Flow-FISH evaluation of telomere length in Philadelphia-negative myeloproliferative neoplasms

Telomeres are repeated DNA sequences localized at the end of chromosomes and involved in maintaining chromosomal stability and integrity.[1] Telomere length (TL) is considered a reliable marker of cell turnover, as telomeres progressively shorten with aging and cell proliferation. In various hematologic malignancies, hematopoietic cells have been shown to be characterized by shortened telomeres.[1] So far, a few studies have evaluated the role of TL in myeloproliferative neoplasms (MPN).[2-6]

We analyzed TL in a cohort of MPN patients in order to investigate its relationship with biological and clinical parameters. We studied 139 consecutive MPN patients, including 42 cases of polycythemia vera (PV), 63 essential thrombocythemia (ET), 18 primary myelofibrosis (PMF), 12 post-PV MF, 4 post-ET MF followed at the Department of Hematology Oncology, Fondazione IRCCS Policlinico San Matteo, University of Pavia, Italy. Results were compared with findings in a cohort of 65 healthy subjects. This study was approved by the Ethics Committee, Fondazione IRCCS Policlinico San Matteo, Pavia, and the procedures followed were in accordance with the Helsinki Declaration of 1975, as revised in 2000.

Demographic and hematologic characteristics of patients are listed in Table 1. TL was measured in both peripheral blood (PB) granulocytes and lymphocytes by flow-FISH, as previously described,[7] with at least 30,000 events acquired for each sample. The age-corrected value of TL in granulocytes (deltaTEL$_{gran}$) was obtained by subtracting the individual patient's measurement from the linear regression line established for healthy subjects: consequently, the shorter the patient's TL value, the more negative the deltaTEL$_{gran}$ value.

As a first step, we evaluated telomere fluorescence in PB granulocytes and lymphocytes, expressed as Molecular Equivalent of Soluble Fluorescence (MESF), from MPN patients and compared it to that of control subjects. In healthy individuals a significant negative linear correlation was found between telomere fluorescence of both granulocytes and lymphocytes and age (Spearman's rho -0.90; P<0.001), whereas in MPN patients this relationship was retained in lymphocytes (r -0.30; P<0.001) but lost in granulocytes (r -0.07; P=0.40) (Figure 1A), suggesting that in MPN patients the impact of a clonal hematopoiesis exceeds the expected effect of demographic parameters on telomere shortening.

When considering the age-corrected value, patients with MPN showed a significantly lower deltaTEL$_{gran}$ (median value -11.14, range -23.99 to 9.78) than healthy individuals (-0.05, - 4.74 to 5.75; P<0.001) (Figure 1B). In keeping with published data,[4,5] we did not find any significant difference in deltaTEL$_{gran}$ among MPN subtypes (P=0.249): median deltaTEL$_{gran}$ in PV patients was –11.09 (25th percentile -16.90, 75th percentile -7.30), in ET -11.43 (-17.04 to -7.01) and in PMF, post-PV MF and post-ET MF -11.44 (-16.16 to -1.18). Moreover, no significant difference was observed between deltaTEL$_{gran}$ in chronic phase of PV and ET and in their relative fibrotic evolution (P=0.64 and P=0.84, respectively). The wide variability in TL observed in PMF patients might reflect the heterogeneity of biological and pathogenetic features of this disease, even if further data from a larger cohort of patients are needed to investigate this observation thoroughly. In contrast, disease duration significantly affected deltaTEL$_{gran}$ (Spearman's rho -0.16; P=0.049), also when adjusted by MPN subtypes in multivariate analysis (P=0.01).

Overall, 97 of 139 (70%) patients carried the *JAK2* (V617F) mutation, evaluated with a quantitative AS-PCR.[8,9] The deltaTEL$_{gran}$ was similarly shortened in *JAK2* (V617F)-positive and *JAK2* (V617F)-negative patients. This result was confirmed both by analyzing MPN patients as a whole (P 0.81) and according to MPN subtype (ET P=0.69, PMF P=0.44). In contrast, no significant relationship was observed between deltaTEL$_{gran}$ and percentage of mutant alleles, either when considering this latter as a continuous variable (Spearman's rho 0.03; P=0.73), or when comparing patients with less than 50% or 50% or more mutant alleles (P=0.56). Furthermore, we did not found any difference in deltaTEL$_{gran}$ between patients carrying an *MPL* mutation[10] (6 out of 99 evaluable patients, 6%) and patients with wild-type *MPL* (P=0.46). Taken together, these data confirm that patients with MPN present shortened telomeres irrespective of their *JAK2* or *MPL* mutational status, implying that different molecular mechanisms may affect stem cells in the same way, and suggest that telomere assessment may be of potential diagnostic value in the work up of suspected MPN.

