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Emergence of Clonal Hematopoiesis in the Majority of Patients with Acquired Aplastic Anemia

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Abstract

Acquired aplastic anemia (aAA) is a non-malignant disease caused by autoimmune destruction of early hematopoietic cells. Clonal hematopoiesis is a late complication, seen in 20–25% of older patients. We hypothesized that clonal hematopoiesis in aAA is a more general phenomenon, which can arise early in disease even in younger patients. To evaluate clonal hematopoiesis in aAA, we used comparative whole exome sequencing of paired bone marrow and skin in 22 patients. We found somatic mutations in sixteen patients (72.7%) with a median disease duration of 1 year; twelve (66.7%) were patients with pediatriconset aAA. Fifty-eight mutations in 51 unique genes

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were primarily in pathways of immunity and transcriptional regulation. Most frequently mutated was *PIGA*, with 7 mutations. Only two mutations were in genes recurrently-mutated in MDS. Two patients had oligoclonal loss of HLA alleles, linking immune escape to clone emergence. Two patients had activating mutations in key signaling pathways (*STAT5B*(p.N642H), *CAMK2G*(p.T306M)). Our results suggest that clonal hematopoiesis in aAA is common, with two mechanisms emerging— immune escape and increased proliferation. Our findings expand conceptual understanding of this non-neoplastic blood disorder. Future prospective studies of clonal hematopoiesis in aAA will be critical for understanding outcomes, and for designing personalized treatment strategies.

Keywords

clonal hematopoiesis; aplastic anemia; bone marrow failure; myelodysplastic syndrome; MDS

Introduction

Acquired aplastic anemia (aAA) is a life-threatening blood disorder, affecting children and adults, caused by immune destruction of hematopoietic stem and progenitor cells and the subsequent failure of the bone marrow to sustain normal blood production [1]. Refractory disease and transformation to myelodysplastic syndrome (MDS) are particularly vexing problems in aAA; 10–15% of patients treated with immunosuppressive therapy (IST) evolve to MDS or acute leukemia [2]. Although aAA is considered to be non-malignant, there is a well-described association with clonal blood disorders. Nearly half of aAA patients have clonal populations of cells lacking cell surface proteins linked to a glycosylphosphatidylinositol (GPI) anchor due to somatic loss-of-function mutations in the *PIG-A* gene; these are called Paroxysmal Nocturnal Hemoglobinuria (PNH) clones due to their susceptibility to complement-mediated lysis [3–4]. More recent reports indicate that ∼10% of aAA patients have acquired copy number-neutral loss of heterozygosity (CN-LOH) in chromosome arm 6p, postulated to emerge by immune selection against specific HLA alleles [5–7]. There are emerging data from targeted sequencing of genes recurrently-mutated in MDS indicating that up to 24% of aAA patients carry somatic mutations in *ASXL1*, *DNMT3A*, and *BCOR*, and may be at a greater risk of malignant transformation [8–11]. Taken together, the available data indicate that somatic mutations in genes other than *PIGA* are limited to a minority of generally older aAA patients. Importantly, beyond targeted sequencing studies, the full spectrum of clonal hematopoiesis in aAA remains undefined, with little data on clonal hematopoiesis in the pediatric population.

Based on the known association of aAA and clonal blood disorders, we hypothesized that clonal hematopoiesis in aAA may be a more general phenomenon, present in the majority of patients, including children and young adults, and can emerge early in the course of the disease. To comprehensively evaluate the landscape of clonal hematopoiesis in aAA, we used an unbiased approach of comparative whole exome sequencing (WES) of paired bone marrow and skin fibroblast DNA, combined with genome-wide single nucleotide polymorphism array (SNP-A) profiling in twenty two aAA patients. We found clonal hematopoiesis in three quarters of patients, including two thirds of patients with

pediatriconset disease. Our results show that even in the younger patients, hematopoiesis in aAA is frequently characterized by somatic mutations, which are distinct from mutations in MDS, and instead bear signatures of immune escape and proliferative signaling, and extend beyond the known association with Paroxysmal Nocturnal Hemoglobinuria.

Materials and Methods

Patients and Study Oversight

The Penn-CHOP Bone Marrow Failure Syndrome (BMFS) cohort is an open prospective/retrospective cohort for the study of molecular mechanisms of BMFS, approved by the Institutional Review Boards of Children's Hospital of Philadelphia (CHOP) and of the University of Pennsylvania (Penn). Written informed consent from all study participants or their legal guardians was obtained prior to study participation in accordance with the Declaration of Helsinki. All patients with aAA, referred to the Penn-CHOP Comprehensive BMFS Center between 2009 and 2014, who had a stored bone marrow aspirate and skin biopsy material were eligible for this analysis. The diagnosis of aAA was established according to the International Study of Agranulocytosis and Aplastic Anemia[12], and required exclusion of congenital BMFS and other conditions mimicking aAA . Patients with morphological evidence of dysplasia according to the 2008 World Health Organization (WHO) classification[13] were excluded. Complete medical histories, peripheral blood counts, bone marrow histology and cytogenetic analysis were available for all patients. In accordance with the American Academy of Pediatrics Council on Child and Adolescent Health, pediatric-onset aAA was defined as a diagnosis of aAA under the age of 22[14].

Cytogenetics and Hematopathology

Cytogenetic analysis and fluorescence in situ hybridization (FISH) were performed according to standard methods. Bone marrow histology was evaluated by a clinical hematopathologist in a blinded fashion, as patients were entered into the study only after completion of the diagnostic review. In accordance with department policy, all controversial cases were subject to a clinical consensus conference.

SNP-A Analysis

Illumina Infinium SNP-A genotyping of bone marrow aspirate DNA was performed using Illumina Quad610, Illumina Omni1 Quad, or Illumina CytoSNP 850 Beadchips at the CHOP Center for Applied Genomics according to the manufacturer's protocol. Arrays were analyzed in GenomeStudio (Illumina, Inc., San Diego, CA), which allows direct visualization of B-Allele Frequency and log R ratio. SNP-A data have been deposited in Gene Expression Omnibus (accession GSE48484).

WES

WES was performed on DNA extracted from the patients' bone marrow aspirate and paired skin fibroblasts using Qiagen DNeasy Blood & Tissue Kit (Qiagen Inc., Valencia, CA) at the BGI@CHOP High Throughput Sequencing Center. Skin fibroblasts were expanded in culture for 3–4 passages prior to DNA extraction. Exome libraries were constructed with Agilent SureSelect All Exon V4 + UTRs kit (Agilent Technologies, Santa Clara, CA).

