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Research paper

Replicative senescence of hematopoietic cells in patients with idiopathic cytopenia of undetermined significance

Dong-Yeop Shin[a,b,1], Jin Kyun Park[c,1], Chih Chiao Li[d], Hee Sue Park[e], Soo Young Moon[f], Sung-Min Kim[g], Kyongok Im[g], Yoon Hwan Chang[h], Sung-Soo Yoon[a,g], Dong-Soon Lee[d,g,*]

[a] Division of Hematology and Medical Oncology, Department of Internal Medicine, Seoul National University Hospital, Seoul, South Korea
[b] Biomedical Research Institute, Seoul National University Hospital, Seoul, South Korea
[c] Division of Rheumatology, Department of Internal Medicine, Seoul National University Hospital, Seoul, South Korea
[d] Department of Laboratory Medicine, Seoul National University Hospital, Seoul, South Korea
[e] Department of Laboratory Medicine, Chungbuk National University Hospital, Cheongju, South Korea
[f] Department of Laboratory Medicine, Pusan National University Hospital, Busan, South Korea
[g] Cancer Research Institute, Seoul National University College of Medicine, Seoul, South Korea
[h] Department of Laboratory Medicine, Korea Cancer Center Hospital, Seoul, South Korea

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ABSTRACT

We hypothesized that a subset of idiopathic cytopenia of undetermined significance (ICUS) is associated with an increased autonomous proliferation with exhaustion of hematopoiesis. The aim of this study was to investigate the cell turnover rate and replicative history of the bone marrow cells of ICUS patients. To this end, we examined telomere length (TL), proliferation, and apoptosis of the bone marrow cells of ICUS patients and healthy controls (HCs) using telomere quantitative fluorescence *in situ* hybridization and immunohistochemical staining for Ki-67 and cleaved caspase-3. We also performed targeted sequencing of 88 myeloid-associated genes. A total of 37 patients with ICUS were enrolled in this study, with a median age of 66 years (range: 31–83). TLs were significantly shorter in patients with ICUS than in the HCs (8.8, interquartile range [IQR] 6.8–12.1 vs 18.4, IQR 14.4–22.0, $p < 0.0001$). Proliferation (Ki-67-positive) and apoptosis (cleaved caspase-3-positive) were significantly increased in patients with ICUS compared to HCs (median = 20.0% vs 5.0%, $p = 0.0003$; 45.0% vs 22.5%, $p = 0.0005$, respectively). The shortening of TL and the increased proliferation and apoptotic activity was also prominent in patients with ICUS without mutation and dysplasia than in HCs ($p < 0.0001$, $p < 0.0001$, and $p = 0.0093$, respectively). TL was not associated with mutational profile and clinical characteristics as well in patients with ICUS. To our knowledge, this is the first study to show that ICUS is associated with premature replicative senescence with increased proliferation and apoptosis of bone marrow cells. Further study is needed to address the cause of replicative exhaustion in ICUS patients.

1. Introduction

Idiopathic cytopenia of undetermined significance (ICUS) has recently been proposed as a distinct disease entity defined as persistent cytopenia of unidentified cause. The diagnosis of ICUS is entertained when the etiology of cytopenia remains unexplained after careful evaluation, including bone marrow examination [1]. Although the pathogenesis of ICUS is largely unknown, recent studies suggest that a subset of ICUS could be regarded as a “premalignant” state of hematologic neoplasms such as myelodysplastic syndrome (MDS) or leukemia, because genetic mutations that are commonly associated with MDS are often also present in ICUS [2]. This ICUS subset tends to progress into overt myeloid neoplasm over time, as genetic mutations accumulate in hematopoietic stem/progenitor cells (HSPC) [2,3]. However, more than a half of patients with ICUS still show the persistent cytopenia despite the absence of overt clonal abnormality. We hypothesized that the premalignant HSPC clones in ICUS may proliferate at a higher rate, resulting in increased cell turnover with a premature replicative senescence. In addition, the increased proliferation may be automatically associated with increased apoptosis due to ineffective hematopoiesis,

* Corresponding author at: Department of Laboratory Medicine, Seoul National University Hospital, Cancer Research Institute, Seoul National University College of Medicine, 101 Daehag-ro, Seoul, 03080, South Korea.
E-mail address: soonlee@snu.ac.kr (D.-S. Lee).
[1] D.-Y.S. and J.K.P. contributed equally to this work.

