Published OnlineFirst April 14, 2010; DOI:10.1158/1078-0432.CCR-09-2112

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*Clin Cancer Res* 2010;16:2246-2256. Published OnlineFirst April 14, 2010.

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Human Cancer Biology

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# *RAS* Mutations Contribute to Evolution of Chronic Myelomonocytic Leukemia to the Proliferative Variant

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## Abstract

**Purpose:** The biological and clinical heterogeneity of chronic myelomonocytic leukemia features renders its classification difficult. Moreover, because of the limited knowledge of the mechanisms involved in malignant evolution, chronic myelomonocytic leukemia remains a diagnostic and therapeutic challenge and a poor prognosis disease. We aimed to verify the biological and clinical significance of the discrimination, based on the leukocyte count, between myelodysplastic chronic myelomonocytic leukemia (MD-CMML) and myeloproliferative chronic myelomonocytic leukemia (MP-CMML).

**Experimental Design:** Peripheral blood samples from 22 patients classified as MD-CMML and 18 as MP-CMML were collected at different time points during disease course, and patients' clinical characteristics were examined. *RAS* mutational screening was done by sequencing and, for each substitution identified, a highly selective allele-specific PCR was set up to screen all specimens.

**Results:** MP-CMML patients showed a significantly poorer survival ($P = 0.003$) and a higher frequency of *RAS* mutations ($P = 0.033$) by sequencing compared with MD-CMML. Overall, five MD-CMML patients progressed to myeloproliferative disease: in two, allele-specific PCR unveiled low levels of the *RAS* mutations predominating in the myeloproliferative phase at the time of myelodysplastic disease, documenting for the first time the expansion of a *RAS* mutated clone in concomitance with chronic myelomonocytic leukemia evolution. Moreover, one of the progressed patients harbored the *FLT3-ITD* and two MP-CMML patients presented with the *JAK2 V617F* substitution. All these lesions were mutually exclusive.

**Conclusions:** Our results strongly suggest *RAS* mutations to function as a secondary event that contributes to development of the chronic myelomonocytic leukemia variant with the poorer prognosis (MP-CMML) and therefore advise their detection to be implemented in chronic myelomonocytic leukemia diagnostics and monitoring. *Clin Cancer Res; 16(8); 2246–56. ©2010 AACR.*

Chronic myelomonocytic leukemia is a rare malignancy of the elderly whose diagnosis entails persistent absolute monocytosis in the blood (monocytes, $>1 \times 10^9$/L). It exhibits highly heterogeneous clinical, hematologic, and morphologic features, varying from mainly myelodysplastic to predominantly myeloproliferative (1, 2). Hence, chronic myelomonocytic leukemia nosology has always represented a hurdle; the French American Group (FAB) classification firstly categorized this disorder as a myelodysplastic syndrome (3) and then, in 1994, based on the WBC count threshold of $13 \times 10^9$/L, proposed to discriminate between myelodysplastic chronic myelomonocytic leukemia (MD-CMML, WBC, $<13 \times 10^9$/L) and myeloproliferative chronic myelomonocytic leukemia (MP-CMML, WBC, $\geq 13 \times 10^9$/L; ref. 4). Yet, the biological and clinical significance of this distinction remains controversial because the two groups have been variably associated with prognosis (5–10) and few cases of MD-CMML progressing to MP-CMML have been reported (1, 2, 8, 11); accordingly, this discrimination was not acknowledged in the last WHO classification of myeloid malignancies, in which chronic myelomonocytic leukemia has been rather included in the new category of the myelodysplastic syndrome/myeloproliferative neoplasms (12, 13).

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**Note:** Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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**doi:** 10.1158/1078-0432.CCR-09-2112

Published OnlineFirst April 14, 2010; DOI:10.1158/1078-0432.CCR-09-2112



## Translational Relevance

To date, chronic myelomonocytic leukemia remains a diagnostic and therapeutic challenge, in most cases with a dismal prognosis. Our findings support the prognostic significance of the classification, based on the leukocyte count, into myelodysplastic and myeloproliferative chronic myelomonocytic leukemia and document for the first time the contribution of *RAS* mutations to chronic myelomonocytic leukemia progression from myelodysplastic to myeloproliferative phase. Our results strongly suggest these aberrations to function as a secondary event that contributes to the expansion of a malignant clone with proliferative advantage. Therefore, we propose *RAS* mutational screening by highly selective, yet simple and low-cost allele-specific PCR, to be implemented as a standard in chronic myelomonocytic leukemia diagnostics and disease monitoring because patients harboring *RAS* mutations are predicted to develop the disease phenotype with the worse prognosis (myeloproliferative chronic myelomonocytic leukemia).

The high variability of the clinical presentation and course of chronic myelomonocytic leukemia reflects the heterogeneity of its pathogenetic features. Since, to date, the recurrent mechanisms involved in its malignant evolution have not been fully elucidated, chronic myelomonocytic leukemia remains a diagnostic and therapeutic challenge, in most cases with a dismal prognosis. Notably, among hematologic malignancies, chronic myelomonocytic leukemia encounters the highest incidence of *RAS* substitutions (up to 50%, usually in codons 12, 13, and 61 of *NRAS* and *KRAS*; ref. 14), which have been associated with features of cell proliferation and monocytosis (1) and with shorter survival (15). Of note, a significantly higher incidence of these mutations has been documented among MP-CMML than MD-CMML patients (1, 14), and the expression of activated *KRAS* and *NRAS* in murine bone marrow cells has been shown to rapidly and efficiently induce a fatal myeloproliferative disease resembling human chronic myelomonocytic leukemia (16, 17). However, *RAS* mutations are not specific for chronic myelomonocytic leukemia (18), and conversely, other genetic lesions have been found in subsets of patients. For example, *RUNX1* aberrations have been detected in MD-CMML and MP-CMML patients, also in concomitance with *RAS* mutations, suggesting implication of these abnormalities in the acquisition of myelodysplastic features (14). More recently, mutations of the putative tumor suppressor gene *TET2* have been identified in a spectrum of myeloid malignancies (19–26), including chronic myelomonocytic leukemia (21–23, 26), MD-CMML and MP-CMML (26). Noteworthy, the ubiquitous nature of *TET2* mutations suggests that they are an early event in hematopoietic differentiation and that at least one additional contributing event is required to determine the disease phenotype (19, 27). Other genetic defects (i.e., *FLT3-ITD* and *JAK2 V617F* substitution; refs. 28–30) have been identified at a higher frequency among MP-CMML than MD-CMML patients, in a manner mutually exclusive with *RAS* mutations, and have been shown to induce a chronic myelomonocytic leukemia-like disease in mice (i.e., the *FLT3-ITD* and *BID* deletion; refs. 28, 31), implying a role in the establishment of myeloproliferation. Therefore, although a key role for *RAS* mutations in chronic myelomonocytic leukemia pathogenesis has been proposed, particularly in those forms with a prominent proliferative component, no straightforward conclusion has been definitely drawn.