Paired-end WES to 150X average depth was performed using the Illumina HiSeq 2500 platform, according to the manufacturer's recommendations. Somatic variant calling on bone marrow-skin biopsy pairs was performed with VarScan2, an algorithm optimized for detection of somatic mutations[15], using parameters *–min-coverage 4,–min-var-freq 0.08,–p-value 0.05,–strand-filter 1–min-avg-qual 20*. Filtering and annotation of somatic mutations was performed using SNP & Variation Suite v8.0 (Golden Helix, Inc., Bozeman, MT). All mutations were manually curated in Integrative Genomics Viewer[16], and were classified into Tiers as described previously[17]. All chromosome coordinates were based on hg19 (NCBI build 37).

Sanger sequencing

All putative Tier 1 and Tier 2 somatic mutations[17] identified with WES were validated with Sanger sequencing. Briefly, amplicons containing putative somatic mutations were subjected to bi-directional sequencing using a 3730 DNA Analyzer (Applied Biosystems, South San Francisco, CA). Mutations were confirmed as somatic if they were present in bone marrow and absent in paired skin DNA.

HLA Typing

Paired-end next-generation sequencing (2×251 bp) of the Human Leukocyte Antigen (HLA) region was performed on the Illumina MiSeq platform at >10,000X average depth as previously described[18]. Sequence alignment and HLA typing were performed using NGSengine™ (GenDx Utrecht, Netherlands) software in the CHOP CLIA-approved Immunogenetics Laboratory.

Telomere Length Measurement

Fifteen patients had lymphocyte telomere length (TL) measurements performed by the CLIA certified TL testing center (Repeat Diagnostics, Inc., North Vancouver, Canada). Five patients, who did not have a clinical TL measurement, but had available material, had TL measured using the FITC-conjugated $(C_3TA_2)_3$ peptide nucleic acid probe, as previously described[19]. Relative TL were obtained by comparison to a control cell line (GM03671C; Coriell Institute, Camden, NJ), which was assigned a TL of 100%. TL were normalized to the clinical lab TL measurement, and were compared to age-matched normal controls (Repeat Diagnostics).

T Cell Receptor (TCR)-γ Gene Rearrangement

TCR γ gene rearrangement analysis was performed by the Penn CLIA-approved Molecular Diagnostics Laboratory, using fluorescent-based amplification of the consensus V and J regions of *TCR* γ, as previously described.[20]

Cell Sorting

Lymphoid- and myeloid-enriched cell fractions were obtained from peripheral blood by immunomagnetic selection for CD3 and CD19-positive cells. Briefly, peripheral blood was subjected to red cell lysis, incubated with anti-CD3 and anti-CD19 microbeads (Miltenyi Biotec, San Diego, CA), and separated using "LS" columns into the myeloid-enriched

(CD3- and CD19-depleted) and lymphocyte (CD3+ and CD19+ selected) fractions. Purity was verified by flow cytometry.

GO Pathway Analysis

Gene Ontology (GO) enrichment analysis was performed using WebGestalt Gene Analysis Toolkit[21], using a Benjamini-Hochberg adjustment for multiple testing[22], and a significant p-value <0.05.

Statistics

Association analysis of clinical characteristics and presence of detectable clonal hematopoiesis was performed using Fisher's exact test for categorical variables, and Mann-Whitney U Test for continuous variables, with a two-tailed significance level of 0.05. Multivariate logistic regression was also performed.

Results

Patient Population

We analyzed a cohort of 22 patients with aAA; 18 had pediatric-onset aAA, and 4 had adult-onset aAA (Table 1). Median age at diagnosis was 14.5 years (range 1.5–61). The majority of patients (21 of 22) were diagnosed with severe or very severe aAA; one patient had moderate aAA. Nineteen patients (86.4%) were treated with immunosuppressive therapy (IST) prior to analysis, and two had a history of eltrombopag therapy. Consistent with the known high frequency of PNH in aAA, 45.5% of patients had a PNH clone of over 1% by flow cytometry. 25% of patients (5 of 20 patients with available telomere length measurement) had telomeres under the 10^{th} percentile. The majority of patients (21 of 22) lacked acquired karyotypic abnormalities; one patient had an acquired deletion of chromosome arm 13q. None of the patients had morphologic evidence of dysplasia by bone marrow histopathology.

The Majority of Pediatric and Adult aAA Patients Have Clonal Hematopoiesis

We used a combination of metaphase cytogenetics, whole genome SNP-A analysis, and WES to comprehensively evaluate the bone marrow of aAA patients for evidence of clonal hematopoiesis (Table 1–Table 3). To ensure accurate identification of somatic mutations, we identified *de novo* mutations in the patients' bone marrow DNA, which were absent in the patients' constitutional DNA (skin fibroblasts). Identified somatic mutations were classified into Tiers as described previously [17], where non-synonymous coding, consensus splice site, or RNA genes were classified as Tier 1, and regions with regulatory potential, such as 5' and 3' untranslated regions (UTR) were classified as Tier 2. All putative Tier 1 and 2 somatic mutations identified by WES were confirmed by bi-directional Sanger sequencing, and, for the HLA alleles, by targeted next-generation deep sequencing.

Six patients (27.3%) were found to have clones with acquired CN-LOH; CN-LOH for four patients was reported previously [6]. The most common region affected by acquired CN-LOH was chromosome arm 6p, seen in three patients (13.6%). Sixteen patients (72.7%) had evidence of clonal hematopoiesis with confirmed somatic mutations in the bone marrow.

Independent evidence of clonal hematopoiesis was present in all sixteen patients even after exclusion of *PIGA* mutations. The median number of Tier 1 and 2 mutations per patient was 2 (range 0–11). Twelve of 18 patients (66.7%) with pediatric-onset aAA had somatic mutations, with a median of 1 (range 0–6) Tier 1 and 2 mutations per patient; one patient had a single identified somatic mutation in an upstream region of the *CHPF2* gene. Six patients (27.3%), all with pediatric-onset aAA, had no detectable clonal hematopoiesis even after expanding analysis to include synonymous, intronic and intergenic mutations.

Six of 16 patients with detectable somatic mutations had peripheral blood material available for cell sorting; in these patients, mutations were evaluated by Sanger sequencing in the immunomagnetically-sorted myeloid and lymphoid cell lineages at a later time point (median interval between two analyses of 1.1 years, range 0.4–2.4 years). In four of the six patients (66.7%), somatic mutations were detected only in the CD3- and CD19-depleted myeloid cell fraction, but not in the lymphoid fraction, indicating that the mutation most likely occurred in a progenitor population with predominant contribution to the myeloid lineage. Two patients had mutations detectable in both myeloid and lymphoid lineages, suggesting that the mutation likely occurred at a stem cell or a multipotent progenitor level, with a potential to contribute to both lineages.

Association analysis of clinical characteristics of the 16 patients with detectable clonal hematopoiesis compared to the 6 patients without detectable clonal hematopoiesis (Figure 1) revealed that patients with clonal hematopoiesis were more likely to be older at diagnosis, with a median age of 16.5 years (range 3–61), as compared to 3.5 years (range 1.5–15) (p=0.012); similarly, they were more likely to be older at the time of WES (median 20 years (range 4–61) compared to 4.5 years (range 2–20)) (p=0.011). Clonal hematopoiesis was detected early in disease, with a median time from diagnosis of 1 year (range 0.08–29). There was no significant association with disease duration, time from immunosuppression, disease severity or telomere lengths. In the multivariate logistic regression including age at diagnosis, disease duration and telomere length simultaneously, age at diagnosis showed a trend toward significant association with clonal hematopoiesis (p=0.0752).