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leading to peripheral cytopenia.

A key feature of cellular senescence is telomeric erosion [4]. The telomere is a region of repetitive nucleotide sequences at the end of a chromosome that maintains chromosomal integrity by protecting sticky ends from fusion with neighboring chromosomes. Telomere length (TL) is determined by telomere attrition during cell division and telomere prolongation by telomerase [5], making it a surrogate marker of replicative history [6,7] Abnormal TL is observed in various hematologic malignancies such as accelerated telomere shortening in MDS [8,9].

In this study, we investigated whether HSPCs in ICUS have increased cell turnover leading to replicative exhaustion with accelerated premature telomere shortening, and whether this is associated with clinical manifestations and long-term prognosis of ICUS patients.

2. Materials and methods

2.1. Patients

We included patients diagnosed with ICUS at Seoul National University Hospital between 1999 and 2013 in this study. A diagnosis of ICUS was defined as follows: cytopenia in at least one lineage for 6 months or longer, exclusion of common causes of cytopenia (offending drug, vitamin deficiency, or viral infection), and MDS or any other hematologic or non-hematologic disease [1].

Peripheral blood cytopenia was defined as absolute neutrophil count < 1800/μL, hemoglobin < 10.0 g/dL, and platelet count < 100 $\times 10^3$/μL. The degree of cytopenia was graded from 0 to 4 by the Common Terminology Criteria for Adverse Events (CTCAE) version 4.0 proposed by the National Cancer Institute. Samples from two other cohorts were used as healthy controls (HCs) as follows: (1) bone marrow mononuclear cells obtained from 29 normal healthy individuals were used to compare with the TL of patients with ICUS. (2) Normal bone marrow biopsy samples from 20 healthy individuals were used as controls to compare immunohistochemical staining results of Ki-67 and cleaved caspase-3.

This study was approved by the institutional review board at our institution. All procedures including study treatment, follow-up, and data collection were conducted in accordance with the Declaration of Helsinki.

2.2. Telomere Q-FISH

Cryopreserved samples were used for telomere analysis by quantitative fluorescence in situ hybridization (Q-FISH). Q-FISH was performed using a Cy3-labeled telomere peptide nucleic acid (PNA) FISH kit (DakoCytomation Denmark A/S, Glostrup, Denmark) and a fluorescein isothiocyanate (FITC)-labeled PNA probe for the centromere of chromosome 2 (kindly provided by DakoCytomation). The centromere probe (1 μL) was added to 10 μL of the telomere probe. Telomere and centromere Q-FISH hybridizations were then performed according to the manufacturer's instructions. Interphase Q-FISH images were captured with a Zeiss Axioplan 2 imaging microscope (Carl Zeiss MicroImaging GmbH, Munchen, Germany) equipped with ISIS software (MetaSystems GmbH, Altlussheim, Germany). The ISIS-Telomere module (MetaSystems) was used to measure TL as previously described [10]. A telomere-to-centromere fluorescence intensity ratio (T/C) of each cell was computed into TL. At least 25 interphase nuclei were scanned for each patient. TLs shorter than 10% the length of those of the normal control were defined as "extremely short".

2.3. Immunohistochemistry

Immunohistochemical staining for caspase-3 and Ki-67 was performed on bone marrow biopsy sections. A paraffin-embedded tissue block was divided into 2-μm sections and each slide was stained using the Ventana BenchMark ULTRA automated staining platform (Ventana Medical Systems Inc., Tucson, AZ, USA). The cleaved caspase-3 (1:100; Cell Signaling Technology, Danvers, MA, USA) and Ki-67 (1:100; DAKO, Glostrup, Denmark) monoclonal antibodies were applied for 15 min at room temperature. Two expert hematopathologists independently reviewed the stained slides and measured the percentage of cells that were positive for Ki-67 and cleaved caspase-3.

