladbach, Germany). All clinical specimens were obtained with written informed consent, and the study was approved by the ethics committees of both the Jichi Medical University and the Nagasaki University. DNA sequencing wafers were designed and processed at Perlegen Sciences. Genes to be interrogated on the wafers were selected from the Entrez Gene database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene)

by searching with various keywords characteristic to each subcategory (such as DNA repair, regulation of chromatin structure, etc.), followed by manual inspection. The final gene list for the wafers is shown in Supplementary Table S6. Construction of the wafers, quality control analysis and data processing are described in Supplementary Text.

JAK3 analysis

Complementary DNAs for JAK3 mutants were generated using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) and ligated into the pMX retroviral vector (Onishi *et al.*, 1996). Ecotropic recombinant retroviruses encoding each mutant were produced in BOSC23 cells transfected with the corresponding pMX-based plasmid and were used to infect BA/F3 or 32D cells as described previously (Choi *et al.*, 2007). Both types of cell were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (both from Life Technologies, Carlsbad, CA, USA) and mouse IL-3 (Sigma, St Loius, MO, USA) at 10 Units/ml; differentiation of 32D cells was induced by culture in the presence of serum and mouse granulocyte colony-stimulating factor (Sigma) at 0.5 ng/ml. A concentrated preparation of a retrovirus with a VSV-G envelope and encoding both JAK3(M511I) and enhanced green fluorescent protein was used to infect $CD34^-$ c-Kit^+ $Sca\text{-}1^+$ Lineage-$marker^-$ ($CD34^-$KSL) hematopoietic stem cells isolated from the bone marrow of C57BL/6 mice, and the infected cells were transplanted into lethally irradiated mice congenic for the *Ly5* locus (Iwama *et al.*, 2004). *CD4*, *JAK2* and *JAK3* mRNAs were quantitated by reverse transcription and real-time PCR analysis using an ABI7900HT system (Life Technologies) and with the primers 5′-CTGGAATCCAACATCAAGGTTCTG-3′ and 5′-AATTGTAGAGGAGGCGAACAGGAG-3′ for CD4, 5′-CTCCAGAATCACTGACAGAGAGCA-3′ and 5′-CCAC TCGAAGAGCTAGATCCCTAA-3′ for *JAK2* and 5′-GAGC TCTTCACCTACTGCGACAAA-3′ and 5′-AGCTATGAAA AGGACAGGGAGTGG-3′ for JAK3; the cDNA for GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was also amplified with the primers 5′-GTCAGTGGTGGACC TGACCT-3′ and 5′-TGAGCTTGACAAAGTGGTCG-3′. The relative abundance of the cDNAs of interest was calculated from the threshold cycle (C_T) for each cDNA and that for *GAPDH* cDNA.

DNMT3A analysis

Recombinant His_6-tagged DNMT3A or DNMT3A(R882H) was expressed in SF9 cells using the Bac-to-Bac baculovirus expression system (Invitrogen, Carlsbad, CA, USA), and each protein was purified by stepwise column chromatography as described previously (Suetake *et al.*, 2003). The enzymatic activity of each protein was assayed with -*S*-adenosyl-L-methionine (GE Healthcare, Waukesha, WI, USA) and dIdC or dGdC as substrates (Suetake *et al.*, 2003). The association between Myc epitope-tagged human DNMT3L and wild-type or R882H forms of human DNMT3A in transfected HEK293 cells was examined by immunoprecipitation and immunoblot analyses.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)

Prevalence and prognostic impact of allelic imbalances associated with leukemic transformation of Philadelphia chromosome–negative myeloproliferative neoplasms

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Philadelphia chromosome–negative myeloproliferative neoplasms (MPNs) including polycythemia vera, essential thrombocythemia, and primary myelofibrosis show an inherent tendency for transformation into leukemia (MPN-blast phase), which is hypothesized to be accompanied by acquisition of additional genomic lesions. We, therefore, examined chromosomal abnormalities by high-resolution single nucleotide polymorphism (SNP) array in 88 MPN patients, as well as 71 cases with MPN-blast phase, and correlated these findings with their clinical parameters. Frequent genomic alterations were found in MPN after leukemic transformation with up to 3-fold more genomic changes per sample compared with samples in chronic phase ($P < .001$). We identified commonly altered regions involved in disease progression including not only established targets *(ETV6, TP53,* and *RUNX1)* but also new candidate genes on 7q, 16q, 19p, and 21q. Moreover, trisomy 8 or amplification of 8q24 *(MYC)* was almost exclusively detected in *JAK2V617F*$^-$ cases with MPN-blast phase. Remarkably, copy number–neutral loss of heterozygosity (CNN-LOH) on either 7q or 9p including homozygous *JAK2V617F* was related to decreased survival after leukemic transformation ($P = .01$ and $P = .016$, respectively). Our high-density SNP-array analysis of MPN genomes in the chronic compared with leukemic stage identified novel target genes and provided prognostic insights associated with the evolution to leukemia. (*Blood.* 2010; 115(14):2882-2890)

Introduction

Philadelphia chromosome–negative myeloproliferative neoplasms (MPNs) including polycythemia vera (PV), essential thrombocytosis (ET), and primary myelofibrosis (PMF) are defined as clonal hematopoietic stem cell disorders and characterized by increased proliferation of terminally differentiated myeloid cells. The tyrosine kinase *JAK2* is directly linked to the pathogenesis of MPN with the identification of *JAK2V617F* as a recurring gain-of-function mutation.[1,2] Almost all cases with PV, and roughly 50% of patients with ET and PMF, carry this specific mutation localized on chromosome 9p24.

The long-term outcome of patients with acute myeloid leukemia (AML) secondary to MPN, myelodysplastic syndrome (MDS), or treatment with cytotoxic agents is relatively poor compared with patients with de novo AML. Patients with de novo and secondary AML have a similar spectrum of cytogenetic abnormalities, but the occurrence of cytogenetic changes associated with unfavorable risk such as 5q−, −7/7q−, trisomy 8, or complex karyotype is higher in secondary AML.[3,4] However, so far only a small number of studies with limited number of cases have explored the chromosomal alterations and/or clinical markers associated with acceleration to blast phase of patients with MPN.

Previously, we developed the copy number analyzer for Affymetrix GeneChip (CNAG) program and the new algorithm allele-specific copy number analysis using anonymous references (AsCNAR).[5,6] These techniques in combination with high-density single nucleotide polymorphism (SNP) array provide a robust and

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An Inside *Blood* analysis of this article appears at the front of this issue.

