pressure. Accordingly, JMML clones with *SETBP1* mutation and/or *JAK3* mutation in addition to *PTPN11* mutation appear to be refractory to 6-MP. Allogeneic HSCT may be capable to eliminate such 6-MP-resitant JMML clones because three of the children are alive and disease free after HSCT. Further large-scale studies are needed to accurately establish the relationship between acquisition of the nonRAS pathway mutations and post-transplant outcomes in patients with *PTPN11* mutations.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank Ms Yumiko Oguchi, Department of Pediatrics, Shinshu University School of Medicine, for her technical support.

AUTHOR CONTRIBUTIONS

KK and KM designed and performed the research, collected the samples, analyzed the data and wrote the paper. YN designed the research. CI performed the research. TK, KH, SS, MT, KY, RY, KS and SS collected the samples and analyzed the data. All the authors read and approved the manuscript.

K Matsuda[1], Y Nakazawa[2], C Iwashita[1], T Kurata[2], K Hirabayashi[2], S Saito[2], M Tanaka[2], K Yoshikawa[2], R Yanagisawa[2], K Sakashita[2], S Sasaki[3], T Honda[1] and K Koike[2]

[1]*Department of Laboratory Medicine, Shinshu University Hospital, Matsumoto, Japan;*

[2]*Department of Pediatrics, Shinshu University School of Medicine, Matsumoto, Japan and*

[3]*Department of Pediatrics, Hirosaki University School of Medicine, Hirosaki, Japan*

E-mail: koikeken@shinshu-u.ac.jp

REFERENCES

1 Koike K, Matsuda K. Recent advances in the pathogenesis and management of juvenile myelomonocytic leukaemia. *Br J Haematol* 2008; **141**: 567–575.
2 Loh ML. Recent advances in the pathogenesis and treatment of juvenile myelomonocytic leukaemia. *Br J Haematol* 2011; **152**: 677–687.
3 Sakaguchi H, Okuno Y, Muramatsu H, Yoshida K, Shiraishi Y, Takahashi M *et al.* Exome sequencing identifies secondary mutations of SETBP1 and JAK3 in juvenile myelomonocytic leukemia. *Nat Genet* 2013; **45**: 937–941.
4 Piazza R, Valletta S, Winkelmann N, Redaelli S, Spinelli R, Pirola A *et al.* Recurrent SETBP1 mutations in atypical chronic myeloid leukemia. *Nat Genet* 2013; **45**: 18–24.
5 Damm F, Itzykson R, Kosmider O, Droin N, Renneville A, Chesnais V *et al.* SETBP1 mutations in 658 patients with myelodysplastic syndromes, chronic myelomonocytic leukemia and secondary acute myeloid leukemias. *Leukemia* 2013; **27**: 1401–1403.
6 Makishima H, Yoshida K, Nguyen N, Przychodzen B, Sanada M, Okuno Y *et al.* Somatic SETBP1 mutations in myeloid malignancies. *Nat Genet* 2013; **45**: 942–946.
7 Meggendorfer M, Bacher U, Alpermann T, Haferlach C, Kern W, Gambacorti-Passerini C *et al.* SETBP1 mutations occur in 9% of MDS/MPN and in 4% of MPN cases and are strongly associated with atypical CML, monosomy 7, isochromosome i(17)(q10), ASXL1 and CBL mutations. *Leukemia* 2013; **27**: 1852–1860.
8 Thol F, Suchanek KJ, Koenecke C, Stadler M, Platzbecker U, Thiede C *et al.* SETBP1 mutation analysis in 944 patients with MDS and AML. *Leukemia* 2013; **27**: 2072–2075.
9 Shiba N, Ohki K, Park MJ, Sotomatsu M, Kudo K, Ito E *et al.* SETBP1 mutations in juvenile myelomonocytic leukaemia and myelodysplastic syndrome but not in paediatric acute myeloid leukaemia. *Br J Haematol* 2013; **164**: 156–159.
10 Matsuda K, Matsuzaki S, Miki J, Hidaka E, Yanagisawa R, Nakazawa Y *et al.* Chromosomal change during 6-mercaptopurine (6-MP) therapy in juvenile myelomonocytic leukemia: the growth of a 6-MP-refractory clone that already exists at onset. *Leukemia* 2006; **20**: 485–490.
11 Matsuda K, Nakazawa Y, Sakashita K, Shiohara M, Yamauchi K, Koike K. Acquisition of loss of the wild-type NRAS locus with aggressive disease progression in a patient with juvenile myelomonocytic leukemia and a heterozygous NRAS mutation. *Haematologica* 2007; **92**: 1576–1578.
12 Tartaglia M, Niemeyer CM, Fragale A, Song X, Buechner J, Jung A *et al.* Somatic mutations in PTPN11 in juvenile myelomonocytic leukemia, myelodysplastic syndromes and acute myeloid leukemia. *Nat Genet* 2003; **34**: 148–150.
13 Kratz CP, Niemeyer CM, Castleberry RP, Cetin M, Bergsträsser E, Emanuel PD *et al.* The mutational spectrum of PTPN11 in juvenile myelomonocytic leukemia and Noonan syndrome/myeloproliferative disease. *Blood* 2005; **106**: 2183–2185.
14 Matsuda K, Sakashita K, Taira C, Tanaka-Yanagisawa M, Yanagisawa R, Shiohara M *et al.* Quantitative assessment of PTPN11 or RAS mutations at the neonatal period and during the clinical course in patients with juvenile myelomonocytic leukaemia. *Br J Haematol* 2010; **148**: 593–599.

