Dysregulation of shelterin factors coupled with telomere shortening in Philadelphia chromosome negative myeloproliferative neoplasms

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Supplementary data
1. Supplementary methods
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Supplementary methods

Blood cell separation

Peripheral blood was collected from all patients in ETDA-tubes and separated (Hetasep, Stem cell technologies) according to the manufacturer's recommendations and the pellets were resuspended in PBS with 10% FCS. Remaining cells were gradient separated by Ficoll (GE Healthcare). Cells were then washed and pelleted into lymphoid cells and granulocytes, respectively.

Flow-FISH for TL analysis

Flow FISH of whole blood was performed as described in previous protocols with slight modifications.[1,2] Calf thymus cells were kindly donated from the butchery Ö-slakt AB (Värmdö, Stockholm) and prepared as previously described.[26] All readings were made with a Gallios flow cytometer (Beckman Coulter) and analyzed using the Kaluza software (Beckman Coulter). Fluorescent MESF-FITC beads (Bangs Laboratories) were used for quantification and the fluorescent signal was quantified using the QuickCal v.2.3 data analysis program (Bangs Laboratories). Telomere length was calculated using the following formula:

$$Telomere\ length, kB = \frac{MESF \times n_{base}}{n_{chr} \times 1000}$$

where MESF is the fluorescent intensity given by QuickCal v.2.3, n_{base} is the number of bases per PNA probe and n_{chr} is the number of chromosomes in the specimen of origin of the cells.

Cell culture

The erythroleukemia cell line HEL (JAK2$^{V617F+/+}$) (DSMZ) was grown in RPMI 1640 complemented with 10% FBS, 1% L-glutamine and 1% penicillin-streptomycin (all from Gibco, Life Technologies). Cells were cultured in the presence of 3μM of the JAK2^{V617F} inhibitor LY2784544 (Sellek Chemicals) for 24 hrs and then harvested for POT1 mRNA and protein analysis.

RNA isolation and quantitative reverse transcription-PCR (qPCR)

Total cellular RNA in granulocytes from MPN patients and healthy controls was isolated using Trizol reagent (Life Technologies) and diluted to 100 ng/µl. Two µg RNA was used for reverse transcription using M-MLV (Life Technologies) according to the manufacturer's recommendation. Real time amplification was performed in triplicate using SYBR Green PCR Master Mix (Life Technologies) with QuantStudio 7 Flex Teal-Time PCR system (Applied biosystems). All primers were purchased from Invitrogen and their sequences are presented in table S1.

Protein expression analyses

Proteins were extracted from granulocytes of 9 patients and 9 healthy controls using Trizol (Life Technologies) according to the manufacturer's protocol. HEL cells were lysed in RIPA buffer (Abcam). The protein concentration was measured with DC protein assay (Biorad) and diluted to equal concentrations. Separation and transfer were performed using mini-proteanTGX gels and transblot turbo system (Biorad), followed by blocking in 5% Blotting grade blocker (Biorad) and 1% BSA in TBS for 1h. Primary antibodies were incubated overnight 4°C and secondary antibodies for 1h at RT. The antibodies used were anti-RAP1 (Active Motif), anti-TPP1 (Abcam), anti-POT1 and anti-TIN2 (Novus Biologicals) anti-actin-HRP and anti-Rabbit-HRP (Santa Cruz) and anti-Mouse-HRP (Biorad). Intensity of blots were measured in the program *ImageJ* (developed at the National Institute of Health and available at http://rsb.info.nih.gov/ij/), using the "Gel Analysis" function.

JAK2 V617F allele burden analysis

The relative quantification of the JAK2 V617F c.1849G>T allele burden in DNA samples from 31 MPN patients was performed using duplicate qPCR. The common forward primer, the reverse primers for wild type and mutant type sequences and common probe are listed in Table S1. The PCR amplification was run with primer concentrations at 300nmol/l and probe

concentration at 200 nmol/l with an standard activation step of 10 min at 95°C, followed by 45 cycles of 15 s at 95°C and 60 s at 60°C on a ABI Prism7000 (Applied Biosystems, Foster City, CA, USA). The levels of the mutated JAK2 V617F allele burden in all samples was compared to the reference genomic DNA levels of the JAK2 V617F homozygous mutated DNA (HEL cells) diluted at tenfold with wildtype DNA.

Statistical analyses

All statistical analyses were made in Excel using 2-tailed Student's t-test for analysis of telomere length and expression of telomere binding proteins. For correlation analyses Pearson's correlation coefficient was generated using the correlation tool in Excel's Analysis toolPak add-in software. A t-value was generated using the following formula;

$$t = \frac{r \times \sqrt{n-2}}{\sqrt{1-r^2}}$$

The T-distribution calculation tool in Excel was then used to generate a p-value. For all calculations two tailed distribution was used. Age adjustment of telomere length was performed in the GraphPad prism 5 software using the ANCOVA-based function "compare slopes and intercepts".

