TELOMERE ATTRITION AND CANDIDATE GENE MUTATIONS
PRECEDING MONOSOMY 7 IN APLASTIC ANEMIA

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Key point: Accelerated telomere attrition precedes chromosomal loss and malignant transformation to MDS/AML arising from aplastic anemia

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Abstract

The pathophysiology of severe aplastic anemia (SAA) is immune-mediated destruction of hematopoietic stem and progenitor cells (HSPC). Most patients respond to immunosuppressive therapies (IST), but a minority transform to myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML), frequently associated with monosomy 7 (-7). Thirteen SAA patients were analyzed for acquired mutations in myeloid cells at the time of clonal evolution to -7. All patients had a dominant HSPC clone bearing specific acquired mutations detected in 50 to 90 percent of circulating granulocytes. However, mutations in candidate genes associated with MDS/AML were present in only four cases. Patients who evolved to MDS and AML showed marked progressive telomere attrition prior to the emergence of -7. Single telomere length analysis (STELA) confirmed accumulation of short telomere fragments of individual chromosomes. Our results indicate that accelerated telomere attrition in the setting of a decreased HSPC pool is characteristic of early myeloid oncogenesis, specifically chromosome 7 loss, in MDS/AML following SAA, and provides a possible mechanism for development of aneuploidy.
Introduction

Human aplastic anemia is characterized by reduced peripheral blood counts due to destruction of hematopoietic stem and progenitor cells (HSPC). Most patients with SAA respond to immunosuppressive therapy (IST), implicating an immune pathophysiology. However, malignant “clonal evolution” to myelodysplasia (MDS) or acute myeloid leukemia (AML) occurs in about 15% of cases, and its pathophysiology is not well understood. The application of next-generation sequencing to de novo MDS/AML has led to definition of recurrently mutated genes as possible drivers of leukemogenesis. In the current study, thirteen SAA patients that developed -7 MDS after IST were analyzed for acquired mutations by deep sequencing and for telomere attrition, as short telomeres may predict clonal evolution in SAA.

Methods

Patients and blood samples
Peripheral blood and bone marrow cells were collected after informed consent according to IRB approved protocols and in accordance with the Declaration of Helsinki. All SAA patients treated between 2001 and 2010 at our institution who had developed -7 MDS and had available serial blood and marrow samples were analyzed. Diagnosis, response to IST, clonal evolution, and relapse criteria have been described.

Telomere length measurements
Mean telomere content of peripheral blood leukocytes by qPCR and TERT/TERC sequencing were performed as previously described. Single telomere length assay (STELA) uses single molecule PCR to amplify chromosome-specific telomeres based on the sequence specificity of subtelomeric regions allowing for direct visualization and quantification of very short telomeres.

Whole exome sequencing (WES) and targeted sequencing
DNA was extracted from peripheral blood and bone marrow mononuclear cells using a Maxwell DNA purification kit (Promega, Wisconsin). DNA extracted from MicroBeads (Miltenyi, Germany)-sorted CD3 T-cells was used as “germline” control DNA. A detailed protocol of WES and analysis algorithm is available in Supplemental Methods. Targeted sequencing for genes associated with de novo MDS/AML (Supplemental Table1) was done.
using SureSelect custom kit from Agilent Technology and sequencing was done using HiSeq 2000 instrument from Illumina with the 100-bp paired-end (PE) read option, according to the manufacturer’s protocol. Acquired mutations were called as previously shown. Deep sequencing of PCR-amplified sequences was performed for validation.

Results and Discussion

Acquired mutations at clonal evolution to -7 MDS

We hypothesized that clonal evolution would be accompanied by mutations in genes previously identified in de novo MDS and AML. We employed a similar strategy to identify and quantify mutations, isolating DNA from marrow mononuclear cells obtained at the time of progression to -7 MDS from a cohort of thirteen SAA patients, followed by WES. We identified 209 somatic mutations in myeloid cells, both single nucleotide variants (SNV) and small insertions/deletions (indels), with variant allele frequency (VAF) >0.1 (Supplemental Table2). All thirteen patients had a dominant HSPC clone, as inferred from SNVs and indels, at VAF 0.25-0.5 (Figure1A). Within the dominant HSPC clone, four patients from this cohort (Supplementary Table2) had somatic mutations in genes known to be recurrently mutated in de novo MDS/AML (Supplemental Table1). Sanger sequencing of serial blood and bone marrow samples confirmed these recurring mutations. No other, novel recurrently mutated genes were identified and mutated genes did not cluster in any pathway associated with cancer.