2.4. Targeted sequencing

Targeted sequencing of 87 hematologic malignancy-associated genes was performed using next generation sequencing with an Illumina HiSeq 2500 platform (Illumina, San Diego, CA, USA). Detailed methods for targeted sequencing and data processing are describe elsewhere [11].

2.5. Statistics

Statistical analyses for clinical parameters were performed using Stata version 13 (Stata Corp., College Station, TX, USA). The Mann-Whitney test was used to compare the TLs and the Ki-67 (%)/cleaved caspase-3 (%) (K/C) ratio. Chi-square test and logistic regression analysis were used to investigate the association between TL and clinical variables.

3. Results

3.1. Patients

A total of 37 patients with ICUS were enrolled in this study. The median age was 66 (range, 31–83) years. Twenty-two patients (59.5%) were female. Baseline characteristics including the clinical information about peripheral cytopenia in our patients was described in Table 1.

3.2. Shortened TL in ICUS

The median TL was shorter in patients with ICUS than in HCs (8.77, interquartile range [IQR]: 6.82–11.72, 95% confidence interval [CI]: 8.19–10.67 vs 18.36, IQR: 14.5–21.64, 95% CI:,16.22–19.80, $p < 0.0001$) (Fig. 1A). Interestingly, the median TL was 23.08 in the 20–40-year age group and decreased with age in HCs ($r^2 = 0.084$), whereas the TLs in ICUS were already short in the 20–40-year age group and decreased with age in ICUS patients more slowly ($r^2 = 0.026$) (Fig. 1B). The median percentage of cells with extremely shortened TL, defined as < 10% the length of normal TL, was 76.47% (IQR: 54.61–87.76, 95% CI: 64.10–78.31) in ICUS vs 18.10% (IQR:

Table 1
Patient characteristics (n = 37).

Variables	Number of patients (%)
Age, years	
median (range)	66 (31–83)
Sex	
Male	15 (40.5%)
Female	22 (59.5%)
Neutropenia (ANC < 1800/mm^3)	28 (70.0%)
Anemia (Hb < 10 g/dL)	30 (75.0%)
Platelets (< 100,000/mm^3)	21 (52.5%)
Lineage involvement of cytopenia	
Uni-lineage	8 (21.6%)
Bi-lineage	22 (59.5%)
Tri-lineage	7 (18.9%)
Grade of cytopenia	
1	5 (13.5%)
2	8 (21.6%)
3	21 (56.8%)
4	3 (8.1%)

ANC, absolute neutrophil count.