References

1. Roos G, Hultdin M. Flow cytometric determination of telomere length. Cytometry. 2001;45(1):79-80.

2. Baerlocher GM, Vulto I, de Jong G, Lansdorp PM. Flow cytometry and FISH to measure the average length of telomeres (flow FISH). Nat Protoc. 2006;1(5):2365-2376.

Table S1. The primers for qPCR, telomere length assessment and JAK2^{V617F} allele burden

qPCR primer sequences

Target	Forward	Reverse
TRF1	5'-GCTGTTTGTATGGAAAATGGC-3'	5'-CCGCTGCCTTCATTAGAAAG-3'
TRF2	5'- GACCTTCCAGCAGAAGATGCT-3'	5'-GTTGGAGGATTCCGTAGCTG-3'
TPP1	5'- CCCGCAGAGTTCTATCTCCA-3'	5'-GGACAGTGATAGGCCTGCAT-3'
TIN2	5'- GGAGTTTCTGCGATCTCTGC-3'	5'-GATCCCGCACTATAGGTCCA-3
POT1	5'-TGGGTATTGTACCCCTCCAA-3'	5'-GATGAAGCATTCCAACCACGG-3'
RAP1	5'-CGGGGAACCACAGAATAAGA-3'	5'-CTCAGGTGTGGGTGGATCAT-3'
KU80	5'-CCCCAATTCAGCAGCATATT-3'	5'-CCTTCAGCCAGACTGGAGAC-3'
hTERT	5'-CGGAAGAGTGTCTGGAGCAA-3'	5'-GGATGAAGCGGAGTCTGGA-3'
β2-M	5'-GAATTGCTATGTGTCTGGGT-3'	5'-CATCTTCAAACCTCCATGATG-3'

Primers for telomere length

Tel1b	5'-CGGTTTGTTTGGGTTTGGGT-TTGGGTTTGGGTTTGGGTT-3'
Tel2b	5'-GGCTTGCCTTACCCTTACCCTTACCC-TTACCCTTACCCT-3'
HBG3	5'-TGTGCTGGCCCATCACTTTG-3'
HBG4	5'-ACCAGCCA-CCACTTTCTGATAGG-3'

Primers and probe for JAK2^{V617F} allele burden assay

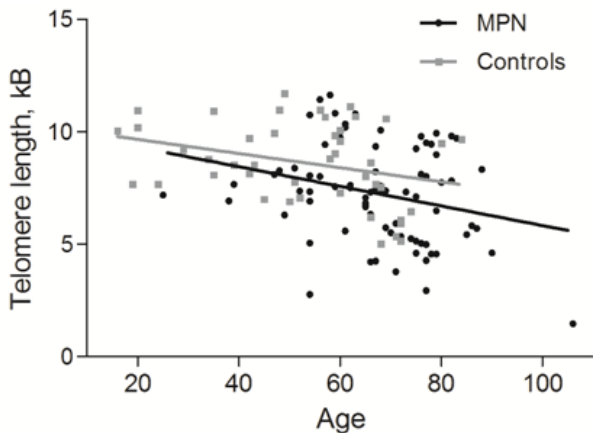|	
Forward	5'-CTTTCTTTGAAGCAGCAAGTATGA-3'
Reverse (wt)	5'-GTAGTTTTACTTACTTACTCTCGTCTCCACAtAC-3'
Reverse (mt)	5'-GTAGTTTTACTTACTTACTCTCGTCTCCACAtAA-3'
Probe	6-FAM-TGAGCAAGCTTTCTCACAAAGCATTTGGTTT-TAMRA