Acquired Mutations in aAA Carry Signatures of Immune Escape and Proliferative Signaling, and Are Distinct from Mutations in MDS

A total of 58 Tier 1 (n=35) and Tier 2 mutations (n=23) in 51 unique genes were identified (Tables 3–4). Excluding *PIGA* mutations, previously reported to be associated with aAA[3–4] and recently reported to arise both as early or late subclonal events in classical PNH[23], there were a total of 50 non-PIGA unique gene mutations. Other recurrent mutations were loss-of-function mutations in HLA class I genes (n=2), and mutations in *ERCC6L* (n=2). Two patients had mutations in genes recurrently affected in MDS, *ASXL1* (n=1) and *BCOR* (n=1); neither patient had evidence of myelodysplasia at the time of analysis.

Activating Mutations in Signaling Pathways

Although none of the other mutations were recurrent within our cohort, several mutations affected functional domains and would be predicted to serve as drivers. Two such mutations

involved critical residues in cellular signaling proteins, both previously reported to be activating [24–25](Figure 2).

Patient 364.01 (diagnosed with aAA in young adulthood, with refractory disease despite IST and a trial of eltrombopag) was found to have 11 Tier 1 and Tier 2 somatic mutations, with a median clone size of 16% (range 11–23%)(Table 3). There was no evidence of myelodysplasia, and no acquired abnormalities detected by metaphase cytogenetics and SNP-A. A somatic p.N642H mutation in the exon 16 SH2 domain of the signal transducer and activator of transcription 5B (*STAT5B)* gene was identified in the patient's bone marrow, with the dominant clone size (calculated as a ratio of mutated to reference allele) of 23%, roughly corresponding to half of the patient's cells carrying a heterozygous mutation (Fig 2A). The *STAT5B* p.N642H mutation is a recurrent, activating mutation found in some large granular lymphocyte leukemias [24]. In contrast, this patient had no lymphocytosis, and no clonal T cell receptor rearrangement. Sanger sequencing of the mutated *STAT5B* region in immunomagnetically-sorted peripheral blood revealed that the p.N642H *STAT5B* mutation was restricted to the myeloid lineage, and was not detected in lymphocytes (Fig 2B).

Patient 20.01 (diagnosed with aAA in adolescence, with partial response to IST, analyzed at relapse while on cyclosporine therapy) was found to have 6 somatic mutations with a median clone size of 12% (range 8–16%), as well as acquired CN-LOH in chromosome arm 5q with an estimated clone size of 15–20%. A somatic p.T306M mutation in the calcium^{2+}/calmodulin-dependent protein kinase II γ (*CAMK2G*) gene in chromosome band 10q22.2 comprised a 12% clone, corresponding to ∼25% of cells carrying this heterozygous mutation (Fig 2B). T306 of CAMK2G is a known phosphorylation site within the autoregulatory domain, loss of which was previously shown to be activating [26].

Oligoclonal Hematopoiesis with Loss of HLA Class I Alleles

Fourteen percent of patients (3 of 22) had acquired CN-LOH for chromosome arm 6p encompassing the HLA locus (Table 3); CN-LOH for two patients was reported previously [6]. All three patients had evidence of multiple breakpoints for the CN-LOH within the 6p region, indicating emergence of different CN-LOH clones within the same patient. Importantly, two patients with acquired CN-LOH were also found to have somatic loss-of-function mutations within HLA class I genes (Figure 3).

SNP-A analysis of patient 281.01 revealed two dominant 6p CN-LOH clones, both encompassing the *HLA-A* gene (Fig 3). HLA-typing by targeted deep sequencing of patient's peripheral blood and skin DNA revealed a bias towards the HLA*A 24:02 allele (60%) in the blood, consistent with the loss of the HLA*A 33:03 locus due to LOH (Fig 3A, 3G). Additionally, WES uncovered a somatic nonsense p.Tyr142* mutation in the HLA-A gene in the patient's bone marrow (Fig 3B). Targeted deep sequencing and HLA typing confirmed that the p.Tyr142* mutation occurred on the HLA*A 33:03 allele, leading to an additional 7% loss of HLA*A 33:03 due to mutational inactivation (Fig 3C).

Similarly, patient 54.01 had two 6p CN-LOH clones detected by SNP-A analysis; the homozygous region encompassed the *HLA-B* gene (Fig 3D). HLA typing of peripheral blood

and skin DNA indicated a bias toward the HLA*B 44:03 allele in the blood (57%), consistent with the loss of the HLA*B 14:02 allele due to LOH (Fig 3F–G). WES revealed a somatic frameshift mutation p.L294fs (Fig 3D), causing an additional 13% loss of the HLA*B 14:02 allele by mutational inactivation (Fig 3D–H).

Significant Enrichment in Pathways Regulating Immunity and Transcription

Gene Ontology (GO) pathway analysis of the 28 genes disrupted by Tier 1 mutations revealed a significant enrichment in pathways regulating immunity and transcription (Table 4). GO categories with significant enrichment included antigen processing and presentation via MHC class I and MHC class I receptor activity, interferon-gamma-mediated signaling and cellular response to interferon-gamma, response to and detection of bacterium, innate immune response, detection of biotic stimulus, as well as transcription and regulatory region, DNA and nucleic acid binding.

Discussion

Using comparative WES and genome-wide SNP-A analysis, we have identified clonal hematopoiesis in the majority of aAA patients, including 67% of patients with pediatric-onset aAA. Our results demonstrate that clonal hematopoiesis in aAA is much more common than previously described, and can emerge early in the disease course—half of the patients with mutations in our cohort were within 1 year of diagnosis. Our data indicate that in the younger patient population, somatic mutations in aAA overlap only to a small degree with mutations typical of MDS. Instead, mutations in aAA are enriched in genes regulating immunity and transcription, with immune escape and proliferative signaling emerging as the main drivers of clonal hematopoiesis.