Finally, we investigated the effect of cytoreductive therapy on telomere shortening. At the time of TL evaluation, 87 of 139 patients were on cytoreductive therapy, mainly hydroxyurea. Our analysis showed that deltaTEL$_{gran}$ was not affected by cytoreductive therapy *per se* (P=0.93) nor by its duration (Spearman's ρ -0.07; P=0.42). Future evaluation of the impact of interferon α[11] or innovative drugs[12] on TL in MPN might be of interest.

Table 1. Demographic and hematologic characteristics of 139 MPN patients at the time of telomere length evaluation.

Variables	Patients
N. of subjects	139
Median follow up, months (range)	73.6 (3.8-365.2)
Median age, years (range)	59.1 (20.1-92.2)
Male/female	62/77
Diagnosis:	
PV	42
ET	63
post-PV MF	12
post-ET MF	4
PMF	18
Disease duration, months (range)*	57.6 (0-328.3)
JAK2 (V617F)-positive patients, n (%)	97/139 (70)
JAK2 (V617F) allele burden (%), median (range)	33.8 (6.4-99.8)
JAK2 (V617F) allele burden ≥50%, n (%)	37/97 (38)
MPL-mutated patients, n (%)	6/99 (6)

*MPN: myeloproliferative neoplasms; PV: polycythemia vera; ET: essential thrombocythemia; post-PV MF: post-polycythemia vera myelofibrosis; post-ET MF: post-essential thrombocythemia myelofibrosis; PMF primary myelofibrosis. *Disease duration was defined from diagnosis to the time of telomere length evaluation*

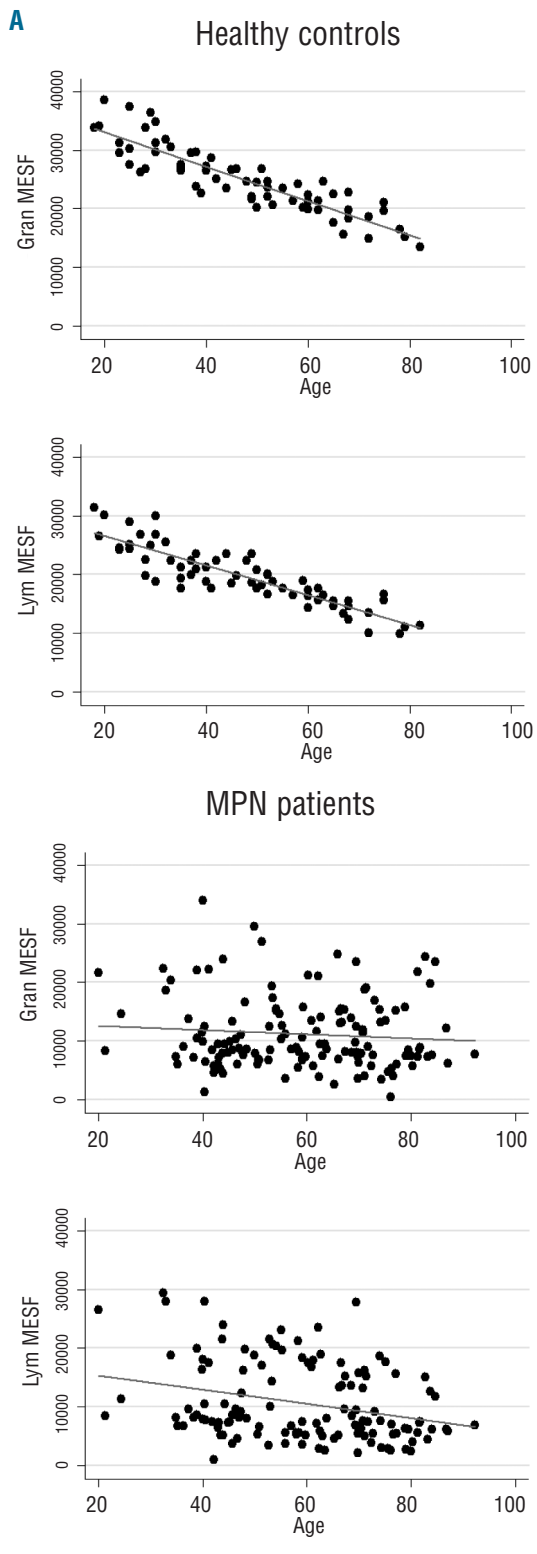

Figure 1. Dynamics of telomere shortening in patients with myeloproliferative neoplasm (MPN) and in healthy subjects. (A) In healthy controls Spearman's rank test revealed a significant negative linear correlation between telomere fluorescence, expressed as Molecular Equivalent of Soluble Fluorescence (MESF), and age both in peripheral blood (PB) granulocytes and lymphocytes (P<0.001). In contrast, in MPN patients this relationship was present in PB lymphocytes (P<0.001) but not in granulocytes (P=0.40). (B) Age-corrected telomere length (DeltaTEL$_{gran}$) is significantly shorter in MPN patients (median value -11.14, range -23.99 to 9.78) than in healthy individuals (-0.05, -4.74 to 5.75; Kolmogorov-Smirnov's test P<0.001).