Because its pathogenesis is mostly unknown, to date, the only possible curative strategy for chronic myelomonocytic leukemia remains allogeneic stem cell transplantation, which, however, may be applied only to a small fraction of patients because of the advanced median age of presentation (32). Hence, to improve patient outcome, it is crucial to define molecular subtypes of chronic myelomonocytic leukemia, which could possibly facilitate the development of novel targeted agents.

In light of these issues, we aimed to investigate the biological and clinical significance of the discrimination between MD-CMML and MP-CMML. Our findings support the prognostic significance of this distinction because MP-CMML patients had a significantly shorter survival than those with myelodysplastic disease. Moreover, we document for the first time the expansion of a *RAS*-mutated clone in concomitance with progression from MD-CMML to MP-CMML, strongly suggesting these aberrations to function as a secondary event that contributes to the expansion of a malignant clone with proliferative advantage. Altogether, our results highlight the clinical relevance of identifying chronic myelomonocytic leukemia patients harboring these aberrations because they are predicted to develop the disease phenotype with the worse prognosis (MP-CMML).

## Materials and Methods

***Patients characteristics.*** Forty patients diagnosed with chronic myelomonocytic leukemia were included in this study between December 2002 and December 2008; according to the FAB proposal (4), 22 were classified as MD-CMML and 18 as MP-CMML. In addition, our series included one patient (patient 38) who developed chronic myelomonocytic leukemia rapidly progressing to the MP-CMML, after achievement of complete remission from a previously diagnosed Philadelphia-positive chronic myelogenous leukemia (CML). Clinical and laboratory characteristics of patients are listed in Tables 1 and 2. Five patients (patients 7, 8, 9, 31, and 33) initially presented as MD-CMML and later on experienced evolution to myeloproliferative disease. Patients 24 and 30 were referred as MP-CMML but had a previous history of MD-CMML.

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**Table 1.** Clinical and laboratory characteristics of the patients included in this study

| Pt. ID | Age | Sex | WBC count (×10$^9$/L) | Monocyte count (×10$^9$/L) | Cytogenetics |
|---|---|---|---|---|---|
| *MD-CMML* | | | | | |
| 2 | 71 | M | 10.3 | 3.6 | 46, XY |
| 3 | 71 | F | 8.4 | 1.6 | 46, XX |
| 4 | 64 | M | 4.5 | 1.6 | 46, XY |
| 5 | 89 | F | 9.2 | 2.0 | 46, XX |
| 6 | 74 | F | 3.9 | 1.1 | 46, XX |
| 7* | 69 | F | 12.3 | 3.3 | 46, XX |
| 8* | 73 | F | 7.6 | 2.7 | 46, XX |
| 9* | 77 | M | 7.6 | 1.8 | 46, XY |
| 10 | 80 | F | 3.5 | 1.0 | 46, XX |
| 11 | 64 | F | 10.1 | 1.3 | 46, XX |
| 13 | 88 | F | 3.2 | 2.0 | 46, XX |
| 15 | 82 | F | 5.2 | 1.9 | 46, XX |
| 17 | 84 | M | 4.0 | 1.2 | 46, XY, del(20)(q11) |
| 18 | 76 | M | 6.6 | 1.8 | 45, X,-Y |
| 19 | 72 | M | 3.9 | 1.1 | 46, XY |
| 20 | 70 | F | 6.0 | 2.2 | n.a. |
| 21 | 69 | F | 10.6 | 4.7 | 46, XX |
| 26 | 66 | F | 4.6 | 1.3 | 46,XX |
| 29† | 71 | M | 5.3 | 1.8 | 46, XY |
| 31* | 79 | M | 6.0 | 1.4 | 46, XY |
| 33*,‡ | 59 | F | 11.4 | 1.1 | 46, XX |
| 41 | 81 | M | 8.3 | 2.0 | 46, XY |
| *MP-CMML* | | | | | |
| 1§ | 76 | M | 14.4 | 4.0 | 46, XY |
| 12 | 80 | F | 13.4 | 4.8 | 46, XX |
| 14§ | 69 | M | 9.4 | 2.0 | 46, XY, inv(9)(p11q12) |
| 16 | 68 | M | 13.4 | 3.6 | 46, XY |
| 22 | 70 | M | 39.5 | 2.2 | 46, XY |
| 23 | 70 | M | 13.9 | 2.3 | 46, XY |
| 24‖ | 71 | M | 14.6 | 3.7 | 46, XY |
| 25 | 84 | F | 16.6 | 3.2 | n.a. |
| 27§ | 77 | M | 16.2 | 5.8 | 46, XY |
| 28 | 78 | M | 21.2 | 3.0 | 46, XY |
| 30‖ | 74 | F | 30 | 7.6 | n.a. |
| 32 | 83 | F | 13.7 | 2.3 | 46, XX |
| 34 | 79 | M | 15.3 | 4.1 | n.a. |
| 35 | 79 | M | 21.5 | 6.1 | n.a. |
| 36§ | 68 | M | 22.8 | 3.1 | n.a. |
| 37§ | 72 | M | 39.4 | 5.7 | 46, XY |
| 39§ | 73 | M | 23.9 | 3.7 | 46, XY |
| 40 | 79 | M | 14 | 1.8 | 46, XY |
| *CML* | | | | | |
| 38*,§ | 69 | M | 88.3 | 3.5 | t(9;22)(q34;q11) |

Abbreviations: Pt., patient; M, male; F, female; n.a., not available.

*Patients who later on developed MP-CMML.

†This patient 10 months after presentation displayed the trisomy of chromosome 8 (47, XY +8 [13] 46, XY [13]).

‡Thirty-six months after first presentation and in concomitance with development of acute myelogenous leukemia, this patient displayed the following cytogenetics: 45, XX, del(6)(q22), -7, add(12)(p13).

§Patients under chemotherapy.

‖MP-CMML patients with a previous history of MD-CMML.

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**Table 2.** Results of RAS mutational screening done by direct sequencing and allele-specific PCR at the time of first presentation, as well as during the course of disease