OPEN

Serial exome analysis of disease progression in premalignant gammopathies

Leukemia (2014) **28,** 1548–1552; doi:10.1038/leu.2014.59

Nearly all human cancers are preceded by precursor states far more prevalent than clinical malignancy itself. Understanding which premalignant lesions are truly biologically premalignant versus those with impending clinical progression (biologically malignant) is a central question in cancer biology. Tracking the progression of human premalignant states (or lack thereof) *in vivo* is not feasible in most tumors because precursor lesions are typically resected at diagnosis. Multiple myeloma (MM) is characterized by progressive growth of malignant plasma cells (PCs) in the bone marrow leading to organ dysfunction.[1] Nearly all cases of clinical MM are preceded by asymptomatic monoclonal gammopathies (AMGs) further classified as either monoclonal gammopathy of undetermined significance (MGUS) or asymptomatic myeloma (AMM).[1,2] MM is a unique model to dissect genetic evolution of early cancer as the precursor state is well defined and not resectable. Several studies have characterized the genetic architecture of malignant PCs in both MM and its precursor states.[3] MM tumor cells carry several cytogenetic abnormalities (most notably, IgH translocations and hyperdiploid karyotypes), and copy number abnormalities leading to genomic gains/losses and loss of heterozygosity (LOH). Comparisons of PCs in cohorts of MM versus MGUS/AMM show greater proportion of cytogenetically abnormal PCs in MM, suggesting the expansion of pre-existing and more proliferative clones during transition to MM.[4,5] However, nearly all of the cytogenetic changes described in MM tumor cells have also been observed in MGUS/AMM, and it has proven difficult to define the malignant phenotype on the basis of genetic changes alone.[4,5] Whole-genome and whole-exome-based sequencing strategies

Accepted article preview online 5 February 2014; advance online publication, 25 February 2014

have recently been applied to human MM and shown that there are about 20–40 non-synonymous variants per MM tumor cell, but no single unifying variant has been identified.[6–8] The most common targets of recurrent mutation were known cancer genes, such as *KRAS* and *TP53*, with *KRAS* mutations in 27% of the cases.[6] Analysis of serial samples from MM patients undergoing therapy recently demonstrated the presence of subclones, with greater changes in patients with cytogenetically high-risk disease.[9,10] Similar findings were made *via* serial genome sequencing in a patient with t(4:14) myeloma evolving to PCs leukemia.[8] In this study, we serially analyzed the exomes of patients with AMGs who progressed to clinical MM and compared the findings with those AMGs that did not progress to clinical MM.