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DNMT3A mutations are rare in childhood acute myeloid leukemia

Childhood acute myeloid leukemia (AML) is a complex disease of the hematopoietic stem cell. Overall survival is relatively low with an overall survival rate of 50-70%. Besides cytogenetic changes and response to induction therapy, molecular aberrations are important prognostic markers that can help to risk stratify children with AML. Molecular aberrations include mutations in *FLT3*,[1] *NPM1*,[2] *CEBPA*,[3] *WT1*[4] with the recent addition of *IDH1* and *IDH2*.[5] In a significant number of pediatric and adult AML patients, no known mutation or cytogenetic aberration can be identified. It is, therefore, believed that a large number of gene mutations in AML are still to be identified. Recently, somatic mutations in *DNA methyltransferase 3A* (*DNMT3A*) have been found in adult AML[6,7] but the incidence and prognostic impact in childhood AML is unknown. DNMT3A is involved in epigenetic regulation of genes by enzymatic *de novo* addition of methyl groups to the cytosine residue of CpG dinucleotides. Mutations in *DNMT3A* occur in approximately 20% of adult AML patients. Interestingly, a mutational hotspot in codon R882 located in the catalytic methyltransferase domain has been reported to present approximately 60% of all mutations while the remaining 40% are located throughout the gene with the main focus in the methyltransferase domain.[6,7] Ley *et al.* described an adverse prognostic impact of the mutation for adult AML patients[6] which has been confirmed by our group and others.[7,8] Hence, mutations in *DNMT3A* appear to play an important role as a novel prognostic marker in adult AML. However, so far little is known about the frequency and prognostic impact of *DNMT3A* mutations in childhood AML. The only study to date which looks at *DNMT3A* mutations in a cohort of 180 children with AML did not identify any *DNMT3A* mutations associated with disease.[9] Here we report the frequency, and clinical and molecular characteristics of *DNMT3A* mutations in a well defined cohort of 195 pediatric AML patients. Bone marrow (BM) or peripheral blood (PB) samples from initial diagnosis were obtained from 195 pediatric AML patients. Details regarding the clinical and molecular characteristics of the study cohort are shown in Table 1. All patients were treated within two prospective multicenter trials: the AML-Berlin-Frankfurt-Münster (BFM) 98 or the 2004 (NCT00111345), as previously described.[10-12] The studies were approved by the protocol review committee of the German Cancer Society and by

Table 1. Main clinical and biological features of the study cohort.

Characteristic	Number (n =195)	% 100
Age, years		
median	8.75	
Sex		
male - n. (%)	98	50.3
female - n. (%)	97	49.7
Study		
AML-BFM 98	30	15.4
AML-BFM 04	165	84.6
FAB-subtype		
M0	4	2.1
M1	26	13.4
M2	35	18
M3	9	4.6
M4	57	29.2
M5	42	21.5
M6	2	1
M7	15	7.7
missing data	5	2.6
M. Down		
no	190	97.4
yes	4	2.1
unknown	1	0.5
Cytogenetic standard risk*		
no	121	62.1
yes	48	24.6
unknown	26	13.3
Bone marrow blasts day 15		
≤ 5%	131	67.2
> 5%	40	20.5
missing data	24	12.3
WBC count		
median - (x10^9/L)	31.4	
range - (x10^9/L)	0.8-585	
Platelet count		
median - (x10^9/L)	64	
range - (x10^9/L)	17-376	
FLT3-ITD – n. (%)		
mutated – n. (%)	25	12.8
wild type – n. (%)	123	63.1
missing – n. (%)	47	24.1
NPM1		
mutated – n. (%)	22	11.3
wild type – n. (%)	116	59.5
missing – n. (%)	57	29.2

AML-BFM, multicenter treatment trials AML-Berlin-Frankfurt-Münster (BFM); FAB: French-American-British classification of acute myeloid leukemia; WBC: white blood cell count; FLT3-ITD, internal tandem duplication of the FLT3 *gene; NPM1, nucleophosmin 1 gene. *Cytogenetic standard risk, chromosomal aberrations including t(8;21), inv 16 or t(15;17).*