| Pt. ID | First presentation | | Course of disease | | Outcome | Follow-up (mo) |
|---|---|---|---|---|---|---|
| | By seq | By ASP | By seq | By ASP | | |
| *MD-CMML* | | | | | | |
| 2 | *WT* | *WT* | *WT* (+11 mo) | *WT* (+11 mo) | MD-CMML; lost to follow-up | 11 |
| 3 | *WT* | *WT* | | | MD-CMML | 82.5 |
| 4 | *WT* | *WT* | | | MD-CMML | 80 |
| 5 | *WT* | *WT* | *WT* (+27 mo) | *WT* (+27 mo) | MD-CMML; lost to follow-up | 35 |
| 6 | *WT* | *WT* | | | MD-CMML | 81 |
| 7 | *WT* | *WT* | *WT* (+23 mo) | *WT* (+23 mo) | Progressed to MP-CMML; deceased | 26 |
| 8 | *WT* | *N G60E* | *N G60E* (+22 mo) | *N G60E* (+22 mo) | Progressed to MP-CMML; deceased | 25 |
| 9 | *WT* | *WT* | *WT* (+23 mo) | *WT* (+23 mo) | Progressed to MP-CMML; lost to follow-up | 30 |
| 10 | *WT* | *WT* | | | MD-CMML | 76.5 |
| 11 | *WT* | *WT* | | | MD-CMML | 75 |
| 13 | *WT* | *WT* | | | Lost to follow-up | 0 |
| 15 | *WT* | *WT* | | | Lost to follow-up | 0 |
| 17 | *WT* | *WT* | | | Deceased | 40 |
| 18 | *WT* | *WT* | | | MD-CMML | 53 |
| 19 | *WT* | *WT* | | | MD-CMML | 58.5 |
| 20 | *WT* | *WT* | | | MD-CMML | 57.5 |
| 21 | *WT* | *WT* | | | Deceased | 42 |
| 26 | *WT* | *WT* | | | MD-CMML | 47 |
| 29 | *WT* | *WT* | *WT* (+10 mo) | *WT* (+10 mo) | Progressed to AML; deceased | 13 |
| 31* | *WT* | *WT* | *WT* (+6 mo) | *WT* (+6 mo) | Progressed to MP-CMML and then AML; deceased | 8 |
| 33 | *WT* | *N G12D* | *N G12D* (+13-18 mo) | *N G12D* (+13 mo) | Progressed to MP-CMML and then AML; deceased | 39.5 |
| 41 | *WT* | *WT* | | | MD-CMML | 13.5 |
| *MP-CMML* | | | | | | |
| 1 | *WT* | *WT* | | | Deceased | 5 |
| 12 | *N G12D* | *N G12D* | *N G12D* (+19 mo) | *N G12D* (+19 mo) | MP-CMML† | 73.5 |
| 14 | *N G12D* | *N G12D* | *N G12D* (+8 mo) | *N G12D* (+8 mo) | Deceased | 15 |
| 16 | *WT* | *WT* | | | Deceased | 24 |
| 22 | *WT* | *N G12D* | | | Deceased | 28 |
| 23‡ | *WT* | *WT* | *WT* (+30 mo) | *WT* (+30 mo) | Deceased | 48 |
| 24 | *WT* | *WT* | *N G12V* (+36 mo) | *N G12V* (+27 mo) | MP-CMML† | 55.5 |
| 25 | *N G12D* | *N G12D* | | | Deceased | 12 |
| 27 | *WT* | *WT* | | | Deceased | 10 |
| 28 | *WT* | *WT* | | | Deceased | 2 |
| 30 | *K G12R* | *K G12R* | | | Progressed to AML; deceased | 1 |
| 32 | *WT* | *N G12D* | | | MP-CMML | 41.5 |
| 34 | *WT* | *WT* | | | MP-CMML | 38 |
| 35‡ | *WT* | *WT* | | | Deceased | 2 |
| 36 | *WT* | *WT* | | | Progressed to AML; deceased | 14 |

(*Continued on the following page*)

Published OnlineFirst April 14, 2010; DOI:10.1158/1078-0432.CCR-09-2112



**Table 2.** Results of RAS mutational screening done by direct sequencing and allele-specific PCR at the time of first presentation, as well as during the course of disease (Cont'd)

| Pt. ID | First presentation | | Course of disease | | Outcome | Follow-up (mo) |
|---|---|---|---|---|---|---|
| | By seq | By ASP | By seq | By ASP | | |
| 37 | *WT* | *WT* | | | Deceased | 28.5 |
| 39 | *WT* | WT | | | Deceased | 7 |
| 40 | *WT* | *WT* | | | Deceased | 14 |
| *CML* | | | | | | |
| 38 | *WT* | *WT* | *N G13V* (+3-25 mo)<br>*N G12R* (+42 mo) | *N G13V* (+3-42 mo)<br>*N G12R* (+12 mo) | Progressed to AML; deceased | 54 |

Abbreviations: seq, sequencing; ASP, allele-specific PCR; N, *NRAS*; AML, acute myelogenous leukemia; K, *KRAS*.
*Patient with *FLT3-ITD*.
†Patients currently under chemotherapy.
‡Patient with the *JAK2 V617F* allele.

***Sample collection and preparation.*** Overall, 62 peripheral blood samples were obtained after informed consent. Peripheral blood mononuclear cells were isolated by Hystopaque 1077 density gradient (Sigma). Genomic DNA extraction was carried out by DNAzol (Molecular Research Center, Inc.); RNA was isolated by TRIreagent (Sigma), and cDNA was synthesized using oligo$(dT)_{12\text{-}18}$ primers and SuperScript II Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions.

***Direct sequencing of KRAS and NRAS.*** Regions spanning codons 12 to 13 (exon 1) and 61 (exon 2) of *NRAS* and codons 12-13 (exon 1), 61 (exon 2), and 146 (exon 3; refs. 14, 33) of *KRAS* were amplified by PCR using M13-tailed primers starting from 200 ng of DNA (Supplementary Table S1 and S2). Amplicons were purified (MinElute QIAquick gel extraction kit, Qiagen) and automatically sequenced (ABI PRISM 310 Genetic Analyzer, Applied Biosystems) using the Big Dye Terminator Cycle Sequencing Kit.

***Construction of green fluorescent protein (GFP)–NRAS chimeras.*** Primers for amplification of the entire human *NRAS* coding sequence (GenBank NM_002524) were designed as previously described (34), with minor modifications. Briefly, two nucleotides were added to the forward primer to insert *NRAS* in frame with *GFP*, and four and two excess nucleotides were added at the 5′ of *Pst*I and *Bam*HI sites, respectively, to increase cleavage efficiency of endonucleases (Supplementary Table S1). The sequence of the final construct was verified by sequencing using the internal primer (*NRAS* coding sequence-forward internal) specified in Supplementary Table S1. *Pst*I/*Bam*HI-digested amplicons corresponding to the entire coding sequence of *NRAS* wild type (*WT*), *G12D*, and *G60E* were inserted into the *Pst*I/*Bam*HI-digested pAcGFP-C1 vector (Clontech Laboratories, Inc.), leading to a chimeric construct of *GFP* fused in frame to the 5′ end of *NRAS*. The resulting plasmids were subcloned into *Escherichia coli*.

***Transient transfection of NIH3T3 cells, confocal analysis, and evaluation of Ras-GTP levels.*** NIH3T3 mouse fibroblasts were maintained in DMEM (Cambrex Corporation) supplemented with 10% of fetal bovine serum (Sigma) and 1% of a penicillin/streptomycin solution (Sigma). Cells were transiently transfected with 1 μg of the pAcGFP-C1 vector, either empty or containing the *GFP-NRAS WT*, *G12D*, or *G60E* constructs, using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

For confocal analysis, NIH3T3 cells were grown on four-well glass chambers, transfected, fixed 48 h later with 4% paraformaldehyde, permeabilized with 1% Triton X-100, and incubated overnight with 1:50 Anti-Pan Ras Ab (Calbiochem). After 30 min of labeling with phycoerythrin, cells were washed, mounted with cover glass, and analyzed by confocal microscopy.

