at three other trinucleotides was also significantly higher in relapsed AML compared to presentation disease (Table 1), suggesting the presence of mutation signatures from other chemotherapy agents.

In summary, these data demonstrate that Ara-C preferentially induces mutation at $5\text{TpGpA}^3/\text{TpCpA}^3$ sequences which are significantly elevated in relapse disease after exposure to Ara-C-containing regimens. Given the relationship between Ara-C dose and mutagenicity reported here, a consideration of chemotherapy-induced mutagenicity could be important when developing strategies for treating AML that maximise the likelihood of remission whilst minimising the risk of mutation in surviving cells which could contribute to evolution of relapse disease.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

ACKNOWLEDGEMENTS
We are grateful to Ding et al.11 for making their primary whole genome sequencing data available for analysis. This work was supported by grants from Leukaemia and Lymphoma Research (#11006 and #13044, to JMA).

AUTHOR CONTRIBUTIONS
SEF and JMA designed and performed research, analysed data and wrote the paper. MC analysed data. JAI designed research. JMA conceived the project.

Supplementary Information accompanies this paper on the Leukemia website (http://www.nature.com/leu)

OPEN

MARIMO cells harbor a CALR mutation but are not dependent on JAK2/STAT5 signaling

Leukemia (2015) 29, 494–497 doi:10.1038/leu.2014.285

Mutations in calreticulin (CALR) were recently described to be present in the majority of patients with a JAK2-unmutated myeloproliferative neoplasm (MPN).12,3 This discovery has had rapid clinical impact, and testing for CALR has been embedded in national and international diagnostic guidelines.3,5 However, a human MPN-derived cell line harboring a CALR mutation has not been reported and the mechanisms by which mutated CALR results in an MPN remain unclear.

To begin to investigate the pathogenetic consequences of mutant CALR, we searched for patient-derived cell lines harboring CALR mutations. None were identified by exome sequencing of 1015 cell lines, including 37 derived from hematopoietic neoplasms.1 We therefore looked for cell lines derived from patients with leukemic transformation of a preceding MPN. Given that CALR and JAK2 mutations are almost completely mutually exclusive,2,6 we focused on four such lines known to lack a JAK2 mutation (MONO-MAC-6, MARIMO, GDM-1 and ELF-153), and also tested a further 52 other predominantly myeloid cell lines (Supplementary Table 1). Mutation screening was done by Sanger sequencing as previously described,1 and details of other methods are in the Supplementary Information.

The only cell line found to harbor a CALR mutation was MARIMO, originally derived from a 68-year-old female with AML-M2, and an antecedent history of ET.2 MARIMO is negative for JAK2V617F and MPL exon 10 mutations (data not shown) and carries a heterozygous 61-basepair (bp) deletion in CALR exon 9 (c.1099_1159del; L367fs*43), which, like all other reported CALR mutations, results in a +1-bp shift in the reading frame and thus generates a novel C terminus (Figure 1A).

In patients, the commonest two CALR mutations, accounting for 85% of cases, are a 52-bp deletion (type 1; c.1099_1150del; L367fs*46) and a 5-bp insertion (type 2; c.1154_1155ins;
K385fs*47). Both the type 1 deletion and the MARIMO deletion are immediately preceded by a nucleotide sequence identical to that at the 3' end of the deletion (Figure 1a). The 61-bp MARIMO deletion is readily detected by fragment analysis and represents a useful positive control for diagnostic clinical testing (Figure 1b).

**CALR** Amino acid sequence | AA
---|---
Wild type | QDEEQLKEEDEKKKKEEEDKEDEDEKKKEDEDEEEDEDEEDVPGQAEDLE 417
Type 1: L367fs*46 | QDEEQR TRPR 411
Type 2: K385fs*47 | QDEEQLKEEDEKKKKEEEDKEDEDEKKKEDEDEEEDEDEEDVPGQAEDLE (MARIMO) 430
MARIMO: L367fs*43 | QDEEQR TRPR HSC-70 408