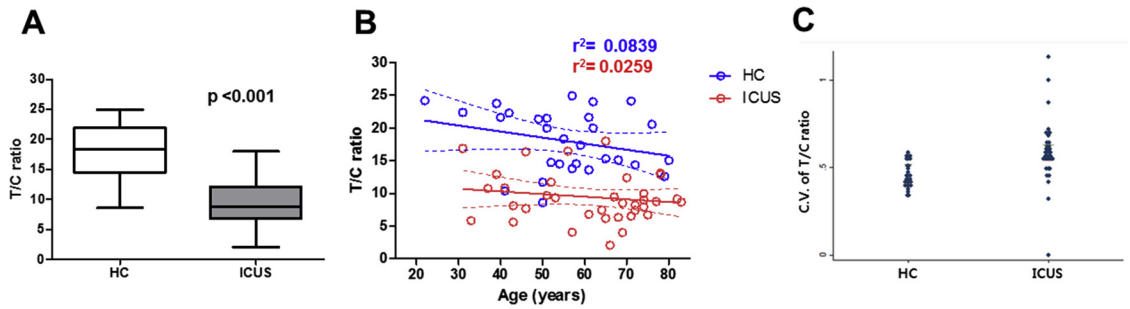

Fig. 1. Telomere length in idiopathic cytopenia of undetermined significance (ICUS) and healthy controls (HC) (A) Telomere length was different between ICUS and HC (median 8.77, interquartile range [IQR]: 6.82–11.72, 95% confidence interval [CI]: 8.19–10.67 vs. 18.36, IQR: 14.5–21.64, 95% CI:,16.22–19.80, $p < 0.0001$) (B) Telomere length by age in ICUS and HC (C) The median value of the coefficient variant of TL was significantly increased in patients with ICUS compared to normal controls (0.58, IQR: 0.54 – 0.63, 95% CI: 0.53 – 0.65 vs 0.44, IQR: 0.41 – 0.52, 95% CI: 0.43 – 0.49, $p < 0.0001$).

12.25–38.40, 95% CI: 19.39–34.59) ($p < 0.001$) in HCs, suggesting that the majority of cells have extremely short TL in ICUS. The median value of the coefficient variant of TL was significantly increased in patients with ICUS compared to normal controls (0.58, IQR: 0.54 – 0.63, 95% CI: 0.53 – 0.65 vs 0.44, IQR: 0.41 – 0.52, 95% CI: 0.43 – 0.49, $p < 0.0001$) (Fig. 1C)

3.3. Increased proliferation and apoptosis in ICUS

Next, we semi-quantitatively measured proliferation and apoptosis among nucleated cells in the bone marrow of ICUS patients and HCs (Fig. 2). The proportion of proliferating cells with Ki-67 expression was significantly higher in patients with ICUS than in HCs (median, 20.0%, IQR 5–35, 95% CI: 17.36–30.93 vs. 5.0%, IQR 5–10, 95% CI: 4.92–11.58, $p = 0.0003$) (Fig. 3A). Similarly, the proportion of apoptotic cells with cleaved caspase-3 was significantly higher in patients with ICUS compared to HCs (median, 45.0%, IQR.: 30–70, 95% CI: 39.67–56.90 vs 22.5%, 5–35, 95% CI: 15.17–30.83, $p = 0.0005$) (Fig. 3B). Interestingly, the ratio of proliferation to apoptosis was similar between patients with ICUS and HCs (Fig. 3C).

3.4. Association between TL and cell turnover

The association between TL and Ki-67, cleaved caspase-3 positivity, or their ratio were analyzed. Shorter telomeres appeared to be associated with a lower proliferation and a higher apoptosis rate than longer telomeres (Fig. 3D–F). For an in-depth analysis, ICUS patients were divided into two groups, short TL and long TL, using a median TL of 8.77 as a cut-off value. Ki-67 and cleaved caspase-3 positivity did not differ between ICUS patients with short TLs (< 8.77) and those with long TLs (≥ 8.77). A K/C ratio did not differ between two sub-groups of ICUS, too (Table 2).

3.5. TL and clinical phenotype of ICUS

ICUS patients with shorter (telomere of ≤ 8.77) and longer (telomere of > 8.77) TLs were compared in terms of their clinical phenotype and mutation profile. Genetic mutations were detected in 6 (33.3%, 95% CI: 19.6–50.4) of 19 ICUS patients with shorter telomeres and in 6 (31.6%, 95% CI: 9.2–57.5) of 19 ICUS patients with longer telomeres ($p = 0.91$). Both groups did not differ in regards to numbers of gene mutations per patient ($p = 0.65$) and variant allele frequency (10.47 vs 11.60, $p = 0.86$) (Supplementary Fig. 1).

The frequency of the presence of dysplasia did not differ between ICUS patients with short and those with long telomeres (44.4% vs 57.9%, $p = 0.41$). Neither the number of involved lineages nor the grade of cytopenia were significantly different between ICUS patients with low and those with a high K/C ratio ($p = 0.49$ and 0.79, respectively). In addition, the grade of neutropenia, anemia, and thrombocytopenia were not different between patients with short vs long telomeres ($p = 0.90$, 0.24, and 0.96, respectively).