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Table 1. Clinical features of MPN/MPN-blast phase cases (unmatched and matched)

	Unmatched MPN	Unmatched MPN-blast phase	Matched MPN	Matched MPN-blast phase
All cases, no. (%)	77 (56)	60 (44)	11 (50)	11 (50)
MPN diagnosis no. (%)				
PV samples	21 (27)	17 (28)	2 (18)	2 (18)
ET samples	31 (40)	18 (30)	1 (09)	1 (09)
PMF samples	25 (33)	25 (42)	8 (73)	8 (73)
Sex, M:F				
PV samples	1:2	1:1	1:1	1:1
ET samples	1:2	1:1	0:1	0:1
PMF samples	2:1	2:1	2:1	2:1
Mean age at diagnosis, y, ± SD*				
PV	57 ± 5	68 ± 5	—	—
ET	59 ± 6	69 ± 7	—	—
PMF	57 ± 6	65 ± 9	59 ± 9	65 ± 7
Mean blast count in bone marrow, ± SD, no. (%)*				
PV samples	< 5%	70 ± 20	—	—
ET samples	< 5%	66 ± 23	—	—
PMF samples	< 5%	70 ± 21	< 5%	66 ± 24
***JAK2V617F* (+) no. (%)**				
PV samples	21/21 (100)	14/17 (82)‡	2/2 (100)	1/2 (50)
ET samples	18/31 (58)	6/18 (33)	0/1 (0)	0/1 (0)
PMF samples	16/25 (64)	12/25 (48)	5/8 (62.5)	4/8 (50)
***c-MPL* mutation positive, no. (%)**				
PV samples	1/21 (5)†	0/17 (0)	0/2 (0)	0/2 (0)
ET samples	0/31 (0)	1/18 (6)	0/1 (0)	0/1 (0)
PMF samples	3/25 (12)	2/25 (8)	1/8 (12.5)	1/8 (12.5)

MPN indicates myeloproliferative neoplasm; PV, polycythemia vera; ET, essential thrombocytosis; M, male; F, female; and PMF, primary myelofibrosis.

*Data are available for 27 unmatched MPN (10 PV, 10 ET, and 7 PMF) and 54 unmatched MPN-blast phase (15 PV, 18 ET, and 21 PMF) cases, and 8 matched MPN (PMF) cases.

†This *c-MPL* mutation in a PV patient has already been validated and reported by Kawamata et al.[8]

‡Significantly fewer cases with *JAK2V617F* in blast phase vs chronic phase ($P = .045$).

detailed approach to detect large and small copy number changes, as well as copy number–neutral loss of heterozygosity (CNN-LOH). To obtain a comprehensive profile of genomic alterations associated with leukemic transformation in MPN, we applied this interrogational method and performed a systemic analysis of 159 samples obtained from patients either in chronic phase or blast phase of MPN.

Methods

Patients and clinical samples

In total, samples from 148 patients were analyzed by SNP-array. One hundred fifty-nine samples were obtained, of which 88 (55%) were diagnosed with MPN in chronic phase (23 PV, 32 ET, 33 PMF) and 71 (45%), with MPN in blast phase (19 PV, 19 ET, 33 PMF). Diagnosis was based on the World Health Organization criteria,[7] and an overview of patients, including clinical data, is given in Table 1. This study received institutional review board approval from the Cedars-Sinai Medical Center, and informed consent was obtained from all patients in accordance with the Declaration of Helsinki. Given the relatively high incidence of homozygous *JAK2V17F* patients diagnosed with ET (3/18 in chronic phase, 2/6 in blast phase), which is usually lower for this disorder,[9] we suggest that at least some cases diagnosed with ET may have been incorrect.

Samples were provided by (1) Department of Hematology, Mayo Clinic (n = 35); (2) Brigham and Women's Hospital, Harvard University, School of Medicine (n = 46); (3) Department of Hematology, Archet Hospital (n = 44); (4) MLL Munich Leukemia Laboratory, (n = 14); (5) Division of Hematology-Oncology, Chang Gung Memorial Hospital (n = 14); and (6) Division of Hematology, Sheba Medical Center and Sackler School of Medicine, Tel-Aviv University (n = 6).

SNP-Chip analysis

A total of 159 tumor specimens (MPN and/or MPN-blast phase) were analyzed on GeneChip SNP genotyping microarrays (GeneChip Mapping 50K and/or 250K arrays; Affymetrix) as described previously.[5,6] After appropriate normalization of mean array intensities, signal ratios were calculated between tumors and anonymous normal references in an allele-specific manner. Genome-wide determination of allele-specific copy numbers (AsCNs) and detection of CNN-LOH at each SNP were inferred from the observed signal ratios based on the hidden Markov model using CNAG/AsCNAR algorithms (http://www.genome.umin.jp).[5,6] For clustering of patient samples with regard to the status of copy number changes, as well as CNN-LOH, CNAG-Graph software (Tokyo University) was used. Size, position, and location of genes were identified with the University of California, Santa Cruz (UCSC) Genome Browser (http://genome.ucsc.edu)[10] and Ensemble Genome Browser (http://www.ensembl.org).[11] Germline copy number changes previously described as copy number variants at Database of Genomic Variants (http://projects.tcag.ca/variation)[12] and UCSC Genome Browser were excluded. SNP-array data used in this study are available in the Gene Expression Omnibus (GEO) database under accession number GSE19647.[13]

Comparison of 50K versus 250K SNP-Chip analysis in MPN chronic phase

SNP-array analysis of 46 of our MPN samples (10 PV, 20 ET, 16 PMF; kindly provided by D.G.G. at Brigham and Women's Hospital, Harvard University) has already been reported by our group.[8] At that time, only 50K arrays were available, whereas later in this study, the 250K arrays were accessible and used to analyze additionally 42 MPN and 71 MPN-blast phase samples. Because no significant differences in either number of deletions, duplications/amplifications, or CNN-LOH per case were found as analyzed by the 50K compared with 250K array (supplemental Table 1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article), we combined the analysis of both platforms in our

results. Supplemental Table 2 lists all individual samples and the array that was used.

Cytogenetics

Routine cytogenetic analysis with conventional banding techniques was performed in 35 of 88 MPN (10/23 PV, 10/32 ET, 15/33 PMF) and 63 of 71 MPN-blast phase (15/19 PV, 18/19 ET, 30/33 PMF) cases according to standard procedures as previously described.[14] No routine fluorescent in situ hybridization (FISH) panel was applied, but in some cases, however, FISH analysis was performed to supplement conventional cytogenetic analysis (supplemental Table 2).