For biochemical analysis, 48 h after transfection, NIH3T3 cells were lysed to pull down the GTP-bound Ras by affinity purification with the Raf-1RBD agarose (Pierce Biotechnology, Inc.) following the manufacturer's instructions. Whole cell extracts and pull-down supernatants were then separated on SDS-PAGE, and immunoblots were incubated overnight with the Anti-Pan Ras Ab (Calbiochem).

***Set up of allele-specific PCR.*** Either forward or reverse primers were designed with specific mismatches in codons 12, 13, and 60 of *NRAS* and in codon 12 of *KRAS* (Supplementary Table S1). To improve specificity, an intentional mismatch on the third nucleotide from the 3′ end was also included. Addition of the M13-tailed primers to the reaction led to the amplification of either the internal control fragment alone (*RAS WT*) or concomitantly with the specific mutant allele (Supplementary Tables S1 and S2). One hundred nanograms of DNA were used in a final volume of 25 μL. Allele-specific PCR specificity was assessed by amplifying multiple DNA from healthy donors (*RAS WT*) and from patients

Published OnlineFirst April 14, 2010; DOI:10.1158/1078-0432.CCR-09-2112



harboring mutant (mut) *RAS* alleles other than that specific for each allele-specific PCR. To determine allele-specific PCR sensitivity, different amounts of *mut*DNA were spiked into DNA *WT* and subjected to the corresponding allele-specific PCR (except for the *NRAS G13V* mutation because of the paucity of starting material).

***Single-colony analysis.*** Peripheral blood mononuclear cells were mixed with a methylcellulose culture medium (MethoCult 4434, StemCell Technologies, Inc.) and single colony–forming unit, granulocytes-macrophages (CFU-GM), and burst-forming unit-erythroid (BFU-E) were harvested in 40 μL of deionized water and heated for 10 min at 95°C to release DNA. Allele-specific PCR for the presence of the mutation previously identified in the total DNA was done.

***Statistical analyses.*** For the survival analyses, Kaplan-Meier plots were generated using the elapsed time between the date of the blood collection and death or the date of the last follow-up, and the survival curves were compared using log-rank testing. Unpaired one-tailed *t* test was used to test the difference for continuous variables. The Fisher's exact test was used to examine the association of two categorical variables. $P < 0.05$ was considered statistically significant. The level of agreement between allele-specific PCR and sequencing was assessed using the $\kappa$ statistics, considering substantial agreement if $\kappa$ was >0.61. Analyses were carried out using GraphPad Prism version 4.00 for Windows (GraphPad Software; www.graphpad.com).

## Results

***Life expectation of patients with chronic myelomonocytic leukemia is significantly shorter in MP-CMML than in MD-CMML.*** The entire cohort of chronic myelomonocytic leukemia patients included 23 men and 17 women, with a median age of 74 years (range, 59-89 years). The median follow-up was 28.3 months (range, 0-82.5 months), and the median survival was 40 months (Fig. 1A). When patients were classified according to the WBC count into MD-CMML ($n = 22$) and MP-CMML ($n = 18$), the median WBC counts resulted $6.3 \times 10^9$/L and $15.8 \times 10^9$/L, respectively. The median age was 73 years for MD-CMML and 75 years for MP-CMML patients. As predicted, the two groups significantly differed for the absolute monocyte count (median, $1.8 \times 10^9$/L in MD-CMML versus $3.7 \times 10^9$/L in MP-CMML; $P < 0.0001$). Importantly, according to the Kaplan-Meier estimates, patients with MP-CMML had a significantly shorter median survival than those with MD-CMML (14.5 months versus not reached; $P = 0.003$; Fig. 1B).

***RAS mutations prevail among patients with the MP-CMML.*** We directly sequenced exons 1 and 2 of *NRAS* and 1 to 3 of *KRAS*. Overall, at first presentation, no mutation was detected in MD-CMML patients, whereas 4 (22.2%) of 18 MP-CMML patients were shown to harbor a mutant allele ($P = 0.033$), the most frequent being the *NRAS G12D* (Table 2). Notably, patient 30, who carried the *KRAS G12R* mutation, was referred to our institution as MP-CMML but had a previous history of MD-CMML; unfortunately, no sample was available at the time of myelodysplastic disease.

During follow-up, sequencing identified the *NRAS G60E* and *G12D* substitutions in two patients (patients 8 and 33, initially presenting as MD-CMML with *RAS WT*) after they progressed to MP-CMML. With regard to the MP-CMML group, sequencing detected the *NRAS G12V* mutation in patient 24 36 months after the time of presentation and the *NRAS G12R* and *G13V* alleles in patient 38 not at the time of presentation as Philadelphia-positive CML but at different times during the course of chronic myelomonocytic leukemia.

Because of the lack of data on the uncommon *NRAS G60E* mutation at the time of its identification in patient 8 and to better understand its role in chronic myelomonocytic leukemia evolution, we expressed the GFP/*NRAS WT*, *G60E*, and *G12D* proteins in NIH3T3 cells and compared their biochemical activity. After verifying by confocal microscopy the expression of chimeric proteins in transiently transfected cells (Supplementary Fig. S1A), we determined the levels of active GTP-bound Ras. As shown in Supplementary Fig. S1B, increased levels of Ras-GTP were observed in cells expressing the mutant proteins compared with the *WT*; in particular, the *NRAS G60E* displayed intermediate levels of active Ras between the *WT* and the *G12D*, in agreement with the recent paper from Tyner et al. (33).

***RAS mutations contribute to chronic myelomonocytic leukemia progression: highly selective allele-specific PCR as a powerful tool for early detection and tracking of disease evolution.*** To gain further insights into the role of activated *RAS* in chronic myelomonocytic leukemia evolution, we set up an allele-specific PCR for each substitution identified by sequencing and rescreened all specimens. In our experimental conditions, allele-specific PCR was able to detect the presence of the corresponding mutant allele in a mixture containing ~1% to 2% of *mut*DNA in a background of DNA *WT* (Supplementary Fig. S2), whereas sequencing required about 20% (results not shown).

Allele-specific PCR confirmed all results obtained by sequencing. Yet, this method led to some interesting findings summarized in Table 2 (the $\kappa$ statistics indicated a substantial agreement between allele-specific PCR and sequencing; $\kappa = 0.6154$). Overall, at presentation, a *RAS* mutation was detected in 2 (9%) of 22 MD-CMML and 6 (33.3%) of 18 MP-CMML patients ($P = 0.065$). Importantly, both MD-CMML patients presenting with a mutant allele (patients 8 and 33) later on progressed to MP-CMML; in our experimental conditions, a small amount of the *NRAS G60E* allele was detected in patient 8's peripheral blood withdrawn 22 months before disease progression (Fig. 2A), and the *NRAS G12D* allele was found at low levels in the peripheral blood obtained from patient 33 at the time of first presentation (Fig. 2B). In this case, three more samples were consecutively collected 13, 18, and 25 months after the first withdrawal; of note, allele-specific PCR allowed to monitor the increase of the level

Published OnlineFirst April 14, 2010; DOI:10.1158/1078-0432.CCR-09-2112



of the oncogenic allele throughout the course of chronic myelomonocytic leukemia (Fig. 2B and Supplementary Fig. S3). Of the other three patients who experienced evolution to myeloproliferative disease, patients 7 and 9 did not display any of the alterations detectable by our assays, whereas patient 31 was shown to harbor the *FLT3-ITD* (results not shown).