The finding of early emergence of clonal hematopoiesis in the majority of young aAA patients is likely to redefine how we view aAA, and helps put into context results of candidate gene sequencing in aAA [8–9, 27]. Our data indicate that a large proportion of blood production in aAA patients comes from clonal or oligoclonal hematopoiesis. It is important to note that a haploid mutant allele frequency of 15% as measured by WES corresponds to a 30% diploid clone harboring a heterozygous mutation, and to an even larger proportion when accounting for cellular heterogeneity of bone marrow aspirates. Using an unbiased approach of WES in a young patient cohort, we found mutations in MDS-associated genes only in a small subset of patients (n=2 (9%), one of whom was a 52-year-old adult). The high prevalence and diversity of somatic mutations in aAA patients in our study is similar to findings from WES of thirteen patients with PNH [23], which reflects the overlap in the underlying immune pathogenesis and selective pressure in these disorders, and highlights the role of *PIGA* as one of a number of drivers of clonal evolution in aAA and PNH. Another important corollary of our findings is that, in the absence of morphologic dysplasia, the presence of somatic mutations cannot be used as a diagnostic tool to distinguish hypoplastic MDS from aAA. Although it is possible that some clones in aAA may be transient, as was previously described for chromosomal abnormalities in aAA [28], our data show that somatic mutations can persist overtime contributing to a significant portion of the patients' hematopoiesis. Taken together, our results suggest that an individual

patient's clinical course is likely to be influenced by their unique somatic alterations, which, for most patients, differ from mutations of hematologic malignancies.

The diversity of mutations in aAA presents a challenge in distinguishing driver from passenger mutations; driver mutations are expected to involve recurrently-mutated genes or pathways, and to confer a proliferative advantage on the mutant clone. Aside from mutations previously implicated in clonal hematopoiesis, MDS, and leukemia (*PIGA*, *BCOR* and *ASXL1*), only one gene (*ERCC6L)* was recurrently affected in our cohort. Among the other mutations, those with translational consequences in pathways of hematopoietic growth and immunity were considered potential drivers: the strongest candidates were mutations in HLA alleles, *STAT5B*, and *CAMK2G*.

Although selection against specific HLA alleles has been proposed to drive 6p CN-LOH emergence[5–7], it has been difficult to exonerate other genes that are in linkage disequilibrium with the HLA locus and are also relevant to hematopoiesis and immunity (e.g. *TNF* or complement)[29]. Our finding of two patients with oligoclonal loss of HLA class I alleles, in whom specific HLA alleles were lost by two different mechanisms—loss-of-function mutation and 6p CN-LOH—strongly argues that in these patients loss of HLA alleles drives oligoclonal hematopoiesis (Figure 3). The recently reported nonsense mutation in the HLA*B 40:02 allele in a 7-year-old aAA patient [30] supports our findings, and provides further evidence that HLA class I loss may be a common and previously underappreciated mechanism behind clonal hematopoiesis in aAA. It is intriguing that oligoclonal hematopoiesis has also been observed with *PIGA* mutations, where multiple inactivating mutations can co-occur [23, 31]; this may be another instance of a more general phenomenon of immune selection leading to oligoclonal hematopoiesis. Future studies using targeted deep sequencing of HLA alleles will help to define the frequency of HLA loss and its prognostic implications [32].

Two other candidate driver mutations in our study—*CAMK2G* p.T306M and *STAT5B* p.N642H—affected key proliferative pathways. The first, *CAMK2G* p.T306M, involves a multifunctional serine-threonine protein kinase critical for Ca^{2+}-based second messenger signaling and for proliferation of myeloid leukemias[33–34]. CAMK2G is inactivated by autophosphorylation of regulatory residues T306 and T307 (numbered T305 and T306 in the α-isoform), which greatly lower its affinity for Ca^{2+}/calmodulin. Mutagenesis of T306 and T307 residues has been shown to abrogate kinase inhibition and increase its activity [26]. It is of interest that this mutation occurred in a patient on long-term cyclosporine therapy; a possible explanation could be that the mutation was selected for by cyclosporine, which activates CAMK2 though inhibition of calcineurin, a Ca^{2+}/Calmodulin-dependent protein phosphatase [35]. The second mutation, *STAT5B* p.N642H, was previously demonstrated to increase activation of transcriptional activity by the *STAT5B* gene [24]. Although the lack of historical banked tissue from this patient precludes the evaluation of the timeline of clone emergence, it is intriguing to consider its potential relationship to the patient's eltrombopag therapy. Enhanced *STAT5B* signaling conferred by the p.N642H mutation could render *STAT5B*–mutant cells hypersensitive to thrombopoietin, leading to clonal expansion. Longitudinal studies of aAA patients treated with thrombopoietin agonists and other

therapies will be important to define effects of therapy on clonal emergence and its prognostic implications.

Our study has limitations. This is a cross-sectional study of a relatively small patient population with limited follow-up and, thus, cannot assess the prognostic implications of specific mutations. However, aAA is a rare disease; and at a median follow-up of 15 months (range 0–38), none of the patients had neoplastic transformation. Due to material availability, clonal architecture analysis was only performed for two patients with oligoclonal loss of HLA I alleles. Future longitudinal studies of multi-institutional patient cohorts, incorporating clonogenic assays and deep sequencing will be needed to determine the frequency and recurrence of mutations, to correlate mutational spectrum with treatment response and disease outcomes, and to evaluate clonal architecture and clonal dynamics. Our study does not include comparative WES of healthy controls. However, recent studies using WES of control populations have shown that only 2–3% of individuals and up to 10% of people over 65 years have clonal mutations; in contrast, healthy children and young adults have none or only rare and non-clonal somatic mutations[36–43]. Thus, clonal hematopoietic expansion in pediatric and young adult aAA patients is strikingly different from healthy hematopoiesis. It is intriguing to consider potential differences in the mutational spectrum between the pediatric patients and older adults, who are more likely to carry age-related somatic mutations associated with malignancy; future longitudinal studies comparing the mutational profiles and disease outcomes in patients of different age groups will help to better understand the prognostic implications of clonal hematopoiesis and its interaction with age. Finally, the low background mutation rate in younger patients may have caused us to underestimate the frequency of clonal hematopoiesis in children. Our study did not identify subclonal mutations with haploid allele frequencies below ∼10% as measured by WES (which corresponds to a ∼20% diploid clone size). Importantly, all of the identified somatic mutations comprise a large (>20%) fraction of patients' hematopoietic production, and are thus likely to influence their hematologic presentation, response to therapy, and disease course.

In conclusion, we have shown that clonal hematopoiesis is present in the majority of aAA patients, including two thirds of patients with pediatric-onset aAA, and can occur early in disease. Our data demonstrate that in the younger patient population somatic mutations in aAA have only a partial overlap with mutations of MDS, and instead bear signatures of immune escape and proliferative signaling. Our finding of frequent clonal hematopoiesis in aAA expands the conceptual understanding of the pathophysiology of this non-neoplastic blood disorder, and demonstrates that clonal hematopoiesis in aAA extends beyond the known association with *PIGA* mutations. Prospective studies of clonal hematopoiesis in aAA and its relationship to aAA therapies and patient outcomes will be critical for understanding the determinants of disease response and transformation, and for designing personalized treatment strategies in aAA.