Whole-exome sequencing was performed on tumor and germline DNA from 10 patients with AMGs (exome run summary in Supplementary Table 1, see Supplementary Methods online). Clinical characteristics of this cohort are shown in Supplementary Table 2 and these patients could be classified as MGUS ($n=3$) or AMM ($n=7$) by current criteria. Four of these patients (P1–P4) progressed to clinical MM requiring therapy, whereas 6 patients

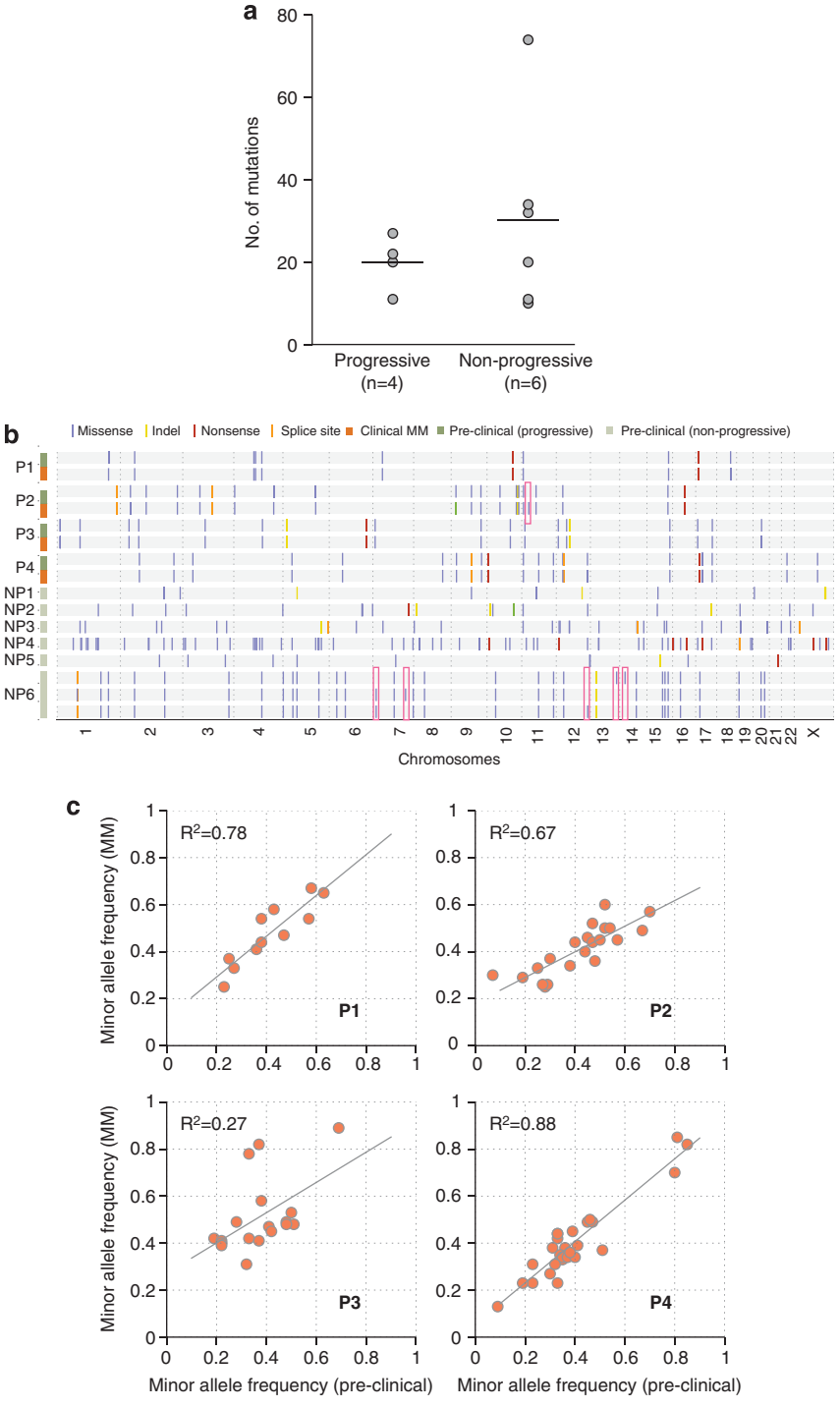

Figure 1. Analysis of somatic variants. (**a**) Number of somatic variants per sample. (**b**) Serial analysis of somatic variants in each patients. (**c**) Analysis of B-allele frequencies in paired samples.

(NP1–NP6) that did not progress with at least 2 years of follow-up were studied as controls. Overall 261 somatic non-synonymous variants (SNVs) were seen in these 10 patients, with a mean of 26 variants per patient (range 10–74). The mean number of SNVs per sample was similar between patients with progressive or non-progressive disease (Figure 1a). In patients who progressed to clinical MM (P1–P4), the sequencing data from baseline was directly compared with that from the corresponding malignancy. In three of the paired samples, there were no new SNVs detected in the progression sample (Figure 1b). In the fourth patient (P2),