Among MP-CMML patients, at presentation, allele-specific PCR identified low levels of the *NRAS G12D* allele in patients 22 and 32, who were both *WT* according to sequencing. During the follow-up, allele-specific PCR could detect the *NRAS G12V* allele in patient 24 27 months after the first presentation, 9 months earlier than sequencing (Fig. 2C). In patient 38, who developed chronic myelomonocytic leukemia as a second malignancy after remission from Philadelphia-positive CML, in our experimental conditions, no mutation was found at the time of CML (Fig. 2D). Later on, allele-specific PCR allowed to accurately follow the consecutive occurrence of two distinct clones harboring the *NRAS G13V* and *G12R* alleles, respectively. In particular, allele-specific PCR identified the presence of the *G12R* allele ~30 months earlier than sequencing and the persistence of the *G13V* mutation 17 months after sequencing could no longer detect it.

Finally, single colony analysis by allele-specific PCR was carried out to assess in which progenitors the *G12D* mutation occurred. We plated peripheral blood mononuclear cells from patient 12 (MP-CMML with *mutRAS* by sequencing) and from patients 32 and 33 (respectively, MP-CMML and MD-CMML at first presentation, with *RAS WT* by sequencing) and found the mutation in colony-forming unit, granulocytes-macrophage, and BFU-E, exception made for patient 32, in which case no BFU-E grew. Interestingly, as shown in Table 3, despite the relatively low number of colonies analyzed, when comparing patients 12 and 33 at time 0 the percentage of cells carrying the *G12D* allele reflected the level of mutation found by allele-specific PCR (Fig. 2B).

***Chronic myelomonocytic leukemia patients with mutRAS might have a trend for a shorter life expectation.*** We analyzed the impact of *RAS* mutations on survival of chronic myelomonocytic leukemia patients. The median age was 74 years in *WT* and mutated groups. The group of patients presenting with *mutRAS* by allele-specific PCR ($n = 8$) had a significantly higher WBC count compared with the group with *RAS WT* ($n = 32$; median, $13.6 \times 10^9$/L versus $9.7 \times 10^9$/L, respectively; $P = 0.035$). Importantly, although median survivals of patients with *mutRAS* and *RAS WT* were not significantly different, possibly because of the limited sample size, a trend for a shorter life expectation, more evident after the first 2 years of follow-up, is suggested for patients harboring a *RAS* mutation in comparison with *WT* patients (median, 26.5 versus 42 months; Fig. 3).

## Discussion

Inclusion of chronic myelomonocytic leukemia into the new category of mixed myelodysplastic syndrome/myeloproliferative neoplasms (12) did not represent a conclusive solution with regard to the diverse clinical phenotypes of this malignancy. Indeed, because the prognostic significance of the distinction between MD-CMML and MP-CMML is still debated upon (5–10) and because the cutoff value of WBC to identify such groups is arbitrary, no general consensus has been reached on this subclassification dilemma. In agreement with previous reports (5, 7–9), our results support its prognostic value because MP-CMML patients had a significantly shorter survival than those with myelodysplastic disease.

We then investigated the biological implication of the myelodysplastic/myeloproliferative distinction, focusing in particular on the role of *RAS* mutations in chronic myelomonocytic leukemia evolution. To this aim, we conducted a longitudinal monitoring of chronic myelomonocytic leukemia course first by sequencing and then by allele-specific PCR. In fact, among the various assays developed for early detection of specific mutations, tracking of disease progression, and evaluation of molecular response to therapy, which are crucial issues in clinical practice, allele-specific PCR represents a simple, fast, and sensitive method (35, 36). Direct sequencing detected a significantly higher incidence of *RAS* mutations in MP-CMML patients, consistent with previous reports (1, 5, 7–9, 14). Next, for each mutation identified, we set up a highly selective yet simple and low-cost allele-specific PCR, which,

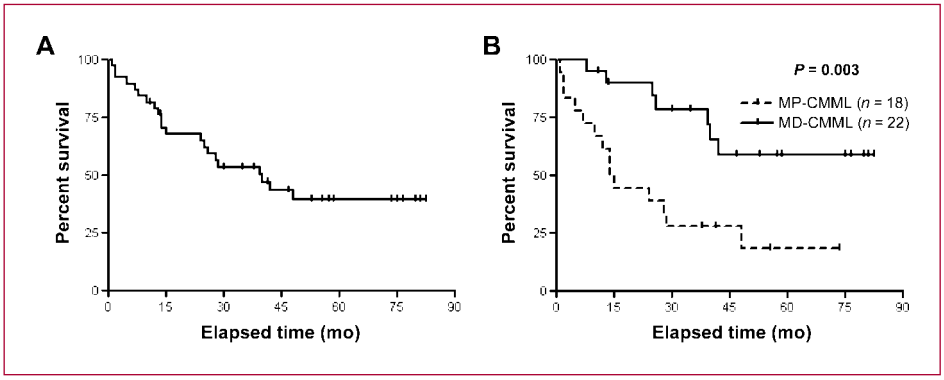


**Fig. 1.** Kaplan-Meier survival curves of chronic myelomonocytic leukemia patients. A, overall survival of all 40 patients with chronic myelomonocytic leukemia. B, survival of chronic myelomonocytic leukemia patients grouped by WBC count ($<13 \times 10^9$/L or $\geq 13 \times 10^9$/L). The log-rank test was used to analyze differences between the survival curves.