During the 47.8-month median follow-up period, 8 patients died and 8 patients experienced a progression to MDS ($n = 6$) or aplastic anemia ($n = 2$). However, TL in patients with ICUS at diagnosis was not associated with overall or event-free survival of ICUS.

3.6. Shortened TL in ICUS without mutation and dysplasia

We compared TL of ICUS without mutation and dysplasia ($n = 14$) and HC to exclude the impact of mutation or dysplasia on TL. The finding of this sub-group analysis was consistent with the previous results. The median TL was shorter in patients with ICUS without mutation and dysplasia than in HCs (8.56, IQR: 6.82–12.39, 95% CI: 7.09–12.15 vs 18.36, IQR: 14.5–21.64, 95% CI:,16.22–19.80, $p < 0.0001$). The median percentage of Ki-67 positivity was significantly higher in patients with ICUS without mutation and dysplasia

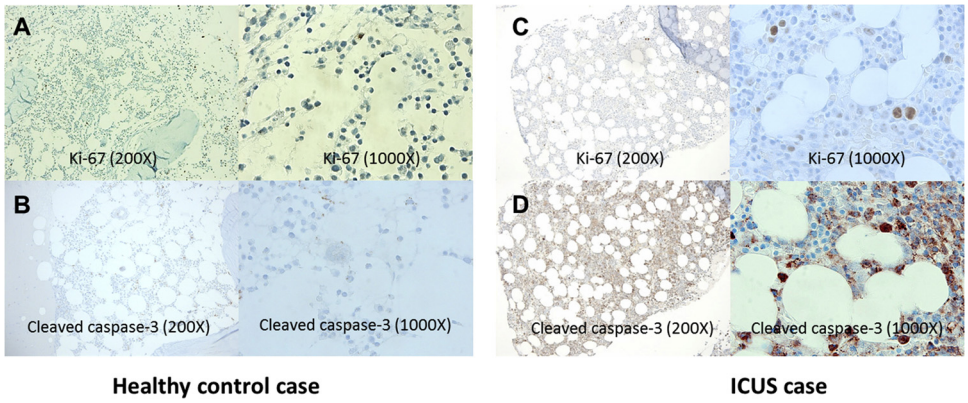

Fig. 2. Representative cases of immunohistochemical stain. (A) Ki-67 positive in < 5% of cells (200X) and (1000X) (B) cleaved caspase-3 positive in < 5% of cells (200X) and (1000X) in healthy control case, and (C) Ki-67 positive in 5% of cells (200X) and 1000X) (D) cleaved caspase-3 positive in 80% of cells (200X) and (1000X) in idiopathic cytopenia of undetermined significance case.