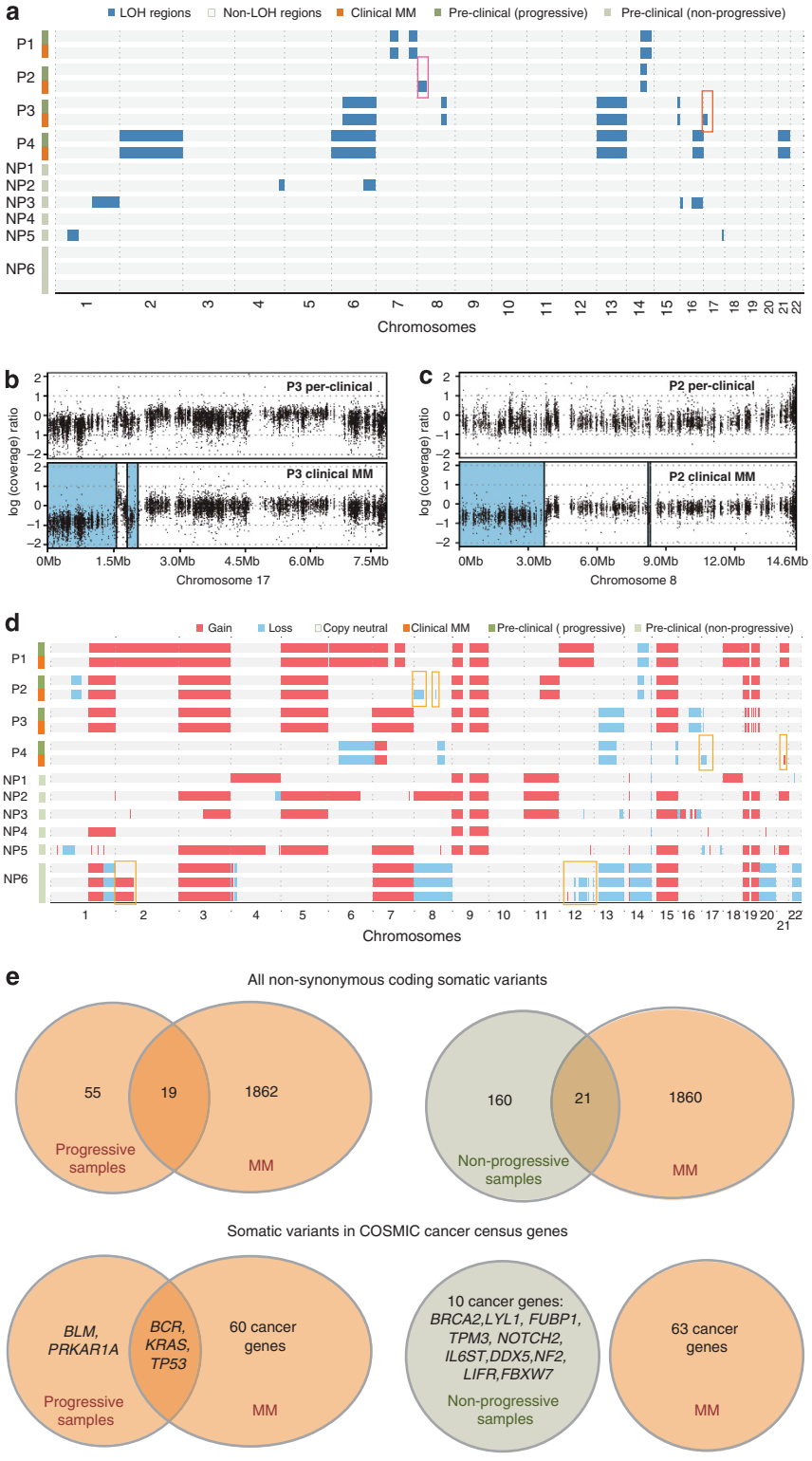

Figure 2. Analysis of genomic gains/losses with disease progression. (**a**) Analysis of LOH patterns. (**b**) Analysis of copy number variation (CNV) pattern in chromosome 17 in patient P3. (**c**) Analysis of CNV pattern in chromosome 8 in patient P2. (**d**) Analysis of CNVs. (**e**) Comparison of mutational profile with published data in patients with clinical myeloma.

a new SNV in a single gene (*BBOX1*) of unclear significance was detected in the progression sample. The lack of significant evolution from baseline to progression was also confirmed through analysis of minor/alternate-allele frequencies in the paired samples (Figure 1c). Variant calls from all progression samples were validated using Sanger sequencing (not shown). Serial samples (at 2 and 4 years of follow-up) were also available from a patient (NP6) without disease progression. These samples again demonstrated stable SNV profile, with balanced loss and gain of two new detectable SNVs at each time point (Figure 1b). Together these data indicated that nearly all of the SNVs found at the diagnosis of clinical MM were also present in the precursor state.

In view of lack of consistent progression-specific SNVs, we turned our attention to regions of somatic copy number alterations (CNAs), intra-clonal variation and pattern of somatic mutations. Analysis of LOH patterns revealed that the degree of LOH at baseline was much greater in patients with Prog-AMG than in NonProg-AMG, although there was no consistent pattern shared by all samples (Figure 2a). In the patients who progressed to MM, the LOH regions at MM stage matched those at baseline with