Published OnlineFirst April 14, 2010; DOI:10.1158/1078-0432.CCR-09-2112



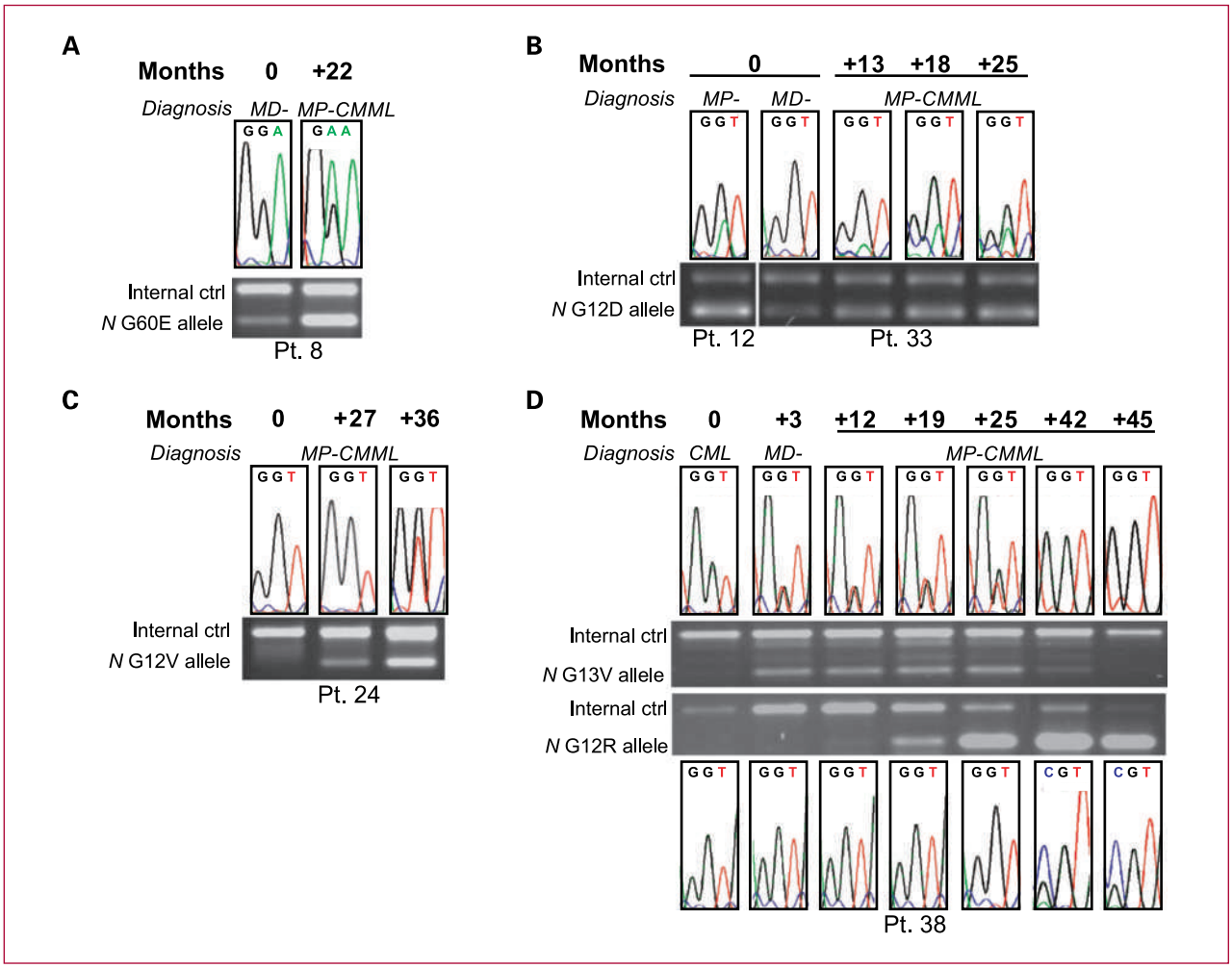


**Fig. 2.** Allele-specific PCR unveils the presence of RAS mutations much earlier than sequencing in a number of cases and provides evidence for the contribution of RAS mutations acquisition in chronic myelomonocytic leukemia progression to myeloproliferative phase. For each sample, the diagnosis is reported along with the results of RAS sequencing. A, allele-specific PCR for the *NRAS G60E* substitution (GGA→GAA) identified the mutant allele in the peripheral blood of patient 8 already at the time of presentation (0 mo; MD-CMML), whereas sequencing could detect it only after progression to MP-CMML (+22 mo). ctrl, control; Pt., patient. B, in patient 33 allele-specific PCR allowed to follow the increase of the *G12D* allele (GGT→GAT) throughout the course of disease, from MD-CMML to blunt MP-CMML, whereas sequencing suggested the presence of the mutation only 13 mo after presentation. Thirty-eight cycles of amplification were done in place of the canonical 39 to enhance the differences between the various samples. C, in patient 24, allele-specific PCR unveiled the presence of low levels of the *NRAS G12V* allele (GGT→GTT) 27 mo after the first increase of WBC to more than the $13 \times 10^9$/L threshold, whereas sequencing could detect the substitution only after further 9 mo (for more details on the clinical situation of patient 24, refer to the Discussion section). D, allele-specific PCR allowed to follow the consecutive occurrence of two clones harboring the *NRAS G13V* (GGT→GTT) and *G12R* mutations (GGT→CGT), respectively, earlier than sequencing in patient 38, who had a previous diagnosis of Philadelphia-positive CML (0 mo).

in our experimental conditions, resulted up to 20 times more sensitive than sequencing and did not require expensive equipment and proprietary reagents. Accordingly, at the time of first presentation or referral, allele-specific PCR detected a higher percentage of patients carrying a *RAS* mutation compared with sequencing. Moreover, allele-specific PCR allowed identification of a *mutRAS* allele in MD-CMML and MP-CMML patients, whereas sequencing only did in the latter group. Importantly, molecular monitoring done on matched presentation/follow-up pair samples from both MD-CMML patients presenting with a *RAS* mutation (patients 8 and 33) who experienced progression to myeloproliferative disease, unveiled at the time of myelodysplastic diseaselow levels of the mutations that became predominant in the myeloproliferative phase. Albeit few cases of MD-CMML progressing to myeloproliferative disease have been reported (1, 2, 8, 11), to the best of our knowledge, this is the first study documenting the appearance of *RAS* mutations in concomitance with chronic myelomonocytic leukemia evolution.

Allele-specific PCR was also able to detect the presence of a mutant allele earlier than sequencing during the course of myeloproliferative disease (patients 24 and 38). Indeed, in the case of patient 24, allele-specific PCR

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could identify the *G12V* mutation in the peripheral blood sample obtained during hydroxyurea (HU) treatment 27 months after the first withdrawal that coincided with the initial increase of WBC to more than the $13 \times 10^9$/L threshold. Because allele-specific PCR for the *G12V* allele has shown the lowest sensitivity out of the six allele-specific PCR developed, we cannot rule out the possibility that the mutated clone was already present at very low levels at the time of the first withdrawal. We may also speculate that HU reduced the mutated clone, thus preventing sequencing but not allele-specific PCR (which is far more sensitive) from identifying the *G12V* allele at the time of the second sample.

Finally, allele-specific PCR provided evidence for the occurrence of the *NRAS G12D* mutation in a progenitor common to the granulocytic/monocytic and erythroid precursors and in a percentage of cells that reflected the level of mutation detected in total DNA. In these regard, it would be interesting to further explore whether the mutation arises in the stem cells compartment because it was shown very recently in mouse models of myeloproliferative diseases (37, 38). Altogether, our findings suggest the occurrence of a *RAS* mutated clone in chronic myelomonocytic leukemia that eventually expanded, taking proliferative advantage on the *RAS WT* cells, and finally led to disease progression along with establishment of a stable myeloproliferative phenotype, which is associated with worse outcome. Notably, in agreement with the findings from the largest chronic myelomonocytic leukemia population ever analyzed (15), also in our cohort of patients the presence of *mutRAS* significantly correlated with higher WBC counts, whereas association with a shorter survival did not reach statistical significance possibly because of the limited sample size.