Allele-specific PCR for *JAK2V617F* mutation

For the detection of *JAK2V617F,* allele-specific polymerase chain reaction (PCR) was performed according to the previously reported method.[15]

Direct mutation screening

Primers were designed to amplify and sequence coding exons and splice junctions of the following genes: *TET2, c-CBL, TP53,* and *RUNX1.* We screened only the 11 matched samples that showed genomic changes in the particular gene regions. Primer details are available from the corresponding author (N.H.T.).

We evaluated all MPN and MPN-blast phase patients with 1pCNN-LOH for the *MPLW515* mutation (exon 10) by direct sequencing. If no mutation was detected in this cohort, we also screened the other coding exons of the *c-MPL* gene previously shown to be mutated in MPN.[8]

Validation of acquired genomic copy number changes including CNN-LOH

To confirm the somatic origin of genomic copy number changes, quantitative genomic real-time (QG RT)–PCR was performed on the genomic DNA from the hybridized MPN and matched MPN-blast phase samples according to the calculation method described by Weksberg et al.[16] For example, we used primers for the *RUNX1* gene (21q22.12; supplemental Figure 1A) as well as *TET2* gene (4q24; data not shown) and a random region on chromosome 21q21.1 and 4p15.1, respectively, as a reference in patient 121.

Detection of acquired CNN-LOH was also validated by QG RT-PCR and subsequently by nucleotide sequencing. Three SNP sequences (rs919275, rs10854117, and rs10854117) on chromosome 19p in case 36 at diagnosis of PV, as well as at leukemic transformation, were determined (supplemental Figure 1B). The genomic region of each SNP site was amplified, and products were purified and sequenced (supplemental Figure 1C). In addition, we confirmed loss of CNN-LOH on 9p after leukemic transformation in matched case 120 using SNP sequences rs3858029, rs1360461, and rs10818814 on chromosome 9 (data not shown).

Homozygous deletions of *CUTL1* and *SH2B2* (case 138) as well as *PIG-A* (case 121) in both MPN and/or MPN-blast phase samples were also confirmed by QG RT-PCR (supplemental Figure 2). Primers for these experiments will be provided upon request.

Statistical analysis

Wilcoxon rank sum tests were used to assess differences in continuous variables, and categoric variables were assessed using chi-square tests, all with a significance level of $\alpha = .05$. The methods of estimations included the standard deviation ($\pm$ SD) of the sampling distribution. Asterisks shown in the figures indicate significant differences of experimental groups in comparison with the corresponding control condition ($^*P < .05$; $^{**}P < .001$). Survival analysis was performed using the Kaplan-Meier method, and survival curves were compared using the log-rank test.

Results

Lower frequency of *JAK2V617F* and 9p alterations after leukemic transformation

In the present study, we examined 159 samples (88 MPN and 71 MPN-blast phase) from a total of 148 patients. An overview of the clinical features of matched and unmatched cases including sex, age, leukemic blast infiltration, and mutational status (*JAK2V617F, c-MPL*) is provided in Table 1. The sex ratio of male and female patients in chronic phase was 1:2 for PV and ET, whereas after transformation, the ratio was balanced with 1:1. For PMF patients, the male-to-female ratio was 2:1 in both MPN chronic and blast phase.

Overall, the incidence of *JAK2V617F* was almost 20% less in the blast phase compared with the chronic phase for both the matched and unmatched MPN cases (unmatched cases: $P < .05$; Figure 1A). Cases that were negative for *JAK2V617F* were also exclusively negative for 9p duplication, trisomy 9, or 9pCNN-LOH in the chronic as well as leukemic stage of MPN. 9pCNN-LOH was noted approximately 3 to 4 times more often than 9p duplication and/or trisomy 9 in *JAK2V617F*$^+$ MPN cases during either the chronic or blast phase (Figure 1A), but the frequency of 9pCNN-LOH was significantly less in the blast crisis compared with the chronic phase of unmatched PMF and PV patients (supplemental Table 3). In contrast, unmatched ET cases had about the same frequency of 9pCNN-LOH in the chronic phase versus the blast phase of the disease. Furthermore, in the analysis of the 11 matched MPN cases, 7 were positive for *JAK2V617F* (64%), 4 had 9p CNN-LOH (37%), and 1 had 9p duplication (9%) at first diagnosis (Figure 1A). In comparison, 2 of these patients were *JAK2V617F*$^+$ with either trisomy 9 or 9pCNN-LOH during their chronic phase (1 PV, 1 PMF), but no longer had detectable *JAK2V617F* with a normal chromosome 9 after leukemic evolution (Figure 1B).

JAK2V617F mutational status had no impact on time to transformation or survival

In the evaluation of clinical data for MPN-blast phase patients, no significant correlation was noted between the prevalence of *JAK2V617F* at transformation and either age, percentage of leukemic blast cells in the marrow, or pretreatment with alkylating agents and/or hydroxyurea (data not shown). Moreover, we found no statistical association between either time to leukemic transformation or overall survival and the *JAK2V617F* status at transformation in PV, ET, or PMF patients. The overall survival of MPN-blast phase patients with *JAK2V617F* versus blast phase patients without this mutation is provided in Figure 2A ($P = .6$). In addition, with respect to the comparably low frequency of *MPLW515*-positive MPN-blast phase patients (6%), we noted no impact of the *c-MPL* mutational status on either time to transformation (data not shown) or the overall survival in MPN patients who underwent leukemic transformation ($P = .5$; Figure 2B).