little change. However, in two patients (P2 and P3), new regions of MM-specific LOH were identified (Figures 2a–c). Interestingly, in one of these patients (P3), the region of new LOH correlated with the site of p53 on chromosome 17 (Figure 2b) and possibly involved a dominant subclone as the contribution of the cells carrying the deletion was 10% lower than the estimated fraction of tumor cells in this sample. Profile of somatic CNAs again revealed several regions of alterations throughout the genome, which were again largely maintained between baseline and progression samples (Figure 2d). Together these data demonstrate that patients with progressive lesions may have a greater degree of genomic gains/losses compared with those that remain clinically asymptomatic.

An important and unique aspect of this study is the concurrent analysis of samples from patients with AMG that did not progress to clinical MM. Therefore, we compared the targets of somatic mutations in this cohort with those published for patients with MM.[6–8] Targets of somatic mutations in Prog-AMGs had a greater overlap than NonProg-AMGs with the MM dataset (19 of 74 (26%) in Prog-AMG versus 20 of 181 (11%) in NonProg-AMG, $P = 0.01$) (Figure 2e). This was also true when mutations in known oncogenes in the COSMIC database overlapping between the current AMG cohorts and the MM datasets were compared (3 of 5 in Prog-AMG versus 0 of 10 in NonProg-AMG) (Figure 2e). Notably, this included *KRAS* and *TP53* wherein mutations were only detected in Prog-AMG, but not in patients with NonProg-AMG. Therefore, the pattern of mutations at baseline may correlate with the risk of progression to MM.