From a molecular point of view, albeit the mechanisms at the basis of chronic myelomonocytic leukemia pathogenesis are not fully understood, evidences from *in vivo* studies along with analysis of patient series suggest that multiple genetic events may contribute to disease development, especially in the proliferative forms. In particular, although *RAS* mutations seem to be the most commonly identified genetic aberration in MP-CMML, the *FLT3-ITD*

**Table 3.** Results of the single-colony analysis carried out by allele-specific PCR for the *NRAS G12D* substitution

| Pt. | No. of *NRAS G12D*$^+$ colonies/tot | |
|---|---|---|
| | CFU-GM | BFU-E |
| 12 | 8/8 | 10/10 |
| 32 | 0/8 | No growth |
| 33 | 4/6 | 2/7 |

Abbreviations: tot,, total; CFU-GM, colony-forming unit, granulocytes-macrophage.

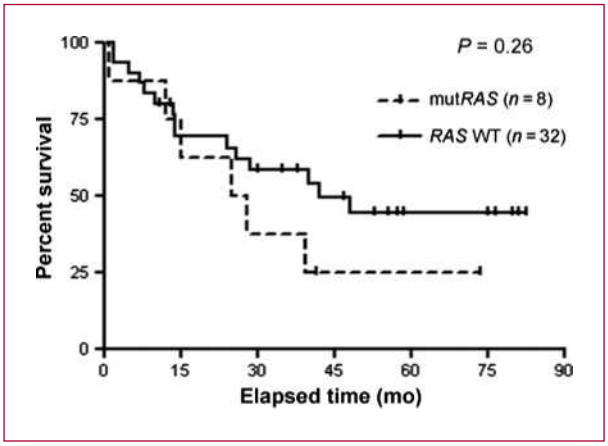


**Fig. 3.** Kaplan-Meier survival curves of patients with chronic myelomonocytic leukemia as a function of RAS mutations. The log-rank test was used to analyze differences between the survival curves.

(28) and the *JAK2 V617F* substitution (29, 30) have also been found in a minority of patients. Indeed, we detected the *FLT3-ITD* in a case of MD-CMML who rapidly progressed to MP-CMML and then to acute leukemia (patient 31) and the *JAK2 V617F* substitution in two cases classified as MP-CMML at the time of first presentation (patients 23 and 35; results not shown). Interestingly, the cumulative median survival of all patients ($n = 11$) presenting with a genetic aberration (*mutRAS*, *FLT3-ITD*, or *JAK2 V617F*, which are mutually exclusive) compared with those without ($n = 29$) was shorter (25 months versus not reached), and the difference reached statistical significance ($P = 0.049$). Given that specific inhibitors are currently under investigation for these aberrations (39, 40), their detection is of clinical relevance.

Based on these data, it may be hypothesized that the myelodysplastic/myeloproliferative distinction reflects different molecular subtypes of chronic myelomonocytic leukemia. In particular, as far as the MD-CMML is concerned, it might be supposed that pathogenetic events other than *RAS* mutations are responsible for its establishment and that acquisition of a secondary event like a *RAS* mutation or, in a minority of patients, the *FLT3-ITD* or the *JAK2 V617F* substitution causes the myeloproliferative phenotype. One possible candidate could be *TET2*, whose mutations have been recently identified in a wide spectrum of myeloid malignancies (19–26), leading to hypothesize that they are an early event in hematopoietic differentiation and that at least one additional event is required to determine the disease phenotype (19, 27). Moreover, Gelsi-Boyer et al. (14) have recently identified *RUNX1* alterations in a series of chronic myelomonocytic leukemia patients with MD-CMML and MP-CMML, whereas *RAS* mutations were absent in the former group. These results, together with the observation that *RAS* and *RUNX1* aberrations were not mutually exclusive, led the authors to speculate that *RUNX1* alterations might be responsible for dysplasia

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and *RAS* mutations for myeloproliferation. In contrast, Tyner et al. (41) found *RAS* mutations in MD-CMML and MP-CMML. One possible explanation for this discrepancy may be the different sensitivity of the methods used. Unfortunately, none of the two groups conducted a longitudinal analysis, neither gave information on the disease course of MD-CMML patients. Clearly, to elucidate the biology of chronic myelomonocytic leukemia, further investigations on larger groups of patients are needed.

In conclusion, albeit not all patients who experienced progression to myeloproliferative phase nor all patients presenting with a *de novo* myeloproliferative phenotype harbored a *mutRAS* allele, our results suggest the negative impact of *RAS* mutations on chronic myelomonocytic leukemia course. Therefore, also in light of the ongoing studies aimed at identifying effective Ras inhibitors (42–45), we believe that detection of *RAS* mutations in chronic myelomonocytic leukemia could be of clinical relevance. We propose screening by highly selective yet simple and low-cost allele-specific PCR to be implemented as a standard in chronic myelomonocytic leukemia diagnostics and disease monitoring to help clinical decision making.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Acknowledgments

We thank Simona Iacobelli, Ph.D. (Centro Interdipartimentale di Biostatistica e Bioinformatica, Università degli Studi di Roma "Tor Vergata," Rome, Italy), for the biostatistical advices.

## Grant Support

Associazione Italiana contro le Leucemie–Linfomi e Mieloma (sezione di Milano).

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Received 08/06/2009; revised 01/25/2010; accepted 02/10/2010; published OnlineFirst 04/06/2010.