Figure S1 Age-related telomere shortening in granulocytes from both normal healthy individuals and MPN patients. Telomere length was determined using Flow-FISH and expressed as Kilobases (KB). *There was a high correlation between age and telomere length (r = -0.34, P <0.001).*
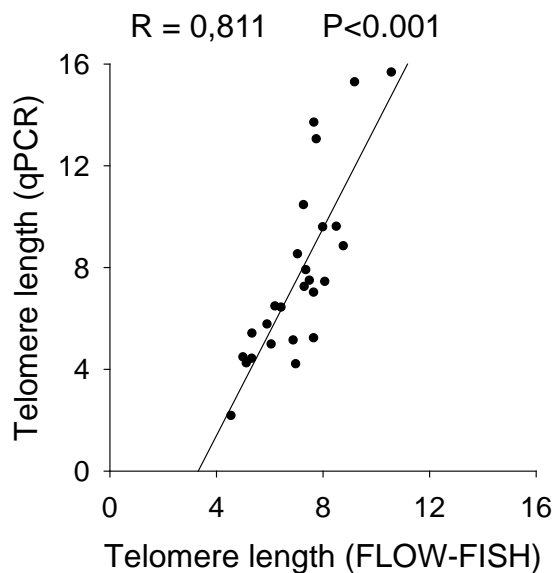
Figure S2. The correlation of telomere length as determined using FLOW-FISH and quantitative PCR (qPCR). Granulocytes derived from 26 MPN patients or healthy individuals were analyzed for telomere length using FLOW-FISH. DNA derived from these same individuals' granulocytes was also analyzed for telomere length using qPCR (Cawthon RM. Nucleic Acids Res 2002; 30: e47). Two methods show a highly consistent results in all the analyzed samples.
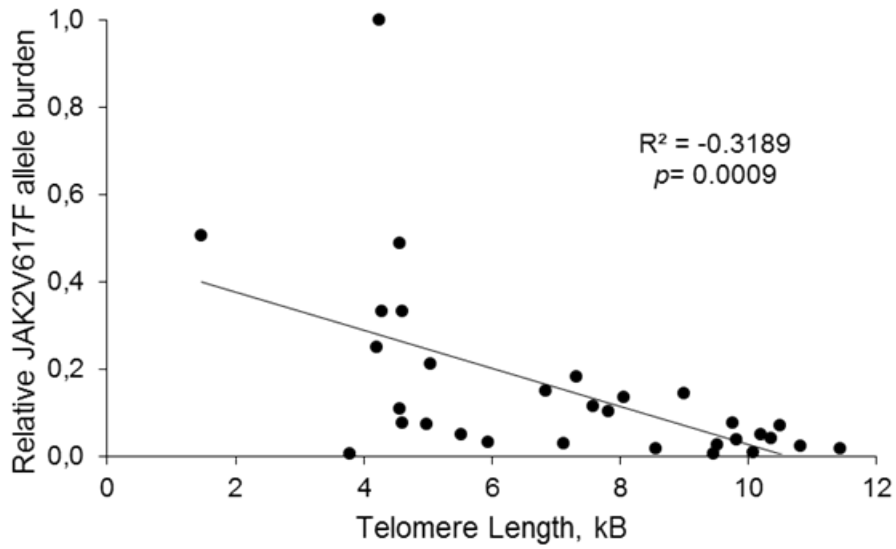
Figure S3 The negative correlation between JAK2^{V617F} burdens and telomere length in MPN patients (N=31). Telomere length was determined using Flow-FISH and expressed as Kilobases (KB). There was a significant correlation between JAK2^{V617F} and telomere length *(r = -0.34, P <0.001)*.
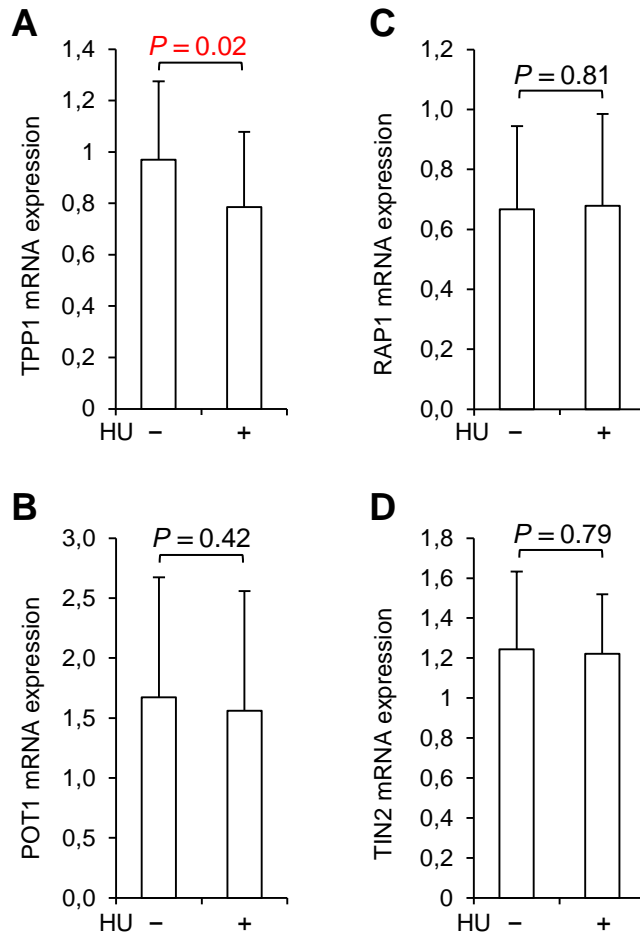
Figure S4 Hydroxyurea treatment leads to the inhibition of TPP1 mRNA expression in granulocytes from MPN patients. The treatment information was available in 74 patients and 50 of them received HU while the rest 24 were either never treated (8) or treated with interferon or other agents (16). TPP1 mRNA expression was significantly lower in HU-treated patients (HU-: 0.97±0.30, HU+: 0.78 ±0.29, P = 0.02) (A). Such a relationship was not observed in POT1 (B) RAP1 (C) and TIN2 (D) expression. HU: Hydroxyurea.