Among the four patients with candidate gene mutations, one (UPN#1) had completely responded to IST, later relapsed, and then responded to cyclosporine (Figure 1B); two years later she developed -7 and blood counts were stable despite 5-10% bone marrow blasts for the next 3 years. At 6 months after initial IST, a clone was characterized by mutations in ASXL1 and DNMT3A, which was unchanged for three years. A subclone emergent at relapse had mutations in SETBP1, DOT1L and STAT3; it increased over 30 months until it was present in 60% of myeloid cells at diagnosis of -7. UPN#2 was refractory to IST before developing -7. He died 6 months later from infection and the last marrow showed 7% myeloblasts (Figure 1C). In two patients (UPN #9, 10) acquired mutations were detected in RUNX1 and
CSF3R, respectively, only at the time of clonal evolution and also present at a lower VAF in the lymphoid lineage.

Targeted sequencing of genes associated with de novo MDS and AML (Supplemental Table1) confirmed all mutations identified by WES and identified small subclones at low VAF mutations in three additional patients (Supplemental Table3).

WES allowed characterization of the oligoclonal architecture of hematopoiesis at development of -7 MDS. Somatic mutations in genes associated with de novo MDS and AML were present in a subset of patients whose disease had evolved but candidate gene abnormalities were not prevalent in the group as a whole.

Accelerated telomere attrition and accumulation of very short telomeres in SAA evolving to MDS

Accelerated telomere attrition causes increased chromosomal instability, aneuploidy, and progression to malignancy in mouse models.11 We have linked short telomeres at SAA diagnosis with later MDS and AML.8 We postulated that accelerated telomere attrition might be pathogenic in the development of myeloid neoplasms after marrow failure. First, we estimated mean telomere content loss from the time of SAA diagnosis until clonal evolution. In the thirteen patients that progressed to -7 MDS, the average attrition rate was 419 bp/year (Fig 2A); among controls, the rate was much lower, 65 bp/year (Fig 2A; p<0.01). (The control group of 30 stable SAA patients was treated in the same time period and matched by age, sex, and IST response to -7 cases; Supplemental Table4). The attrition rate was significantly higher in the clonal evolution group compared to the control SAA group, starting at 6 months after IST (Fig 2A). STELA confirmed increased attrition and revealed that chromosome-specific very short telomeres accumulated in SAA patients with clonal evolution and were present before development of a cytogenetic abnormality and diagnosis of MDS. In the stable SAA group, XpYp profiles showed no increase in shorter telomeres to 48 months after IST (Figs 2B and C). A similar shift in telomere XpYp STELA profiles was observed with cultured bone marrow cells; accumulation of shorter telomeres was observed in samples from SAA patients who developed MDS (Fig 1SA) but not in cells from stable SAA patients (Fig 1SB).
In this report we show that SAA patients who progress to -7 MDS have oligoclonal hematopoiesis and increased telomere attrition due to accumulation of very short telomeres before development of aneuploidy. Rapid telomere attrition did not appear to be genetic, as these patients did not have clinical features of a telomeropathy, they had telomeres within the normal range at diagnosis, and they lacked mutations in TERT and TERC. Accelerated telomere loss likely is secondary to restricted clonal hematopoiesis and proliferative stress leading to chromosomal instability. The increased average number of acquired mutations identified by WES in this cohort (16/case) compared to 5 to 10 mutations that were previously reported in healthy volunteers of similar ages also would result from a long replicative history.

In the thirteen patients who suffered clonal evolution, no novel recurrently mutated genes were identified and acquired mutations in genes postulated to be drivers of myeloid malignancies were present only in four patients in the dominant HSPC clone. Acquired low VAF mutations in these genes were identified in three additional patients by targeted sequencing. However, due to their low VAF these subclones were unlikely to be drivers of progression to aneuploidy. We cannot exclude novel unique gene mutations, for the remaining patients. Oncogenesis might be driven by aneuploidy through haploinsufficiency of genes on chromosome 7. Moreover, acquired mutations in the same gene set were recently reported by targeted sequencing in 10-19% of AA patients, and not all had progressed to overt MDS. In the largest reported cohort of 150 AA patients, 17 had clonal evolution, but only four patients had -7 MDS.