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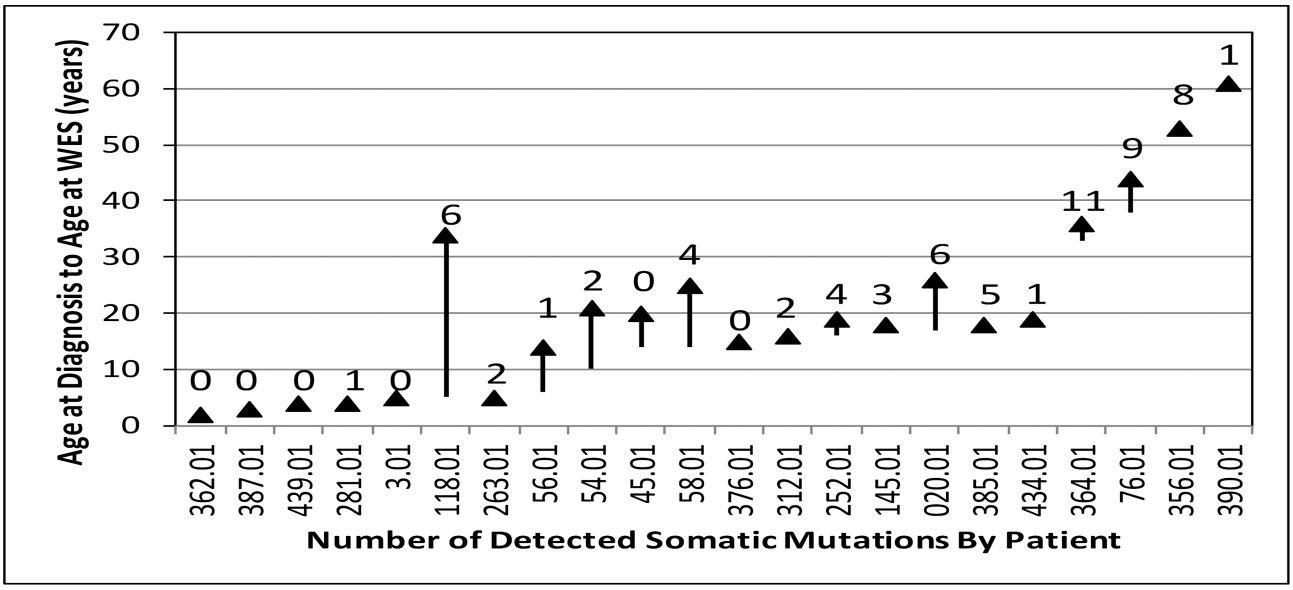
A



B

Clinical Characteristic	Clonal Hematopoiesis (n=16)	No Clonal Hematopoiesis (n=6)	p-value
Age at diagnosis (years)	16.5 (3-61)	3.5 (1.5-15)	**0.012**
Disease onset (n)			0.541
Pediatric-onset	12	6	
Adult-onset	4	0	
Age at WES (years)	20 (4-61)	4.5 (2-20)	**0.011**
Use of Immunosuppressive Therapy (IST)			0.533
Yes	13	6	
No	3	0	
Time from IST (years)	1 (0-18)	0.75 (0.3-6)	0.803
Duration of disease (years)	1 (0.08-29)	0.88 (0.5-6)	0.682
AA severity (n)			0.341
NSAA	1	0	
SAA	10	6	
VSAA	5	0	
Telomere length (n)			1.000
<10th Percentile	4	1	
>10th Percentile	10	5	
n/a	2	0	

Figure 1. Clinical Characteristics Associated with Presence or Absence of Clonal Hematopoiesis in aAA

A. The distribution of the total number of identified somatic mutations per patient as it relates to the patients' age and disease duration. For each patient, listed on the X-axis, the age is plotted as a vertical line, with the beginning of the line corresponding to age at diagnosis and an arrowhead depicting age at WES. The number above the line corresponds to the total number of somatic mutations. B. Association analysis of clinical characteristics with presence or absence of clonal hematopoiesis in aAA.