However, regardless of the mutational status of MPN-blast phase patients, we noted that the time from diagnosis of MPN to leukemic transformation was significantly shorter in those with pre-existing PMF (median, 58 months) compared with patients with either prior PV (median, 98 months) or ET (median, 110 months; $P = .01$). This earlier transformation resulted in a decreased overall survival from the time of diagnosis of the underlying MPN in leukemic patients with preceding PMF patients compared with preceding PV or ET ($P = .02$; Figure 2C), which is congruent with previously published results.[17]

Increased number of additional genomic changes after leukemic transformation

Altogether, a relatively low number of genomic alterations was found by SNP-array analysis in the chronic phase of the MPN samples (Figure 3A). In contrast, 2 to 3 times more abnormalities per sample were detected after leukemic evolution in both matched and unmatched cases with MPN (both $P < .001$; Figure 3A). We

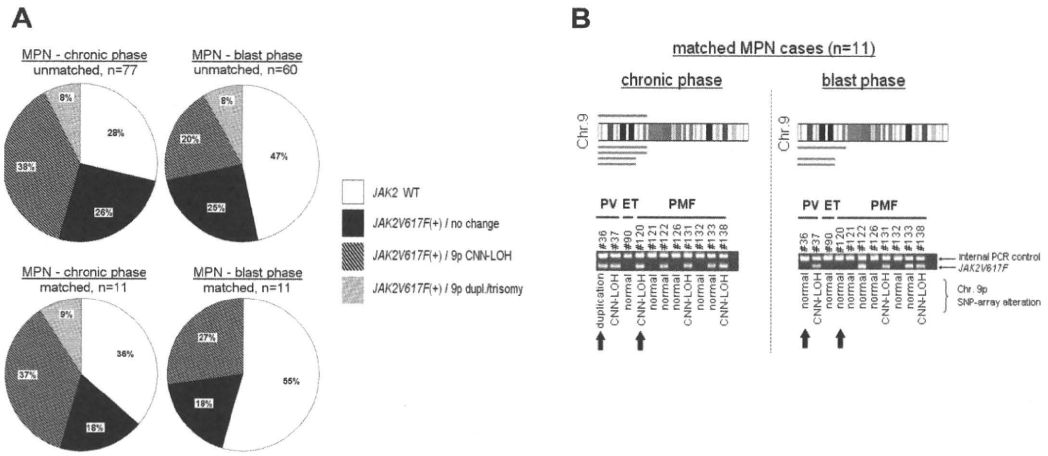

Figure 1. Frequency of *JAK2V617F* and associated alterations on chromosome 9. (A) Diagrams represent matched and unmatched MPN cases in chronic versus blast phase. Indicated are frequencies of *JAK2V617F* and association to 9p duplication (dupl)/trisomy 9 or 9pCNN-LOH. Data and statistical evaluation for underlying MPN subgroups are shown in supplemental Table 3. (B) CNAG software represents duplication (red) and CNN-LOH (green) on 9p detected in 11 patients with matched samples (chronic MPN vs MPN-blast phase). In addition, allele-specific PCR for the detection of *JAK2V617F* was performed in these samples. Arrows indicate 2 MPN patients who were initially positive for *JAK2V617F* in association with 9p imbalances; leukemic transformation was accompanied with loss of *JAK2V617F* and a normal chromosome 9.

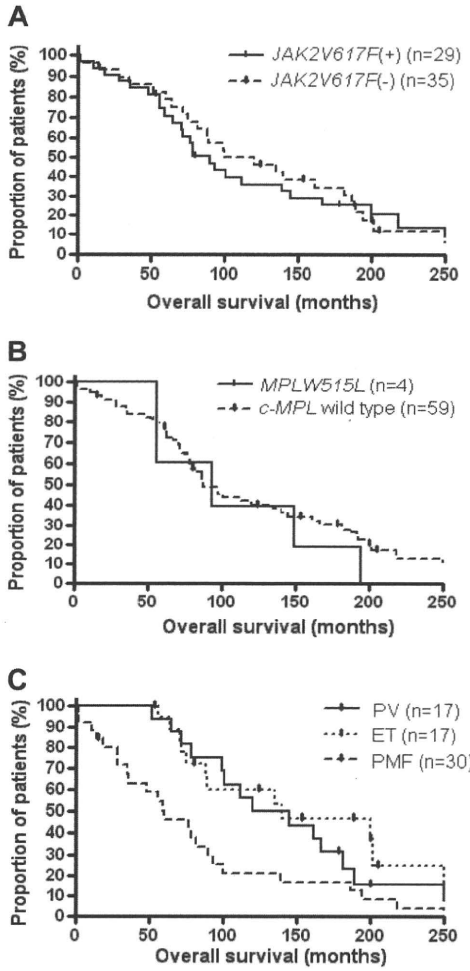

Figure 2. Overall survival of MPN patients with subsequent transformation to blast crisis. Kaplan-Meier plots of all MPN-blast phase patients from the diagnosis of pre-existing MPN were stratified for (A) the presence or absence of a *JAK2V617F* mutation at transformation, (B) the presence or absence of a *MPLW515L* mutation at transformation, and (C) the underlying type of MPN.

found no statistical relationship between the *JAK2V617F* status and the number of genomic changes in matched as well as unmatched samples (data not shown). However, samples from ET patients had fewer copy number changes than those from either PV or PMF patients in the chronic phase, which was highly significant in the unmatched cases ($P < .001$; Figure 3A, supplemental Figure 3A). After leukemic transformation, a similar number of SNP-array changes occurred in cases with prior ET compared with those with pre-existing PV and PMF (unmatched cases: $P = .59$). Statistical evaluation of the matched samples divided into each subentity was not possible because of the small number of cases (Figure 3Aii and supplemental Figure 3B). A subanalysis of the number of either deletions, duplications/amplifications, or CNN-LOH per case, matched and unmatched, is shown in supplemental Figure 3.

Compared with the cytogenetic data, SNP-array analysis detected more than 2-fold of additional chromosomal changes in the MPN samples of either chronic or blast phase, whereas SNP-array practically captured all cytogenetic abnormalities (Figure 3B).

Candidate genes involved in leukemic transformation of MPN patients

SNP-chip analysis detected several additionally altered regions in patients after leukemic evolution compared with the MPN chronic phase in both unmatched (Figure 4; supplemental Figure 4) and matched (Figure 5A) cases. The altered regions included chromosome 8q *(MYC)*, 12p *(ETV6)*, 17p *(TP53)*, and 21q *(RUNX1)*, which are already known to be involved in leukemogenesis.[18-22] Trisomy 8 was detected in 12% of unmatched and 9% of matched cases in MPN-blast phase; interestingly, almost all these samples were negative for *JAK2V617F*. PMF patient 148, who was also *JAK2V617F*$^-$, showed amplification of 8q24.21 in blast crisis involving the *MYC* gene. MPN-blast phase patients with trisomy 8 did not show an inferior outcome compared with cases without this abnormality ($P = .11$; data not shown).