We previously reported that mean telomere content of leukocytes at diagnosis of SAA was the only known predictor of malignant progression. Aneuploidy would be expected if chromosomal instability were responsible, and indeed cytogenetic clonal evolution in SAA is almost invariably accompanied by whole or partial loss of chromosome 7, and less frequently by gain of chromosome 8, deletions of 13q and 20q, and similar aberrations. Moreover, telomerase-deficient bone marrow cells in culture show increased -7, linking this aneuploidy to telomere pathology.

While our study is limited by sample size and, as with most investigations of human biology, shows association rather than causation, it allows some cautious inferences. Accelerated telomere attrition preceded aneuploidy and
malignant transformation at an early stage of oncogenesis arising from bone marrow failure. Chromosome instability, as the molecular mechanism of aneuploidy, has been suggested by many cell culture experiments and animal models.\textsuperscript{18,19} Identification of critically short telomeres before development of clinical progression might allow timely management of patients at risk of clonal evolution. Therapeutic upregulation of telomerase, as for example by sex hormones\textsuperscript{20} or other pharmacological agents, might reduce the risk of clonal evolution in the setting of a reduced stem cell pool, in bone marrow failure syndromes or after intensive chemotherapy.

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\textbf{Contribution:} BD, XF, DMT, RC, NSY designed experiments; BD, XF, YU, TY, YY, YW, SK performed experiments; BD, XF, TY, YY, SO, JZ, NSY analyzed the data; BD and NSY wrote the paper. Authors have no conflicts of interest.

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between hematologic response and long-term outcome. JAMA. 2003;289(9):1130–1135.


**Figure legends:**

**Figure 1.** A. Dominant hematopoietic clone VAF in 13 SAA patients at the time of clonal evolution to MDS with -7. Each patient’s mutations with VAF>0.25 are shown. B and C. VAF of acquired mutations in UPN#1 and #2, with neutrophil count. Direct Sanger sequencing confirmed the presence of mutations and VAF. The upper part of the graph shows representative hematoxylin-eosin staining of the core biopsy and CD34 antigen staining at the time of clonal evolution. The images were taken on an Olympus BX41 microscope with an Olympus DP72 camera, using a 4× UPlanFL N Olympus objective (original magnification ×20). Neutrophil count (left y-axis) is shown by blue bars. VAF of acquired mutations (right y-axis) at various time points from diagnosis of SAA until diagnosis of MDS are shown in red and green line graphs. Arrows on the x-axis mark ISTs and time to progression to MDS.

**Figure 2.** A. Mean telomere content for clonal evolution group (red line, n=13) and SAA stable controls (blue line, n=30) measured by qPCR. For each patient in both groups mean telomere content at each available time point was normalized to mean telomere content at presentation. B. Examples of XpYp STELA profile of serial peripheral blood leukocytes for patients from the time of diagnosis of SAA until progression to -7 MDS (left) or SAA controls (right). C. Quantification of telomere length measured by XpYp STELA. For each of the two groups, all available samples at various time points after IST were assayed by both qPCR and STELA. For the clonal evolution group, samples up to the time of -7 diagnosis were used; thus for each time point at least four patients samples were used. Similarly
for the SAA control group, 30 patients’ samples were available to 24 months after initial IST; each time point included more than 15 patient samples. For serial blood samples’ telomere length measured by XpYp STELA, average lengths were mean-centered by patient. Discrete bands lengths were quantified using ImageQuant TL software (GE Healthcare Life Sciences, Piscataway, NJ). Bands were quantified based on a standard DNA ladder. A one-way Wilcoxon test on equal length of the telomeres of the patients over all time points was statistically significant (p=0.0027) for the clonal evolution group but not for controls (p=0.62).
Telomere attrition and candidate gene mutations preceding monosomy 7 in aplastic anemia

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