**Figure 1.** Identification of a CALR-mutated human cell line. (a) Top panel shows the mutated region in CALR exon 9 (red bases). The commonest CALR mutations are shown above the DNA sequence. Solid gray line shows type 1 (52-bp deletion; c.1099_1150del; L367fs*46) and gray arrow shows type 2 (5-bp insertion; c.1154_1155_ins; K385fs*47 mutations). The CALR mutation in human cell line MARIMO is shown below the DNA sequence. Dashed gray and red lines represent the homologous sequence flanking the deleted regions in type 1 and MARIMO mutations, respectively, also highlighted in the capillary sequencing image (pale blue) for MARIMO. Lower panel shows the predicted protein sequence of the commonest CALR mutations and of MARIMO with total protein sizes. Amino acids (AA) in the new reading frame are shaded blue and the common novel peptide sequence shared by the different CALR variants are in bold blue. (b) PCR amplification of CALR exon 9 followed by fragment size analysis, as used for diagnostic testing for CALR mutations. Vertical heights of peaks represent dye signal intensity and horizontal position of peaks reflect the fragment size of the PCR amplicon. Wild type (wt) peak occurs at 132-bp. Left panel shows wt and mutated alleles of MARIMO (61-bp separation in peaks), middle panel shows Type 1/L367fs*46 with peak separation of 52 bp and right panel shows Type 2/K385fs*47 peaks separated by 5 bp. (c) Agarose gel image showing wt (upper band) and mutated-CALR (lower band) in MARIMO DNA and cDNA. (d) Quantitative real-time PCR of total CALR mRNA levels expressed as a fold change relative to house-keeping RPLP0 levels, for the cell lines MARIMO, the BCR-ABL1+ CML cell line K562, and the JAK2V617F+ cell lines HEL, UKE-1 and SET-2. Graph depicts all data points generated in two independent experiments performed in duplicate. ***P < 0.001 (e) Western blot showing total CALR protein levels of MARIMO and four other myeloid cell lines.
Allele-specific PCR demonstrated expression of the mutant CALR allele (Figure 1c). Compared with other cell lines derived from patients with JAK2V617F (HEL, UKE-1 and SET-2) or CML (K562) total CALR mRNA levels were 10-fold higher in MARIMO (Figure 1d) and total CALR protein levels were also increased albeit more modestly (Figure 1e).

MARIMO cells expressed cell surface marker CD15 but not other progenitor or lineage-affiliated markers (Supplementary Table 2). The proliferation and cell cycle status of MARIMO was unremarkable compared with other myeloid lines (Supplementary Figure 1). We next analysed cellular calcium stores since CALR has an important role in endoplasmic reticulum (ER) mediated calcium homeostasis and mutant CALR protein lacks variable numbers of calcium binding sites present in the wild-type C terminus. No significant differences in basal cytoplasmic calcium levels were found amongst the six cell lines tested (Figure 2a). Cell lines were then treated with 1 μM thapsigargin, which blocks ER Ca2+-ATPase channels resulting in ER calcium depletion and increased cytosolic calcium levels. MARIMO cells showed the slowest rate of increase of cytosolic calcium levels upon addition of thapsigargin (Figure 2b), consistent with the concept that mutant CALR alters ER dependent calcium homeostasis.

The mutual exclusivity of JAK2 and CALR mutations argues that they may share pathogenetic mechanisms and has been used to suggest that CALR mutations may activate JAK2/STAT5 signaling. This concept is supported by expression profiling of patient-derived granulocytes together with a report that expression of CALR in Ba/F3 cells confers interleukin-3 independence and is accompanied by increased STAT5 phosphorylation. However other studies have...
reported distinct transcriptional signatures in JAK2V617F-mutated and JAK2V617F-unmutated MPNs. Interpretation of these apparently conflicting results is complicated by several issues including limitations of overexpression systems, the uncertain relevance of granulocytes to disease pathogenesis and difficulties inherent to studies of signaling in primary cells containing variable proportions of mutant cells. To circumvent some of these issues, and to gain insight into the consequences of CALR mutations, we explored the properties of MARIMO cells.