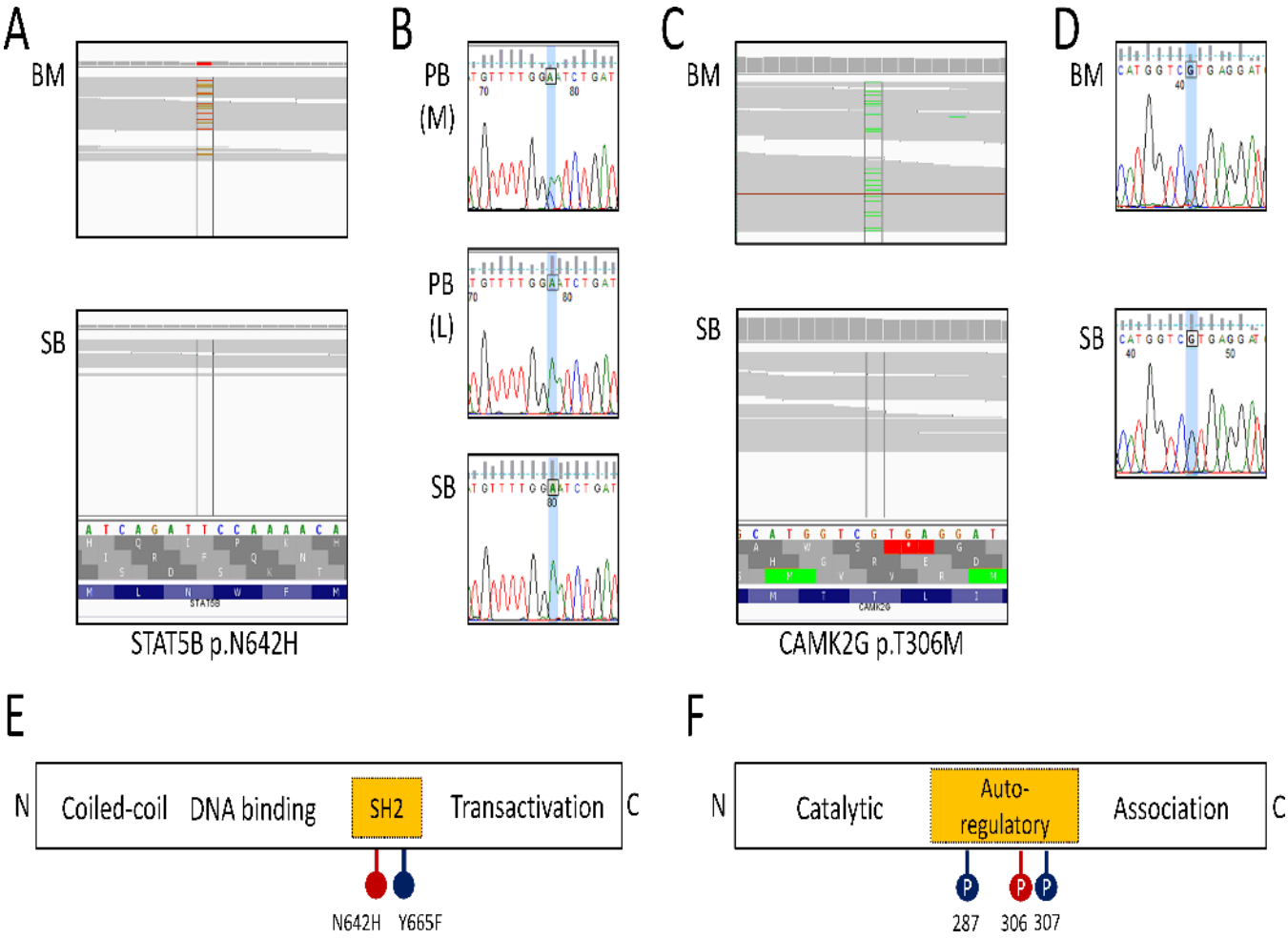
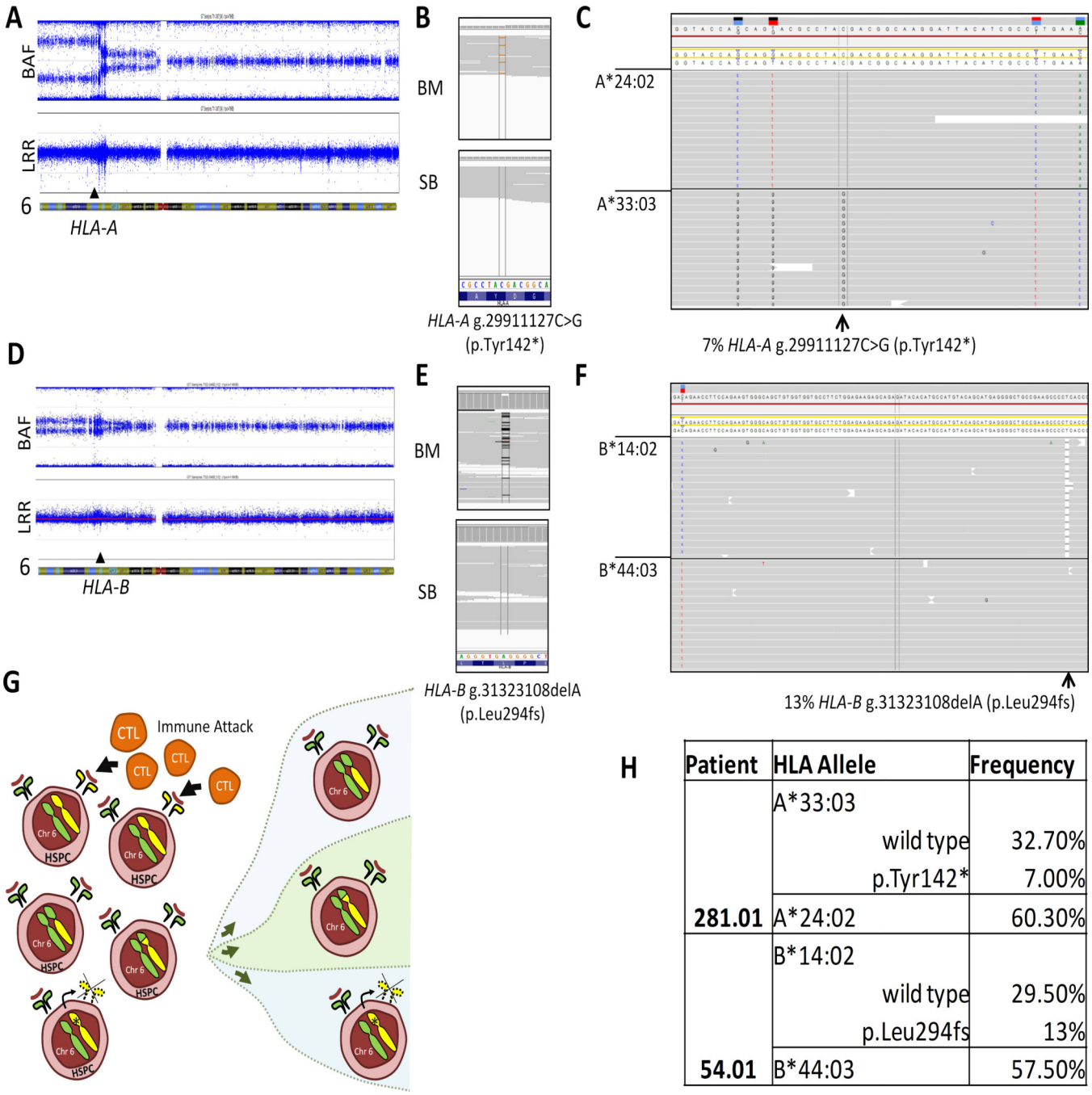


Figure 2. Acquired mutations in the bone marrow of aAA patients

A. IGV screenshots of WES of bone marrow(BM) and constitutional DNA (skin biopsy, SB) showing a g.40359729T>G (p.N642H) mutation in *STAT5B* gene present in the bone marrow but not in the paired constitutional DNA. B. Sanger sequencing chromatograph showing that the acquired g.40359729T>G (p.N642H) mutation is detected in the immunomagnetically sorted myeloid cell fraction of peripheral blood, PB (M), but is absent from the lymphocyte fraction, PB (L), and skin fibroblast DNA (SB); the chromatograph shows reverse complement sequence. C. IGV screenshots of WES of bone marrow (BM) and constitutional DNA (skin biopsy, SB) showing a g.75601956G>A (p.T306M) mutation in *CAMK2G* present in the bone marrow but not in the paired constitutional DNA. D. Sanger sequencing chromatograph validating that the acquired g.75601956G>A (p.T306M) mutation in *CAMK2G* is detected in the bone marrow (BM), but is absent in the skin fibroblast DNA (SB). E. A schematic of the STAT5B protein illustrating the location of previously reported activating mutations in the SH2 domain. F. A schematic of the CAMK2G protein showing the locations of regulatory phosphorylation sites.