## References

1. Onida F, Beran M. Chronic myelomonocytic leukemia: myeloproliferative variant. Curr Hematol Rep 2004;3:218–26.
2. Bowen DT. Chronic myelomonocytic leukemia: lost in classification? Hematol Oncol 2005;23:26–33.
3. Bennett JM, Catovsky D, Daniel MT, et al. Proposals for the classification of the myelodysplastic syndromes. Br J Haematol 1982;51:189–99.
4. Bennett JM, Catovsky D, Daniel MT, et al. The chronic myeloid leukaemias: guidelines for distinguishing chronic granulocytic, atypical chronic myeloid, and chronic myelomonocytic leukaemia. Proposals by the French-American-British Cooperative Leukaemia Group. Br J Haematol 1994;87:746–54.
5. Nosslinger T, Reisner R, Gruner H, et al. Dysplastic versus proliferative CMML—a retrospective analysis of 91 patients from a single institution. Leuk Res 2001;25:741–7.
6. Onida F, Kantarjian HM, Smith TL, et al. Prognostic factors and scoring systems in chronic myelomonocytic leukemia: a retrospective analysis of 213 patients. Blood 2002;99:840–9.
7. Beran M, Wen S, Shen Y, et al. Prognostic factors and risk assessment in chronic myelomonocytic leukemia: validation study of the M.D. Anderson Prognostic Scoring System. Leuk Lymphoma 2007;48:1150–60.
8. Voglova J, Chrobak L, Neuwirtova R, Malaskova V, Straka L. Myelodysplastic and myeloproliferative type of chronic myelomonocytic leukemia—distinct subgroups or two stages of the same disease? Leuk Res 2001;25:493–9.
9. Gonzalez-Medina I, Bueno J, Torrequebrada A, Lopez A, Vallespi T, Massague I. Two groups of chronic myelomonocytic leukaemia: myelodysplastic and myeloproliferative. Prognostic implications in a series of a single center. Leuk Res 2002;26:821–4.
10. Germing U, Gattermann N, Minning H, Heyll A, Aul C. Problems in the classification of CMML—dysplastic versus proliferative type. Leuk Res 1998;22:871–8.
11. Wang SA, Galili N, Cerny J, et al. Chronic myelomonocytic leukemia evolving from preexisting myelodysplasia shares many features with *de novo* disease. Am J Clin Pathol 2006;126:789–97.
12. Orazi A, Germing U. The myelodysplastic/myeloproliferative neoplasms: myeloproliferative diseases with dysplastic features. Leukemia 2008;22:1308–19.
13. Emanuel PD. Juvenile myelomonocytic leukemia and chronic myelomonocytic leukemia. Leukemia 2008;22:1335–42.
14. Gelsi-Boyer V, Trouplin V, Adelaide J, et al. Genome profiling of chronic myelomonocytic leukemia: frequent alterations of *RAS* and *RUNX1* genes. BMC Cancer 2008;8:299.
15. Beran M. Chronic myelomonocytic leukemia. In: Ansell SM, editor. Rare hematological malignancies. New York (NY): Springer; 2008, p. 107–32.
16. Parikh C, Subrahmanyam R, Ren R. Oncogenic NRAS rapidly and efficiently induces CMML- and AML-like diseases in mice. Blood 2006;108:2349–57.
17. Parikh C, Subrahmanyam R, Ren R. Oncogenic NRAS, KRAS, and HRAS exhibit different leukemogenic potentials in mice. Cancer Res 2007;67:7139–46.
18. Braun BS, Shannon K. Targeting Ras in myeloid leukemias. Clin Cancer Res 2008;14:2249–52.
19. Delhommeau F, Dupont S, Della Valle V, et al. Mutation in TET2 in myeloid cancers. N Engl J Med 2009;360:2289–301.
20. Tefferi A, Levine RL, Lim KH, et al. Frequent TET2 mutations in systemic mastocytosis: clinical, KITD816V and FIP1L1-PDGFRA correlates. Leukemia 2009;23:900–4.
21. Tefferi A, Lim KH, Abdel-Wahab O, et al. Detection of mutant TET2 in myeloid malignancies other than myeloproliferative neoplasms: CMML, MDS, MDS/MPN and AML. Leukemia 2009;23:1343–5.
22. Abdel-Wahab O, Mullally A, Hedvat C, et al. Genetic characterization of TET1, TET2, and TET3 alterations in myeloid malignancies. Blood 2009;114:144–7.
23. Jankowska AM, Szpurka H, Tiu RV, et al. Loss of heterozygosity 4q24 and TET2 mutations associated with myelodysplastic/myeloproliferative neoplasms. Blood 2009;113:6403–10.
24. Couronne L, Lippert E, Andrieux J, et al. Analyses of TET2 mutations in post-myeloproliferative neoplasm acute myeloid leukemias. Leukemia 2009.
25. Langemeijer SM, Kuiper RP, Berends M, et al. Acquired mutations in TET2 are common in myelodysplastic syndromes. Nat Genet 2009;41:838–42.
26. Kosmider O, Gelsi-Boyer V, Ciudad M, et al. *TET2* gene mutation is a frequent and adverse event in chronic myelomonocytic leukemia. Haematologica 2009.
27. Mullighan CG. TET2 mutations in myelodysplasia and myeloid malignancies. Nat Genet 2009;41:766–7.
28. Lee BH, Tothova Z, Levine RL, et al. FLT3 mutations confer enhanced proliferation and survival properties to multipotent progenitors in

Published OnlineFirst April 14, 2010; DOI:10.1158/1078-0432.CCR-09-2112



a murine model of chronic myelomonocytic leukemia. Cancer Cell 2007;12:367–80.
29. Jelinek J, Oki Y, Gharibyan V, et al. JAK2 mutation 1849G>T is rare in acute leukemias but can be found in CMML, Philadelphia chromosome-negative CML, and megakaryocytic leukemia. Blood 2005; 106:3370–3.
30. Pich A, Riera L, Sismondi F, et al. JAK2V617F activating mutation is associated with the myeloproliferative type of chronic myelomonocytic leukaemia. J Clin Pathol 2009;62:798–801.
31. Zinkel SS, Ong CC, Ferguson DO, et al. Proapoptotic BID is required for myeloid homeostasis and tumor suppression. Genes Dev 2003; 17:229–39.
32. Emanuel PD. Mixed myeloproliferative and myelodysplastic disorders. Curr Hematol Malig Rep 2007;2:9–12.
33. Tyner JW, Erickson H, Deininger MW, et al. High-throughput sequencing screen reveals novel, transforming RAS mutations in myeloid leukemia patients. Blood 2009;113:1749–55.
34. Niv H, Gutman O, Henis YI, Kloog Y. Membrane interactions of a constitutively active GFP-Ki-Ras 4B and their role in signaling. Evidence from lateral mobility studies. J Biol Chem 1999;274:1606–13.
35. Pao W, Ladanyi M. Epidermal growth factor receptor mutation testing in lung cancer: searching for the ideal method. Clin Cancer Res 2007;13:4954–5.
36. Archambeault S, Flores NJ, Yoshimi A, et al. Development of an allele-specific minimal residual disease assay for patients with juvenile myelomonocytic leukemia. Blood 2008;111:1124–7.
37. Zhang J, Wang J, Liu Y, et al. Oncogenic *Kras*-induced leukemogeneis: hematopoietic stem cells as the initial target and lineage-specific progenitors as the potential targets for final leukemic transformation. Blood 2009;113:1304–14.
38. Sabnis AJ, Cheung LS, Dail M, et al. Oncogenic *Kras* initiates leukemia in hematopoietic stem cells. PLoS Biol 2009;7:e59.
39. Small D. Targeting FLT3 for the treatment of leukemia. Semin Hematol 2008;45:S17–21.
40. Sayyah J, Sayeski PP. Jak2 inhibitors: rationale and role as therapeutic agents in hematologic malignancies. Curr Oncol Rep 2009;11: 117–24.
41. Tyner JW, Loriaux MM, Erickson H, et al. High-throughput mutational screen of the tyrosine kinome in chronic myelomonocytic leukemia. Leukemia 2009;23:406–9.
42. Rowell CA, Kowalczyk JJ, Lewis MD, Garcia AM. Direct demonstration of geranylgeranylation and farnesylation of Ki-Ras *in vivo*. J Biol Chem 1997;272:14093–7.
43. Feldman EJ, Cortes J, DeAngelo DJ, et al. On the use of lonafarnib in myelodysplastic syndrome and chronic myelomonocytic leukemia. Leukemia 2008;22:1707–11.
44. Chow LQ, Eckhardt SG, O'Bryant CL, et al. A phase I safety, pharmacological, and biological study of the farnesyl protein transferase inhibitor, lonafarnib (SCH 663366), in combination with cisplatin and gemcitabine in patients with advanced solid tumors. Cancer Chemother Pharmacol 2008;62:631–46.
45. Ganguly AK, Doll RJ, Girijavallabhan VM. Farnesyl protein transferase inhibition: a novel approach to anti-tumor therapy. The discovery and development of SCH 66336. Curr Med Chem 2001;8:1419–36.