In 20% of unmatched cases in MPN-blast phase, deletions (12%) or CNN-LOH (8%) occurred on chromosome 17 including *TP53* at p13.1. Deletions on the short arm of chromosome 17 were detected significantly often in MPN-blast phase patients who received prior treatment with hydroxyurea with or without the addition of alkylating agents ($P = .035$, Table 2). Supplemental

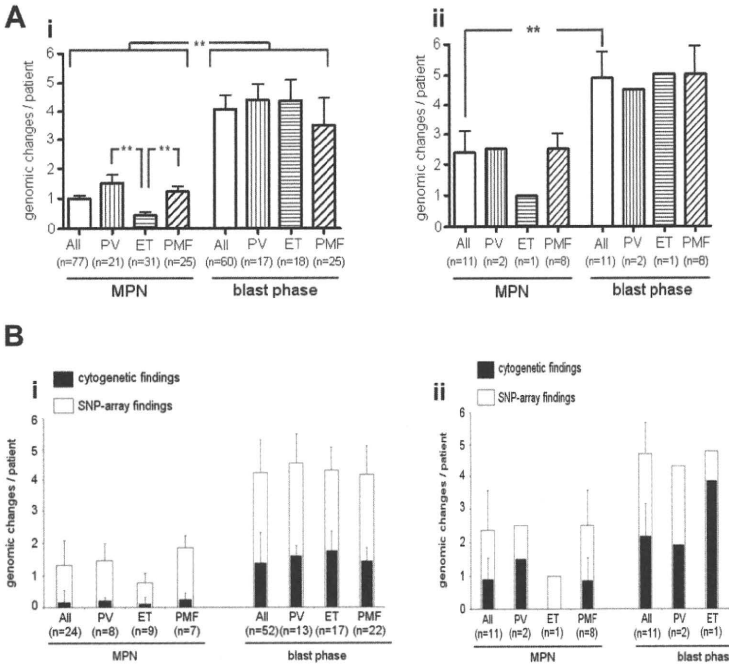

Figure 3. Genomic alterations per MPN patient in chronic versus blast phase. (A) Mean of SNP-array alterations per patient in MPN versus MPN-blast phase with (i) unmatched samples and (ii) matched samples (± SD); $^{**}P < .001$. (B) Mean of SNP-array aberrations compared with cytogenetic alterations per patient in chronic versus blast phase with (i) unmatched samples and (ii) matched samples (± SD).

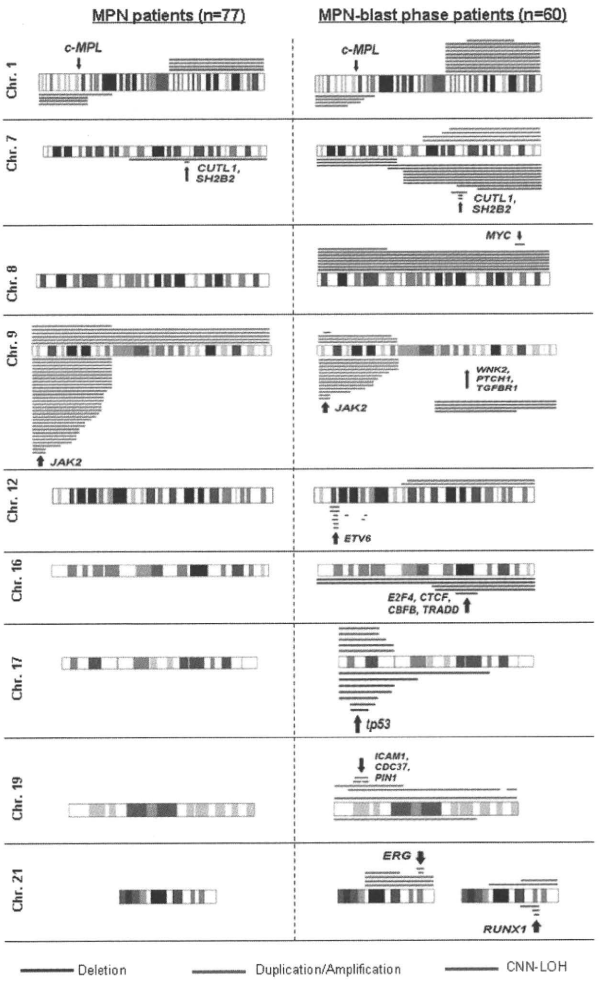

Figure 4. Overview of gains and losses detected by CNAG software. Indicated are the most common altered regions in unmatched MPN-blast phase patients (n = 60; right-sided cytobands) compared with unmatched MPN patients (n = 77; left-sided cytobands). Each line represents 1 sample with either deletion (blue), duplication/amplification (red), or CNN-LOH (green). Candidate genes of the minimal altered regions are highlighted by arrows.

Table 4 indicates pretreatment (hydroxyurea and/or alkylating agents) of 47 MPN-blast phase patients and their individual SNP-array findings. Deletion or CNN-LOH on 17p in unmatched blast phase cases was associated with either complex karyotype or isochromosome 17 ($P = .01$), and significantly decreased survival (with 17p deletion: $P = .012$; with 17p CNN-LOH: $P = .018$). One of the 11 matched MPN samples (case 120) acquired a 17p deletion at diagnosis of blast phase (Figure 5A), resulting in a hemizygous mutant *TP53* (M133K; Figure 5Bi).

On chromosome 21, SNP-chip analysis revealed either deletions or CNN-LOH in 8% of unmatched cases in MPN-blast phase involving the transcription factor *RUNX1* at q22.12. Patient 121 acquired a small deletion of that locus in the leukemic sample (Figure 5A) associated with a mutation of the Runt domain of the *RUNX1* gene on the remaining allele (Q392Stop codon; Figure 5Bii).

Deletion or CNN-LOH on 4q24 spanning the *TET2* gene was detected in 6% of unmatched blast phase cases and 1% in chronic phase. One *TET2* mutation was found by nucleotide sequencing in the matched MPN samples. *JAK2V617F*$^-$ case 121 had no genomicimbalances on 4q at diagnosis of PMF, but acquired a microdeletion (1 Mbp) on 4q24 (*TET2*) after leukemic evolution 1 year later (Figure 5A). The remaining allele had a *TET2* frameshift mutation (N486T; Figure 5Biii), and the mutation was absent in the matched PMF sample.

CNN-LOH involving 11q23.3, which has been shown to be strongly associated with *c-CBL* mutations,[23] had an even lower frequency, with only 2% of unmatched MPN cases in either chronic phase or blast crisis. The *JAK2V617F*$^-$ patient 132 had 11q CNN-LOH with a homozygous *c-CBL* missense mutation (Y371H) in the MPN-blast phase sample. Both the CNN-LOH and the mutation were absent in the corresponding chronic phase, 2 years before disease progression (Figure 5A-Biv).