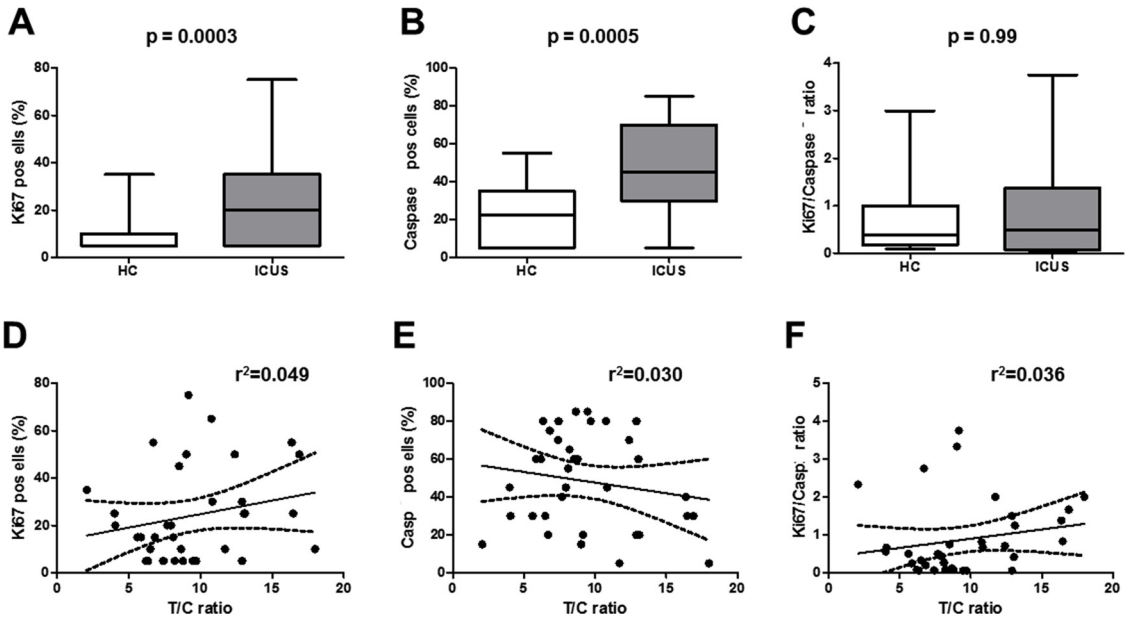

Fig. 3. High cellular turnover in ICUS compared to healthy control (HC) (A) Ki-67 positivity is higher in ICUS patients (n = 35) compared to HCs (n = 20) (median 20.0%, IQR: 5.0–35.0, 95% CI: 17.36–30.93 vs. 5.0, IQR: 5.0–10.0, 95% CI: 4.92–11.58, $p = 0.0003$) (B) Cleaved caspase-3 positivity is increased in ICUS compared to HCs (median 45.0%, IQR: 30.0–70.0, 95% CI: 39.67–56.90 vs. 22.5%, IQR: 5.0–35.0, 95% CI: 15.17–30.83, $p = 0.0005$) (C) the ratio of Ki-67%/cleaved caspase-3% is not different between ICUS and HC (p = 0.99) (D–F) Ki-67 and cleaved caspase-3 (casp) positivity according to telomere/centromere length ratio (T/C) among patients with ICUS.

Table 2
Ki-67 and cleaved caspase-3 positivity according to TL in patients with ICUS.

	Short telomere (≤8.77)[1]	Long telomere (> 8.77)[1]	P[2]
Ki-67 positivity (%)	15.0 (5.0 -20.0)	25.0 (10.0 – 50.0)	0.35
Caspase-3 positivity (%)	57.5 (30.0 – 70.0)	40.0 (20.0 – 70.0)	0.99
K/C	0.30 (0.083 – 0.56)	0.83 (0.42-1.67)	0.18

[1] Presented as the median (IQR).
[2] Bonferroni correction was done.

than in HCs (median, 35.0%, IQR 15.0–50.0, 95% CI: 21.49–48.51 vs. 5.0%, IQR 5–10, 95% CI: 4.92–11.58, p < 0.0001). Similarly, the proportion cleaved caspase-3 positivity was significantly higher in patients with ICUS compared to HCs (median, 40.0%, IQR.: 20.0–60.0, 95% CI: 28.21–56.41 vs 22.5%, 5–35, 95% CI: 15.17–30.83, p = 0.0093) (Supplementary Fig. 2).

4. Discussion

To the best of our knowledge, this study is the first to show that TLs were markedly shortened in the hematopoietic system of ICUS patients compared to those of HCs. Relatively shorter TLs were associated with increased cell turnover as demonstrated by increased proliferation and apoptosis. However, there was no significant association between TL and degree of peripheral cytopenia, somatic mutational profile on 88 recurrently affected genes in hematologic malignancies, or long-term clinical outcome.

The pathophysiology of ICUS remains largely unknown. Recent investigations demonstrate that the putative diagnosis of ICUS can be divided into a clonal cytopenia of undetermined significance (CCUS) and non-clonal ICUS according to the presence of genetic mutations frequently observed in myelodysplasia [12]. As MDS, ICUS, and clonal hematopoiesis of indeterminate potential (CHIP) share similar genetic mutations, a subset of ICUS with increased cell turnover could be a preceding state to overt abnormal clonal hematopoiesis, ranging from CHIP or CCUS to MDS or leukemia. Further studies are needed to define whether ICUS patients with a higher baseline proliferation rate (possibly higher proliferation potential) are at an increased risk to progress to a malignant condition.