The dependence of MARIMO cells on JAK signaling was initially assessed using the JAK inhibitors Tofacitinib (a JAK2/3 inhibitor) and JAK-inhibitor-I (a pan-JAK inhibitor) (Supplementary Figure 2). MARIMO cells were more resistant to both inhibitors than seven cell lines harboring mutant JAK2 or JAK3. Dose response studies using the clinically approved JAK- inhibitor Ruxolitinib (INCBO18424, a JAK1/2 inhibitor) showed that HEL and UKE-1 (both JAK2V617F positive) had IC50 values of 217 and 430 nm, respectively (Figure 2c). In marked contrast the IC50 value for MARIMO was greater than 10 000 nm, demonstrating that MARIMO was not dependent on JAK2 signaling.

Consistent with these data, western blot analysis showed that, compared with JAK2-mutant cells, MARIMO cells contained markedly reduced levels of JAK2, phosphorylated-JAK2 (pJAK2), STAT5 and pSTAT5 (Figure 2d). The lack of JAK2-STAT5 signaling was not accompanied by a compensatory increase in STAT1 or STAT5 and pSTAT5 (Figure 2d). The lack of JAK2-STAT5 signaling markedly reduced levels of JAK2, phosphorylated-JAK2 (pJAK2), JAK2

Consistent with these data, western blot analysis showed that, compared with JAK2-mutant cells, MARIMO cells contained markedly reduced levels of JAK2, phosphorylated-JAK2 (pJAK2), STAT5 and pSTAT5 (Figure 2d). The lack of JAK2-STAT5 signaling was not accompanied by a compensatory increase in STAT1 or STAT5 and pSTAT5 (Figure 2d). The lack of JAK2-STAT5 signaling markedly reduced levels of JAK2, phosphorylated-JAK2 (pJAK2), JAK2

ACKNOWLEDGEMENTS

We are grateful to Hitoshi Kiyoi (Nagoya University) for provision of cell line MARIMO. We thank Barbara Newman, Haematology-Oncologic Diagnostic Service, Addenbrooke’s Hospital, Cambridge, UK. Work in the Green lab is supported by Leukaemia and Lymphoma Research, Cancer Research UK, the NIHR Cambridge Biomedical Research Centre, the Cambridge Experimental Cancer Medicine Centre, and the Leukaemia and Lymphoma Society of America. WW is supported by the Austrian Science Foundation (J 3578-B21). JN is supported by a Kay Kendall Leukaemia Clinical Fellowship.

AUTHOR CONTRIBUTIONS

KK performed western blots and reverse transcriptase–PCR. WW performed calcium release-, inhibitor-, proliferation- and cell cycle assays; JN performed cell line screen; HQ and HD prepared cell lines; AB, EB performed clinical assays. All authors wrote and reviewed the manuscript. ARG directed the research.

K Kollmann 1,5, J Nangalia 1,2,3,5, W Warsch 1,5, H Quentmeier 4, A Bench 1, E Boyd 1, M Scott 1, HG Dreixel 1 and AR Green 1,3

1 Cambridge Institute for Medical Research, Wellcome Trust/MRC Stem Cell Institute and Department of Haematology, University of Cambridge, Cambridge, UK;

2 Wellcome Trust Sanger Institute, Hinxton, Cambridge, UK;

3 Cambridge University Hospitals NHS Foundation Trust, Cambridge, UK and

4 Leibniz-Institute DSMZ, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany

Correspondence: E-mail: vvw305@cam.ac.uk or arg1000@cam.ac.uk

These authors contributed equally to this work.

REFERENCES

1 Nangalia J, Massie CE, Baxter EJ, Nice FL, Gendum G, Wedge DC et al. Somatic CALR mutations in myeloproliferative neoplasms with nonmutated JAK2. N Engl J Med 2013; 369: 2391–2405.

2 Klampf T, Gisslinger H, Harutyunyan AS, Nivarthi H, Rumi E, Milosevic JD et al. Somatic mutations of calreticulin in myeloproliferative neoplasms. N Engl J Med 2013; 369: 2379–2390.