Besides these already well-known targets, SNP-array analysis detected commonly altered regions on chromosomes 1, 7, 16, 19, and 21 encompassing potentially new candidate genes involved in MPN transformation. These imbalances were either absent or at least very infrequent in the chronic phase of the disease (Figures

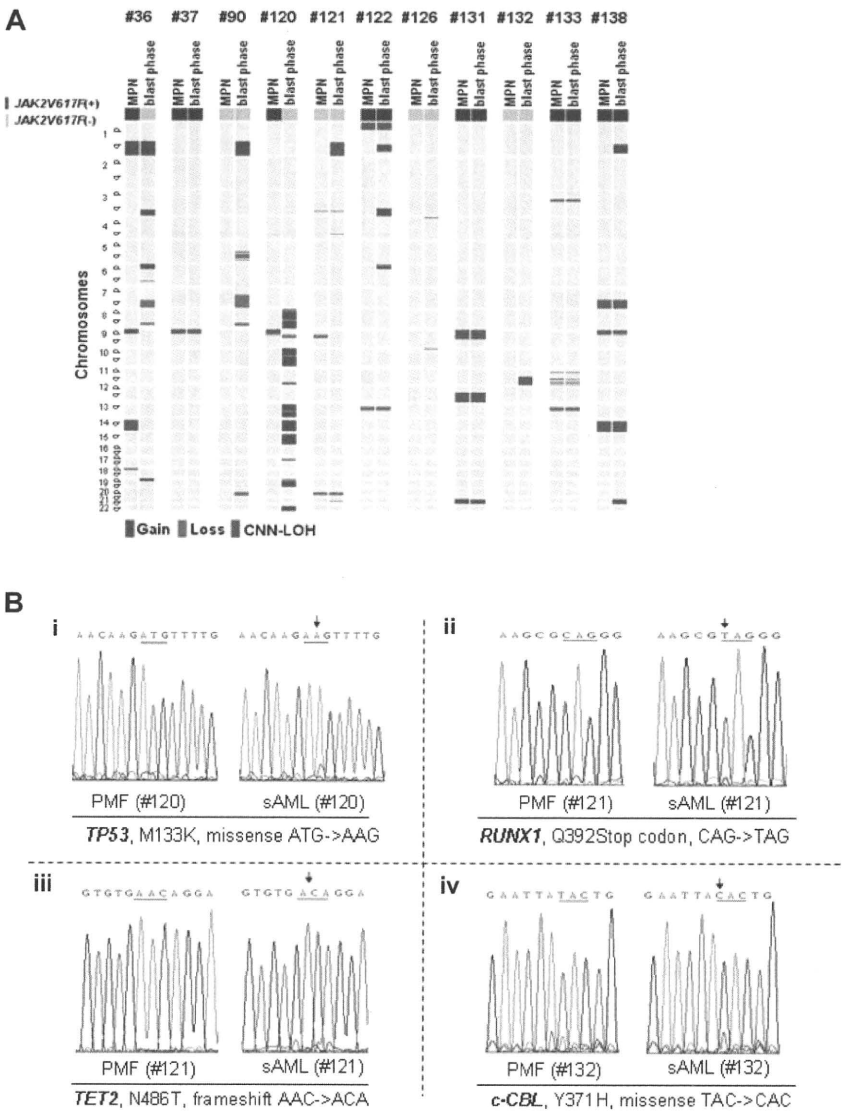

Figure 5. Gains and losses in matched MPN samples and mutational analysis. (A) Most commonly altered genomic regions in MPN samples (left sample column) compared with matched blast phase samples (right sample column) evolved from 11 patients (2 PV, 1 ET, 8 PMF). Each line represents 1 sample with either deletion (blue), duplication/amplification (red), or CNN-LOH (green). (Bi) Hemizygous *TP53* mutation detected in MPN-blast phase sample of case 120 associated with acquired 17q deletion, which was not present in the MPN phase of case 120. (ii) Hemizygous *RUNX1* mutation detected in MPN-blast phase sample (case 121) associated with acquired deletion at 22q22.1, which was not present in the MPN phase of case 121. (iii) Hemizygous *TET2* mutation detected in MPN-blast phase sample of case 121 associated with acquired cryptic deletion on 4q24, which was not present in the MPN phase of case 121. (iv) Homozygous *c-CBL* mutation detected in MPN-blast phase sample of case 132 associated with acquired 11q CNN-LOH, which was not present in the MPN phase of case 132.

4 and 5A, supplemental Figure 4). Ten percent of unmatched and 18% of matched MPN-blast phase cases had either duplication/amplification or CNN-LOH on 19p. The commonly involved region spanned a small locus (2 Mbp) at 19p13.2, where, among others, the genes *PIN1, ICAM1,* and *CDC37,* which have been associated with carcinogenesis, are located.[24-26] In addition, the minimal region (1.8 Mbp) of amplifications/duplications/trisomy on chromosome 21 detected in 8% of unmatched and 9% matched MPN-blast phase samples harbored the oncogenic transcription regulator *ERG* (q22.2).

Table 2. Pretreatment in 47 MPN-blast phase cases and frequency of 17p and 7q deletions

Pretreatment				
Hydroxyurea	−	+	−	+
Alkylating agents	−	−	+	+
SNP-array alteration (17p vs 7q)				
No *del(17)(p)*, no *del(7(q)*	17	16	0	1
del(7)(q)	2	2	2	2
del(17)(p)	0	4*	0	1*
del(17)(p) and *del(7)(q)*	0	0	0	0

Numbers of blast-phase patients are presented.

MPN indicates myeloproliferative neoplasm; and SNP, single nucleotide polymorphism.

*A total of 5 cases with *del(17)(p)* pretreated with hydroxyurea ($P = .035$).