H

Patient	HLA Allele	Frequency
	A*33:03	
	wild type	32.70%
	p.Tyr142*	7.00%
281.01	A*24:02	60.30%
	B*14:02	
	wild type	29.50%
	p.Leu294fs	13%
54.01	B*44:03	57.50%

Figure 3. Oligoclonal Loss of HLA class I alleles in aAA

A. SNP-A genotyping of patient 281.01 depicted as two scatter plots. The top plot shows B-allele Frequency (BAF, a relative frequency of the minor allele); the bottom plot shows Log R Ratio (LRR, a measure of normalized total signal intensity for both alleles). The chromosomal location is depicted on the X-axis. In a region with acquired CN-LOH, the copy number (indicated by the LRR) remains constant, while there is a decreased frequency of the heterozygous alleles (indicated by the change in the left part of the BAF plot). The location of the *HLA-A* gene is shown by the arrowhead. B) IGV screenshots of WES of bone

marrow (BM) and constitutional DNA (skin biopsy, SB) showing the HLA-A g. 29911127C>G (p.Tyr142X) mutation in the bone marrow but not in the paired constitutional DNA. C. Next generation sequencing of the HLA locus, showing the location of the nonsense *HLA-A* g.29911127C>G (p.Tyr142*) mutation (arrow) occurring *in cis* to the *HLA-A**33:03:01 allele. D. SNP-A genotyping of patient 54.01. The top plot shows B-allele Frequency (BAF, a relative frequency of the minor allele); the bottom plot shows Log R Ratio (LRR, a measure of normalized total signal intensity for both alleles). In a region with acquired CN-LOH, the copy number (indicated by the LRR) remains constant, while there is a decreased frequency of the heterozygous alleles (indicated by the change in the left part of the BAF plot). The location of the *HLA-B* gene is shown by the arrowhead. E. IGV screenshots of WES of bone marrow (BM) and constitutional DNA (skin biopsy, SB) showing the frameshift HLA-A g.31323108delA (p.Leu294fs) mutation in the bone marrow but not in the paired constitutional DNA. F. Next generation sequencing of the HLA locus, showing the location of the *HLA-B* g.31323108delA (p.Leu294fs) mutation (arrow) occurring *in cis* to the *HLA-B**14:02:01 allele. G. A model schematic depicting recurrent loss of HLA class I alleles in patients with aAA. Cytotoxic T lymphocytes (CTL) cause autoimmune depletion of hematopoietic stem and progenitor cells (HSPC) due auto-antigen presentation in the context of particular HLA class I alleles. Thus, cells with loss of particular HLA class I alleles either via an inactivating mutation, or by loss of heterozygosity favoring the opposite allele, lead to a growth advantage and resultant clonal expansion of the mutant hematopoietic cells. H. Table showing HLA typing results of the peripheral blood for patients 281.01 and 54.01.

Table 1

Patient Characteristics

	Overall (n=22)	Pediatric-Onset (n=18)	Adult-Onset (n=4)
Patient Characteristics			
Gender, female (%)/male (%)	13 (59) / 9 (41)	9 (50) / 9 (50)	4 (100) / 0 (0)
Age at diagnosis (years), median (range)	14.5 (1.5–61)	12 (1.5–19)	45.5 (33–61)
Age at sequencing (years), median (range)	18.5 (2–61)	17 (2–34)	48.5 (36–61)
Disease duration (years), median (range)	1 (0.1–29)	1 (0.1–29)	1.7 (0.25–6)
Duration of follow-up (months), median (range)	14.8 (0–38.2)	13.3 (0–38.2)	20.3 (0–25.6)
Disease severity (n)			
Moderate	1	0	1
Severe	16	15	1
Very Severe	5	3	2
Post-immunosuppression (IST), n (%)	19 (86.4)	16 (88.9)	3 (75.0)
Time from IST (years), median (range)	1 (0–18)	1 (0–18)	0.25 (0.25–3)
Eltrombopag use (n)	2	1	1
Transformation to MDS (n)	0	0	0
Telomere length			
≥ 10th Percentile of Age-Matched Controls	15	11	4
< 10th Percentile of Age-Matched Controls	5	5	0
N/A	2	2	0
% of patients with PNH clone (>1%)	45.5	44.4	50.0
Summary of Acquired Genetic Changes			
Acquired CN-LOH, n (%)	6 (27.3)	5 (27.8)	1 (25.0)
6pLOH, n (%)	3 (13.6)	3 (16.7)	0 (0.0)
Patients with somatic mutations, n (%)	16 (72.7)	12 (66.7)	4 (100.0)
Tier 1 and 2 gene mutations per patient, median (range)	2 (0–11)	1 (0–6)	8.5 (1–11)
Acquired cytogenetic abnormality, n	1	0	1

Pediatric-Onset aAA: diagnosis under 22 years of age; Adult-onset aAA: diagnosis at 22 years and older.

Tier 1: nonsynonymous coding, consensus splice site, or RNA genes; Tier 2: regions with regulatory potential, such as 5’ and 3’ untranslated regions (UTR).

Table 2

Clinical and Genetic Findings in Pediatric- and Adult-Onset aAA

Patient Number	Age Diagn. (yrs)	Age WES (yrs)	Sex	AA Duration (yrs)	Severity	IST (Y/N)	Time After IST (yrs)	TL	TCR	Marrow Cellularity (%)	WBC (10^3/μl)/ANC (cells/μl)	Hgb (g/dL)	Plt (10^3/μl)	Disease Status at WES	Karyotype	CN-LOH	PNH Clone	# Tier 1 & 2 Muts.	Tier 1 Mutations	Tier 2 Mutations	Other
Pediatric-Onset aAA																					
362.01	1.5	2	F	1.2	SAA	Y	1.2	N	n/a	50–60	4.6/2521	9.6	146	PR	Normal	None	None	0	None	None	
387.01	2	3	M	0.5	SAA	Y	0.5	N	Nl	60–70	5.5/1282	7.9	52	PR	Normal	None	None	0	None	None	
439.01	3	4	F	0.8	SAA	Y	0.3	N	Nl	30–50	4.2/546	9.1	60	PR	47,XX, +der(15)c[20]	None	None	0	None	None	
281.01	3	4	M	0.7	SAA	N	n/a	L	n/a	80–90	5.7/1140	11.1	37	PR	Normal	6p	None	1	*HLA-A* (Y142X)	None	
3.01	4	5	M	1.0	SAA	Y	1.0	N	n/a	70–80	5.5/3267	10.7	39	PR	Normal	None	None	0	None	None	
118.01	5	34	F	29.0	SAA	Y	18.0	n/a	Clonal	n/a	3.6/2500	8	39	PR	Normal	None	Large	5	*HTR4* (K220N), *TAF1C* (D21N), *GZMM* (R37C), *PIGA* (V432fs)	*BFAR* (3'UTR)	*PTPRD* (syn)
263.01	5	5	F	0.5	SAA	Y	0.5	N	n/a	30–40	3.0/990	9.6	15	PR	Normal	None	Subclinical	2	None, PIGA[#]	*CYP4F24P* (NCExonic)	
56.01	6	14	M	8	VSA A	Y	8	L	n/a	80–90	5.1/1647	10.9	35	Rel.	Normal	6p	None	0	None	None	*LCT* (syn)
54.01	10	21	M	11.0	SAA	Y	7.0	L	Nl	15	2.7/713	12.2	100	PR	Normal	6p	None	1	*HLA-B* (Leu294fs)	None	*LYPD8* (upstr)
45.01	14	20	M	6.0	SAA	Y	6.0	L	Nl	10–70	3.7/1628	15.5	114	PR	Normal	None	None	0	None	None	
58.01	14	25	F	11.0	SAA	Y	10.0	N	n/a	50–60	4.0/1750	9.9	246	CR	Normal	None	Moderate	4	*CDC14A*(V166M), *ERCC6L*(R372X), *PIGA* (ex 2 splice)	*CYCS* (3'UTR)	
376.01	15	15	F	0.5	SAA	Y	0.5	N	Nl	60–70	3.6/1418	11.5	115	PR	Normal	None	None	0	None	None	
312.01	15	16	M	1.0	SAA	Y*	1.0	N	Clonal	50–60	5.1/3254	15.7	151	Rel.	Normal	15q	Moderate	2	*PIGA*[#]	*MOSPD1* (3'UTR)	
252.01	16	19	F	2.0	SAA	Y	2.0	n/a	n/a	80–90	3.3/1752	10.3	196	CR	Normal	None	Large	4	*BCOR* (V594I), *PIGA* (A313fs)	*MSRA* (3'UTR), *EPG5* (3'UTR)	
145.01	17	18	F	0.6	VSA A	Y	0.3	N	Indetr.	30–40	4.5/1080	9.7	14	NR	Normal	None	Subclinical	3	*ZSCAN5B* (Q322X), *PIGA* (F259fs)	*IL22RA2* (3'UTR)	
020.01	17	26	F	9.0	SAA	Y	6.0	L	Nl	40	4.8/1890	11.2	41	Rel.	Normal	5q	Moderate	6	*CAMK2G* (T306M), *BPTF* (D312H), *WDR18* (A156T), *PIGA*[#]	*PDE10A* (3'UTR), *SLC2A13* (3'UTR)	