Our finding clearly demonstrated that TLs were shortened in patients with ICUS compared to age-matched HCs. Strikingly, the mean TL in relatively young ICUS patients was even shorter than those in elderly HCs (Fig. 1). The proportion of cells with extremely shortened telomeres (defined as TL < 10% the length of an HC TL) and coefficient variant also suggested the pathologic erosion of TL in ICUS patients. These data suggest that a large number of hematopoietic cells have undergone extensive replication in ICUS patients as a result of abnormally increased replicative stress (autonomous proliferation as premalignant cells and/or ineffective hematopoiesis with premature differentiation block and cell death), impaired telomere repair by telomerase, or both.

Therefore, we investigated the turnover of HSPC in bone marrow cells. We found that the proliferation rate was clearly increased in ICUS patients (Fig. 3A). This higher proliferation rate may indicate an attempt of bone marrow cells to compensate for peripheral cytopenia. However, the higher proliferation was also associated with equally increased apoptosis (Fig. 3B), suggesting that cells die shortly after proliferation due to ineffective hematopoiesis/differentiation, leading to peripheral cytopenia.

Strikingly, ICUS patients with even shorter TLs had a lower K/C ratio, suggesting that as telomeres shorten, the rate of proliferation slows as well (Fig. 3F and Table 2). This result is consistent with a recent animal study demonstrating that HSPCs with dysfunctional telomeres arrest in quiescence and could enter the cell cycle upon stimulation and then die out [13].

Based upon our findings, we propose that a subset of ICUS is driven by a primary ineffective hematopoiesis with peripheral cytopenia. At an early stage, HSPCs proliferate (autonomously or in response to peripheral cytopenia) and generate sufficient mature cells (despite ineffective hematopoiesis). However, as telomere attrition progresses following excessive replication, HSPCs with critical TL no longer meet the peripheral need, leading to overt cytopenia. In a later stage, the telomere is so short that proliferation of HSPCs may begin to slow, and

relatively more cells undergo apoptosis as a result of ineffective hematopoiesis. An accelerated apoptosis rate (cleaved caspase-3) has also been observed in MDS and Fanconi anemia [14,15].

Unexpectedly, ICUS with shorter telomeres was not associated with more genetic mutations or worse clinical outcome. Therefore, one might speculate that critical mutations as a second hit are required to promote ICUS into more advanced hematologic malignancies such as CCUS, which is recently considered a low-risk MDS in terms of genetic mutational profile [16]. Indeed, loss of ASXL1, an epigenetic regulator, in cord blood-derived CD34+ cells reduces the number of hematopoietic stem cells, particularly in the myeloid lineage [17]. A somatic mutation in genes involved in epigenetic regulation of transcriptional expression could impair the stem cell function in hematopoiesis, which causes an ICUS-like phenotype of cytopenia induced by a senescence in HSPCs. However, hematopoietic senescence associated with the aging process could induce genetic mutation and associated disease [18,19]. Perhaps the pathophysiology of pro-apoptotic activity according to the aging process and hematopoietic senescence may be intermingled with the etiology of accumulation of genetic mutations in ICUS.

Our study has several limitations. First, we did not examine the functionality of telomerase, the loss of which can result in rapid telomeric erosions. Second, despite being one of the largest studies in ICUS, the number of enrolled patients is rather small for a robust statistical analysis due to the very low prevalence of ICUS. Third, the cause of the ineffective hematopoiesis increased turnover requires further investigation.

To our knowledge, this is the first study to demonstrate that hematopoietic cells in ICUS have premature replicative senescence with shorter TLs as a result of increased cell turnover. Further studies are warranted to investigate the cause of the increased cell turnover for premature senescence.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.leukres.2019.02.004.

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