Complete or partial deletion (−7/7q−), as well as CNN-LOH of the long arm of chromosome 7, was one of the most common abnormalities detected by SNP-array analysis in up to 25% of unmatched and 27% matched samples evolved in the blast phase. SNP-array also revealed 3 unmatched cases (32, 87, and 116) with a heterozygous microdeletion encompassing the 7q22.1 locus, which was not detectable by cytogenetic analysis. Moreover, case 138 with 7qCNN-LOH had a homozygous deletion on 7q22.1 in both the matched MPN and MPN-blast phase samples (supplemental Figure 2A). The minimally deleted region spanned a small region of 0.88 Mbp at 7q22.1 covering only 2 target genes, *CUTL1* and *SH2B2*. The homozygous deletion of these genes in patient 138 was confirmed by QG RT-PCR (supplemental Figure 2B). Deletions of the long arm of chromosome 7 were found more often in MPN-blast phase patients pretreated with hydroxyurea and/or alkylating agents, but the findings were not statistically significant ($P = .2$; Table 2).

Also worth mentioning, 1 microdeletion encompassing the chromosome X–linked *PIG-A* gene occurred in male patient 121 at

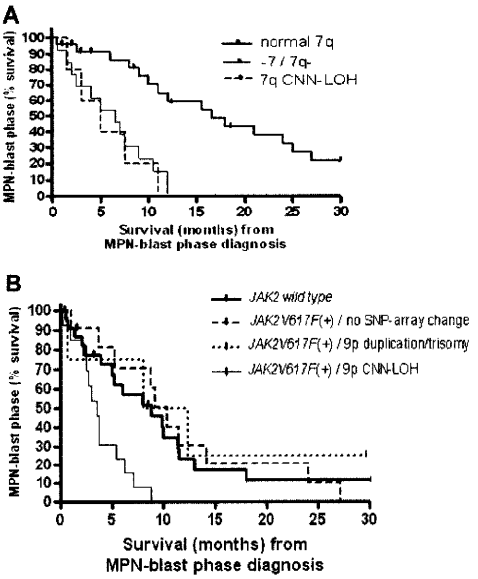

Figure 6. Survival analysis in MPN-blast phase. (A) Survival from the time of diagnosis of blast phase in transformed MPN patients with normal chromosome 7 (normal 7q) compared with either monosomy 7 (−7)/deletion of 7q (7q−) or 7qCNN-LOH. (B) Survival from the time of diagnosis of blast phase in transformed MPN patients with homozygous *JAK2V617F*$^+$ associated with 9pCNN-LOH compared with either heterozygous *JAK2V617F*$^+$ with 9p duplication/trisomy 9 or no abnormality, or patients without the mutation (*JAK2 wild type*). Median survival (months) and the case numbers for each group (transformed PV, ET, or PMF) are listed in supplemental Table 5.

leukemic transformation (supplemental Figure 2C). This patient had a normal chromosome X in his chronic phase of PMF.

CNN-LOH is a marker of poor survival in MPN patients after leukemic evolution

SNP-array technology provides efficient and effective detection of segmental CNN-LOH. In the present study, the most prominent regions for CNN-LOH besides chromosome 9p *(JAK2)* were on 7q and 17p *(TP53)* in patients with MPN-blast phase. In marked contrast to CNN-LOH on 9p, CNN-LOH on 7q or 17p almost never occurred in the chronic phase of the disorder in matched and unmatched samples. As mentioned previously, cases with CNN-LOH and/or deletion of 17p were associated with either complex karyotype or isochromosome 17 and decreased survival.

As also expected, survival in the MPN-blast phase was significantly decreased in patients with −7/7q− (median, 3.75 months) compared with those without chromosome 7 alterations (median, 9 months; $P = .008$). In addition, the unbalanced translocation, der(1;7)(q10;p10), a nonrandom chromosomal abnormality rarely found in AML, was detected by SNP-chip and FISH in 7% of unmatched samples after leukemic evolution and was also associated with an inferior outcome compared with patients without chromosome 7 imbalances ($P = .014$). Strikingly, survival continued to be significantly decreased in MPN-blast phase, when cases with only 7qCNN-LOH were compared with those with a normal 7q ($P = .01$; Figure 6A; supplemental Table 5).

The *JAK2V617F* mutational status in terms of heterozygosity or homozygosity appeared to have no influence on the duration to leukemic evolution. Regardless of the underlying MPN subgroup, no statistical difference in the time to leukemic transformation was found comparing *JAK2V617F*$^+$ patients with normal chromosome 9 to mutant positive blast phase patients with either 9p duplication/trisomy 9 ($P = .28$) or 9pCNN-LOH ($P = .21$). Instead, we found that homozygous *JAK2V617F* had an impact on survival after MPN transformation. Blast phase patients with 9pCNN-LOH resulting in a homozygous *JAK2* mutation had a worse outcome (median, 4 months) compared with *JAK2V617F*$^+$ MPN-blast phase patients with either 9p duplication/trisomy 9 (median, 7.5 months) or no abnormality on 9p (median, 9 months), as well as patients without *JAK2V617F* (median, 7 months, $P = .016$; Figure 6B; supplemental Table 5). Homozygous *JAK2V617F* in association with CNN-LOH diagnosed at leukemic transformation was independent of known risk factors such as 5q−, −7/7q−, or complex karyotype ($P > .05$).

Discussion

Oncogenic *JAK2* signaling is an important event in MPN.[1,2] Recently, we and others showed that homozygosity for *JAK2V617F* is closely related to chromosome 9pCNN-LOH in MPN patients.[1,6,8,9] However, the transformation process of MPN to MPN-blast phase is not well understood.

Recent findings suggested that transition from heterozygosity to homozygosity for *JAK2V617F* is associated with a hyperproliferative disease profile and may be important for disease progression, at least from PV to secondary myelofibrosis.[27] Moreover, Barosi et al showed in a longitudinal prospective study that the presence of a *JAK2V617F* hematopoietic clone was significantly associated with leukemic transformation in PMF.[28] This is in contrast to our present findings showing that not only the mutational status of *JAK2V617F,* but also 9pCNN-LOH with homozygous *JAK2V617F,* had no impact on the time to leukemic transformation in patients with MPN-blast phase. In addition, 2 of the 11 matched MPN samples, initially positive for *JAK2V617F* with either trisomy 9 or 9pCNN-LOH, became negative for these abnormalities after leukemic transformation. Although only tested in unpaired samples, PMF and PV samples also showed a significantly smaller number of both *JAK2V617F*$^+$ and 9pCNN-LOH in the blast phase compared with the chronic phase. Interestingly, and also contrary to the previously cited studies, Tefferi et al noted a significant association between a low *JAK2V617F* allelic burden and evolution to blast phase in a large cohort of PMF patients.[29] Even though these data are not completely congruent with our findings, the results of Tefferi et al and our results point to the coexistence of a more dominant *JAK2V617F*-negative clone with a higher propensity to undergo clonal evolution. This is congruent with recent studies indicating that *JAK2V617F*$^+$ MPN can result in *JAK2V617F*$^-$ MPN-blast phase.[30,31] But still, some of our matched cases with *JAK2V617F*$^+$ had no change in abnormalities including *JAK2* mutational status as well as 9pCNN-LOH, allowing the existence of a common pre-*JAK2V617F* clone. Taken together, the presence of *JAK2V617F* appears not to be a prerequisite for leukemic transformation of MPN, suggesting that additional genetic events are required for full transformation.