These data demonstrate that the vast majority of somatic mutations in MM are present at least several months and possibly years before the onset of symptoms indicative of the diagnosis of clinical malignancy. Similar findings were recently reported by Walker *et al.*[11] who compared exomes from four patients with AMM and corresponding MM. Interestingly, the net mutation load was similar between progressive and non-progressive lesions, demonstrating that long periods of clinical stability are feasible in AMGs in spite of the level of mutational load comparable to that seen in clinical MM. Instead of the number of mutations, the pattern of mutations and genomic changes at baseline may impact the risk of developing clinical malignancy. In such a model, lesions that lack mutations in critical genes are unable to mediate progressive growth needed for malignant phenotype. The list of targets preferentially mutated in patients with Prog-AMGs consisted of well-known cancer genes, such as *KRAS* and *TP53*.[6] The presence of mutations in *KRAS* in myeloma has been previously correlated with an aggressive course and disease progression.[12] Cytogenetic deletion of chromosome 17p (carrying *TP53*) has also been well-documented as a feature of high-risk MM. Mutations in *TP53* have also been described in MM and correlate with the presence of del17p.[13,14] Further studies in asymptomatic patients are needed to identify additional mutations that may predict the increased risk of malignancy.

Somatic LOH and CNA analyses indicated that the genomes in Prog-AMG also carried greater regions of genomic loss/gains than in patients with NonProg-AMG. These data suggest that the degree of genomic instability may itself be a marker of the risk for malignant transformation. In some instances, it is possible that these secondary changes may contribute more directly to malignant transformation through evolution of a subclone. For example, in patient P3, progression to MM was accompanied with the appearance of new LOH interval in chromosome 17p, including *TP53*.

To our knowledge, these data provide the first direct comparison of serial exomes in progressive versus non-progressive precursor states in human cancer. In contrast to recent studies with myelodysplasia and acute leukemia, the precursor state in this setting was observed without any specific therapy, therefore, the findings are not impacted by possible therapy-induced effects.[15] Our findings demonstrate that most somatic mutations and genome alterations predate clinical malignancy and that mutational loads comparable to clinical malignancy could be associated with long periods of dormancy in premalignant states. Instead of net mutational load, the pattern of mutations and the degree of genomic instability may predict malignant fate. Our findings should be considered as preliminary due to the small numbers of AMG exomes sequenced thus far. Analysis of spectrum of mutations in precursor states may identify lesions that are more likely to progress to clinical cancer.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

S Zhao[1,2], M Choi[1,2,3], C Heuck[4], S Mane[5,6], B Barlogie[4], RP Lifton[1,2,6] and MV Dhodapkar[6,7]
[1]*Department of Genetics, Yale University, New Haven, CT, USA;*
[2]*Howard Hughes Medical Institute, Yale University, New Haven, CT, USA;*
[3]*Department of Biomedical Sciences, Seoul National University College of Medicine, Seoul, Korea;*
[4]*Myeloma Institute for Research and Therapy, Little Rock, AR, USA;*
[5]*Yale Center for Genome Analysis, Yale University, New Haven, CT, USA;*
[6]*Yale Cancer Center, Yale University, New Haven, CT, USA and*
[7]*Section of Hematology, Yale University, New Haven, CT, USA*
E-mail: madhav.dhodapkar@yale.edu