3 Tefferi A, Thiele J, Vannucchi AM, Barbi T. An overview on CALR and CSF3R mutations and a proposal for revision of WHO diagnostic criteria for myeloproliferative neoplasms. Leukemia 2014; 28: 1407–1413.

4 Reilly JT, McMullin MF, Beer PA, Butt N, Conneally E, Drumcombe AS et al. Use of JAK inhibitors in the management of myelofibrosis: a revision of the British Committee for Standards in Haematology Guidelines for Investigation and Management of Myelofibrosis 2012. Br J Haematol 2014; e-pub ahead of print 25 June 2014; doi:10.1111/bjh.12985.

5 Harrison CN, Butt N, Campbell P, Conneally E, Drummond M, Green AR et al. Modification of British Committee for Standards in Haematology diagnostic criteria for essential thrombocythaemia. Br J Haematol 2014; e-pub ahead of print 17 June 2014; doi:10.1111/bjh.12986.

6 Tefferi A, Lasho TL, Finke CM, Knudson RA, Ketterling R, Hanson CH et al. CALR vs. JAK2 vs MPL-mutated or triple-negative myelofibrosis: clinical, cytogenetic and molecular comparisons. Leukemia 2014; 28: 1472–1477.

7 Yoshida H, Kondo M, Ichihashi T, Hashimoto N, Inazawa J, Ohno R et al. A novel myeloid cell line, Marimo, derived from therapy-related acute myeloid leukemia during treatment of essential thrombocythemia: consistent chromosomal abnormalities and temporary C-MYC gene amplification. Cancer Genet Cytogenet 1998; 100: 21–24.

8 Guglielmelli P, Nangalia J, Green AR, Vannucchi AM. CALR mutations in myeloproliferative neoplasms: hidden behind the reticulum. Am J Hematol 2014; 89: 453–456.

9 Michalaki M, Groenendyk J, Szabo E, Gold LI, Opsa M, Calreticulin, a multi-process calcium-buffering chaperone of the endoplasmic reticulum. Biochem J 2009; 417: 651–666.

10 Wiemme C, Davis AS, Frost R, Lemmer J, Rada JI et al. A multi-process calcium-buffering chaperone of the endoplasmic reticulum, Biochem J 2009; 417: 651–666.

11 Wirome C, Henderson I, Lee AG, East JM. Mechanism of inhibition of the calcium pump of sarcoplasmic reticulum by thapsigargin. Biochem J 1992; 283: 525–529.

12 Rampal R, Al-Shahour F, Abdel-Wahab O, Patel JP, Brunel JP, Merrell CH et al. Integrated genomic analysis illustrates the central role of JAK-STAT pathway activation in myeloproliferative neoplasm pathogenesis. Blood 2014; 123: e123–e133.

13 Schwemmers S, Will B, Waller CF, Abdulkarim K, Johansson P, Andreasson B et al. JAK2V617F-negative ET patients do not display constitutively active JAK/STAT signaling. Exp Hematol 2007; 35: 1695–1703.

14 Puicdeganet E, Espinet B, Lozano JJ, Sumoy L, Bellosillo B, Arenillas L et al. Gene expression profiling distinguishes JAK2V617F-negative from JAK2V617F-positive patients in essential thrombocythemia. Leukemia 2008; 22: 1368–1376.

15 Passamonti F, Caramazza D, Maffolli M. JAK inhibitor in CALR-mutant myelofibrosis. N Engl J Med 2014; 370: 1168–1169.

16 Verstovsek S, Mesa RA, Gotlib J, Levy RS, Gupta V, D’Império JF et al. A double-blind, placebo-controlled trial of ruxolitinib for myelofibrosis. N Engl J Med 2012; 366: 799–807.

17 Verstovsek S, Kantarjian H, Mesa RA, Pardanani AD, Cortes-Franco J, Thomas DA et al. Safety and efficacy of INCBO18424, a JAK1 and JAK2 Inhibitor, in myelofibrosis. N Engl J Med 2010; 363: 1117–1127.

© 2015 Macmillan Publishers Limited

Leukemia (2015) 491–514

Supplementary Information accompanies this paper on the Leukemia website (http://www.nature.com/leu)