SNP-array analysis was able to capture practically all cytogenetic abnormalities and to uncover additional lesions with potentially important clinical implications. The number of genomic alterations was more than 2 to 3 times greater in the blast phase as in the chronic phase of matched and unmatched cases with MPN. Noticeably, ET patients had fewer alterations in their chronic phase samples compared with the PV and PMF cases, whereas the number was comparable in all 3 MPN subgroups after their transformation. Being aware of the increased number of new

genomic changes enables investigators to focus on the identification of causative genes associated with the evolution of MPN to leukemia.

Commonly altered regions in blast crisis samples were detected on chromosomes 8, 12, 17, and 21 encompassing *MYC, ETV6, TP53,* and *RUNX1,* respectively, which are already known to be involved in the development of de novo and secondary AML.[18-22] Gain of chromosomal material at 8q24.21 was almost exclusively found in *JAK2V617F*$^-$ samples, suggesting that increased activity of *MYC* might allow selection of clones that do not require the *JAK2* gain-of-function mutation. Furthermore, deletion of 17p *(TP53)* was significantly associated with prior exposure to hydroxyurea as well as a complex karyotype in samples with MPN-blast crisis, which is in accordance with recent results.[32,33] Interestingly, not only deletion, but also 17pCNN-LOH, was associated with a complex karyotype, a poor prognostic marker in myeloid malignancies.

In addition, regions on chromosomes 1q, 7q, 16q, 19p, and 21q were frequently altered in the evolution to the leukemic phase and may harbor promising new candidate genes. Abnormalities involving chromosome 7 are frequently detectable in de novo and secondary AML,[34-37] and preceding studies have found a critical breakpoint region involving a locus at centromeric band 7q22, whereas the telomeric breakpoint varies from q32 to q36. Interestingly, the minimal deleted region in our cohort was located at 7q22.1 encompassing only 2 promising target genes, *SH2B2* (previously named *APS*) and *CUTL1. SH2B2* regulates and enhances *JAK2*-mediated cellular responses,[38] and the *CUTL1* gene encodes for a *CUT* family member of the homeodomain proteins that can repress the expression of developmentally regulated myeloid genes.[39] Moreover, genome-wide inspection for minimal regions of duplications/amplifications and CNN-LOH revealed several interesting genes, such as *PIN1, ICAM1,* and *CDC37* on 19p as well as *ERG* on 21q. Whereas the latter 3 targets have been shown to possess potential progrowth activity in de novo AML and/or MDS,[25,26,40] *PIN1* is known to be overexpressed in a variety of cancers and may act as an oncogene via promotion of cell cycle progression and proliferation.[24]

Mutations of the *c-CBL* gene are tightly associated with 11qCNN-LOH and are commonly diagnosed in patients with chronic myelomonocytic leukemia.[23,41,42] Although MPN shares clinical as well as hematologic features with chronic myelomonocytic leukemia, we detected 11qCNN-LOH only in a minority of our study population, suggesting that *c-CBL* mutations are rare events leading to transformation of chronic MPN to leukemic blast phase.

In contrast to recent findings showing frequent LOH on 4q associated with *TET2* mutations in patients diagnosed with MDS/MPN,[43] we detected CNN-LOH or deletions at 4q24 *(TET2)* only in a minority of our patients in the chronic as well as blast phase of MPN. Nevertheless, our study was not sufficient to explore these findings in more detail and make conclusions on tumor suppressor *TET2* and its potential role in leukemic transformation.

However, with regard to the variety of detected allelic imbalances, we suggest that no single candidate gene or molecular pathway is sufficient and necessary to cause transformation of chronic MPN to blast phase. Like de novo AML, MPN-blast phase appears to be a heterogeneous disease prone to have evolved multiple mechanisms to provide a proliferative advantage to the abnormal leukemic clone.

CNN-LOH involving chromosomal regions that are also frequently affected by deletions may have prognostic implications similar to the deletions visible by karyotyping. In our study, prognostic evaluation was based mainly on SNP-array data from blast phase samples without the incorporation of SNP-array results from the matched chronic phase. Moreover, we implied the survival and clinical outcome only of MPN patients who underwent leukemic transformation, without comparison with survival and outcome in untransformed chronic phase. However, as expected, blast phase patients with loss of chromosomal material on 7q showed poor survival, because this is known to be predictive for rapid progression and poor response in AML therapy.[35-37] MPN-blast phase patients with cytogenetically undetectable 7qCNN-LOH had comparable survival rates to those with −7/7q− in their leukemic cells, which is in accordance with previously published data.[44]

In addition, 9pCNN-LOH with homozygous *JAK2* mutation was also linked to an inferior outcome in MPN-blast crisis in comparison with patients with either heterozygous *JAK2V617F* or wild-type *JAK2*. In contrast to LOH on 17p, the prognostic impact of 9pCNN-LOH was independent of established risk factors such as −7/7q−, 5q−, or complex karyotype. Although *JAK2V617F* in association with 9pCNN-LOH appeared to have no impact on the time to MPN transformation, we suggest that the homozygous driver mutation in combination with additional newly acquired aberrations in terms of a second hit may have an implication on the clinical course of MPN-blast phase patients.

In conclusion, high-density SNP-array technology allowed precise identification of chromosomal aberrations, including CNN-LOH, and complemented conventional cytogenetic techniques in patients with chronic and transformed MPN. Our analysis provided prognostic details to further improve clinical prognosis, as well as novel interesting candidate genes potentially involved in the transformation of MPN.

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Authorship

Contribution: N.H.T and U.O.K. performed the research, analyzed the data, and wrote the paper; D.H.T.L., N.K., G.B.I., T.L., T.W., D.N., M.K.-M., M.K., M.S., L.-Y.S., A.N., and S.D.R. assisted with the research; C.M.-T., R.M., T.H., D.G.G., and A.T. designed and performed the research; and S.O. and H.P.K. directed the overall study.

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