REFERENCES

1 Landgren O, Kyle RA, Rajkumar SV. From myeloma precursor disease to multiple myeloma: new diagnostic concepts and opportunities for early intervention. *Clin Cancer Res* 2011; **17**: 1243–1252.
2 Dhodapkar MV, Sexton R, Waheed S, Usmani S, Papanikolaou X, Nair B *et al.* Clinical, genomic and imaging predictors of myeloma progression from asymptomatic monoclonal gammopathies (SWOG S0120). *Blood* 2013; **123**: 78–85.
3 Morgan GJ, Walker BA, Davies FE. The genetic architecture of multiple myeloma. *Nat Rev Cancer* 2012; **12**: 335–348.
4 Lopez-Corral L, Gutierrez NC, Vidriales MB, Mateos MV, Rasillo A, Garcia-Sanz R *et al.* The progression from MGUS to smoldering myeloma and eventually to multiple myeloma involves a clonal expansion of genetically abnormal plasma cells. *Clin Cancer Res* 2011; **17**: 1692–1700.
5 Lopez-Corral L, Sarasquete ME, Bea S, Garcia-Sanz R, Mateos MV, Corchete LA *et al.* SNP-based mapping arrays reveal high genomic complexity in monoclonal gammopathies, from MGUS to myeloma status. *Leukemia* 2012; **26**: 2521–2529.

6 Chapman MA, Lawrence MS, Keats JJ, Cibulskis K, Sougnez C, Schinzel AC *et al.* Initial genome sequencing and analysis of multiple myeloma. *Nature* 2011; **471**: 467–472.
7 Walker BA, Wardell CP, Melchor L, Hulkki S, Potter NE, Johnson DC *et al.* Intraclonal heterogeneity and distinct molecular mechanisms characterize the development of t(4;14) and t(11;14) myeloma. *Blood* 2012; **120**: 1077–1078.
8 Egan JB, Shi CX, Tembe W, Christoforides A, Kurdoglu A, Sinari S *et al.* Whole genome sequencing of multiple myeloma from diagnosis to plasma cell leukemia reveals genomic initiating events, evolution and clonal tides. *Blood* 2012; **120**: 1060–1066.
9 Keats JJ, Chesi M, Egan JB, Garbitt VM, Palmer SE, Braggio E *et al.* Clonal competition with alternating dominance in multiple myeloma. *Blood* 2012; **120**: 1067–1076.
10 Weston-Bell N, Gibson J, John M, Ennis S, Pfeifer S, Cezard T *et al.* Exome sequencing in tracking clonal evolution in multiple myeloma following therapy. *Leukemia* 2013; **27**: 1188–1191.
11 Walker BA, Wardell CP, Melchor L, Brioli A, Johnson DC, Kaiser MF *et al.* Intraclonal heterogeneity is a critical early event in the development of myeloma and precedes the development of clinical symptoms. *Leukemia* 2013; **28**: 384–90.
12 Neri A, Murphy JP, Cro L, Ferrero D, Tarella C, Baldini L *et al.* Ras oncogene mutation in multiple myeloma. *J Exp Med* 1989; **170**: 1715–1725.
13 Chng WJ, Price-Troska T, Gonzalez-Paz N, Van Wier S, Jacobus S, Blood E *et al.* Clinical significance of TP53 mutation in myeloma. *Leukemia* 2007; **21**: 582–584.
14 Xiong W, Wu X, Starnes S, Johnson SK, Haessler J, Wang S *et al.* An analysis of the clinical and biologic significance of TP53 loss and the identification of potential novel transcriptional targets of TP53 in multiple myeloma. *Blood* 2008; **112**: 4235–4246.
15 Walter MJ, Shen D, Ding L, Shao J, Koboldt DC, Chen K *et al.* Clonal architecture of secondary acute myeloid leukemia. *N Engl J Med* 2012; **366**: 1090–1098.

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

Prognostic effect of calreticulin mutations in patients with myelofibrosis after allogeneic hematopoietic stem cell transplantation

Leukemia (2014) **28,** 1552–1555; doi:10.1038/leu.2014.66

Recently, somatic frameshift mutations in exon 9 of *calreticulin* (*CALR*) have been identified in a large proportion of *JAK2* and *MPL* negative myelofibrosis and essential thrombocythemia (ET) patients.[1,2] The *calreticulin* gene encoding a 48 kDa protein is located on chromosome 19p13.3, and regulates key cellular functions like proliferation and apoptosis.[3,4] *CALR* mutations have been identified in 15.5 to 31.4% of unselected ET patients corresponding to 48.9–82% of *JAK2/MPL* negative ET patients,[2,5] and in 15.6–35% of unselected myelofibrosis patients, corresponding to 56–88% of *JAK2/MPL* negative myelofibrosis patients.[1,2] Thus, *CALR* mutations will become an important diagnostic marker for myeloproliferative neoplasms.[6]

Klampfl *et al.*[1] showed that *CALR* mutated primary myelofibrosis (PMF) patients have lower white blood cell counts and higher platelet counts compared with *JAK2* mutated patients and that these patients survive significantly longer than *JAK2* and *MPL* mutated patients. In addition, Tefferi *et al.*[7] reported an association of mutated *CALR* with younger age, lower Dynamic International Prognostic Scoring System (DIPSS)-plus risk score and low frequency of spliceosome mutations, and showed that *CALR/JAK2/MPL* triple negative patients had the worst prognosis.

Allogeneic hematopoietic stem cell transplantation (alloHSCT) is the only curative treatment option in patients with myelofibrosis, but is restricted to patients who have higher risk PMF and who can tolerate allogeneic transplantation.[8,9] It is yet unclear whether mutated *CALR* has a prognostic effect in patients with myelofibrosis undergoing alloHSCT. We therefore evaluated the incidence and prognostic impact of *CALR* mutations in 133 patients with primary or secondary myelofibrosis who underwent alloHSCT.

A total of 133 patients with PMF ($n = 97$, 73%) or post-ET/post-polycythemia vera myelofibrosis ($n = 36$, 27%) according to WHO classification criteria underwent allogeneic HSCT in two German university medical centers (Hamburg and Hannover). Bone marrow or peripheral blood samples were obtained before transplantation. This study was conducted in accordance with the Declaration of Helsinki, and the scientific analysis of the samples was approved by the institutional review board of Hannover Medical School. *CALR* was amplified using the following primers: *CALR* forward 5′-CTGGTCCTGGTCCTGATGTC-3′ and *CALR* reverse 5′-CCAAATCCGAACCAGCCTG-3′. The PCR fragments were directly sequenced and analyzed using Mutation Surveyor software (SoftGenetics, State College, PA, USA). Additional details of genetic and statistical analyses are found in the data supplement.

The median age at transplant in the whole cohort was 57 years (range 18–75). The majority of patients ($n = 90$, 67%) were transplanted with a matched donor (29 from related and 61 from unrelated donors). A total of 43 patients (32%) received a mismatched unrelated allograft. The conditioning regimen was of reduced intensity in all but one patient. Two patients received bone marrow (2%) and 131 patients peripheral blood stem cells (98%). After HSCT, 53 patients (40%) developed stage I–II acute graft-versus host disease (GvHD), 22 patients (17%) stage III–IV acute GvHD, and 68 patients (51%) suffered from chronic GvHD. A total of 45 patients died by the time of last follow-up (34%). The median follow-up was 4.04 years after transplantation. Donor match had no significant impact on overall survival (OS), cumulative incidence of relapse (CIR) and non-relapse mortality (NRM) (Supplementary Figures S1A–C). OS was similar between patients with primary and secondary myelofibrosis ($P = 0.91$, Supplementary Figure S2), and therefore we combined these patient groups for outcome analysis.

A total of 28 patients had mutated *CALR* (21%), corresponding to 52% patients with wild-type *JAK2* and wild-type *MPL*. The most frequent mutations were the previously described 52 basepair deletion (61%) and the 5 basepair insertion (13%) in exon 9 of *CALR* (Supplementary Table S1). Comparing patients with ($n = 28$) and without ($n = 105$) *CALR* mutations, there were no significant differences for patient and disease characteristics (patient age,

Accepted article preview online 7 February 2014; advance online publication, 21 February 2014