Patient Number	Age Diagn. (yrs)	Age WES (yrs)	Sex	AA Duration (yrs)	Severity	IST (Y/N)	Time After IST (yrs)	TL	TCR	Marrow Cellularity (%)	WBC (10^3/μl)/ANC (cells/μl)	Hgb (g/dL)	Plt (10^3/μl)	Disease Status at WES	Karyotype	CN-LOH	PNH Clone	# Tier 1 & 2 Muts.	Tier 1 Mutations	Tier 2 Mutations	Other
385.01	18	18	M	0.1	VSA A	N	0.0	N	Nl	0–50	3.4/942	10.9	24	Diagn.	Normal	None	n/a	4	*EMX2* (A172V), *DSG1* (M208V), *PIGA* (K88N)	*LARP* (3'UTR)	*OR10A 7* (syn)
434.01	19	19	M	0.3	SAA	Y	0.3	N	Nl	80	3.8/2481	12.8	162	CR	Normal	None	None	0	None	None	*CHPF2* (upstr)
Adult-Onset aAA																					
364.01	33	36	F	3.0	SAA	Y	3.0	N	Nl	60	7/3730	8.7	62	Refr.	Normal	None	None	11	*SLC22A6* (R336C), *STAT5B* (N642H), *WFDC12* (G27C), *DNAJC24 (*ex4 splice)	*OSBPL11* (3'UTR), *SENP6*(3'UTR), *MET*(3'UTR), *NFIB*(3'UTR), *ZNF747*(3'UTR), *BLCAP*(3'UTR), *CLCN5* (3'UTR)	
76.01	38	44	F	6.0	NSA A	N	n/a	N	Nl	75	3.2/2260	8.1	124	NSAA	Normal	None	Large	9	*CACNB3* (T347M), *SLC12A5*(M1073R), *HHIP* (R434G), *ERCC6L* (P947T), *PIGA* (I61fs)	*MUL1* (3'UTR), *HSPB2* (3'UTR), *SCN2B* (3'UTR), *RBL1* (3'UTR)	
356.01	53	53	F	0.3	VSA A	Y	0.3	N	Nl	35	4.1/2390	10.2	190	PR	Normal	None	None	8	*SYNPO2*(Q709K), *IL20RA*(Q473K), *FOS*(G97R), *NINL*(R667C), *SEPT3*(R165H), *ASXL1*(C687fs)	*LMTK2* (5'UTR), *ATP2B3* (3'UTR)	
390.01	61	61	F	0.4	VSA A	Y	0.3	N	Nl	25	3.2/1920	8.3	32	PR	46,XX,del(13)(q12q14)[2]/46,XX[18]	6 WC	Subclinical	1	*PIGA*(F438del.L439del)	None	

SAA, severe aAA; VSAA, very severe aAA; NSAA, non-severe aAA. TL, telomere lengths (N: ≥10^{th} percentile of normal, L: <10^{th} percentile of normal). Marrow cellularity, bone marrow cellularity at the time of WES, performed at the corresponding disease duration (AA Duration), time from immunosuppression (Time After IST), and Disease Status (CR, complete response; PR, partial response; Rel., relapse, Refr., refractory aAA, Diagn., diagnosis). TCR, T-cell receptor rearrangement (Nl: polyclonal; Clonal; Inder.: indeterminate). PNH Clone (None (<1%), Subclinical (1–10%), Moderate (10–50%), Large (>50%)).

#: In agreement with published studies of *PIGA* gene sequencing in PNH [1, 2], three patients had flow-cytometric evidence of PNH for which we were unable to identify a mutation by WES or bi-directional Sanger sequencing of the *PIGA* exons and their immediately flanking regions. These were likely caused by multiple independent mutations in *PIGA* below the level of detection of WES and Sanger sequencing [3].

Table 3

Clonal Hematopoiesis in Pediatric- and Adult-Onset aAA

Patient Number	Acquired Structural Abnormalities			PNH Clone	Acquired Mutations									
	Type	Chr	Clone		Chr	Position	Ref/Alt	Depth	Freq	Classification	Gene	Exon	Protein	Lineage
Pediatric-Onset aAA														
281.01	CN-LOH CN-LOH	6pterp22.1 6pterp12.1	~30% ~10%	None	6	29911127	C/G	56	9%	Stopgain	*HLA-A*	3	p.Tyr142*	-
118.01	None			92–98%	X	15339787	TCTGGAGTC ATCCATCTCA AGAAAATGA GGAAGAGGA AGTTGAAAA/−	364	13%	Frameshift Del	*PIGA*	6	p.Val432fs	-
					5	147889435	C/G	262	28%	Nonsyn SNV	*HTR4*	5	p.Lys220Asn	-
					16	84218534	C/T	87	27%	Nonsyn SNV	*TAF1C*	2	p.Asp21Asn	-
					9	8518131	G/T	250	25%	Synonymous	*PTPRD*	9	p.=	-
					16	14761829	G/T	134	21%	UTR3	*BFAR*	n/a	n/a	-
					19	547333	C/T	76	18%	Nonsyn SNV	*GZMM*	2	p.Arg37Cys	-
263.01	None			3%[1]	19	15882928	G/A	49	12%	NCExonic	*CYP4F24P*	n/a	n/a	-
56.01	CN-LOH CN-LOH CN-LOH CN-LOH	6pterp21.31 6pterp21.1 6pterp12.1 6pterp11.1	~10% ~15% ~45 ~15	none	2	136575226	C/T	216	11%	Synonymous	*LCT*	6	p.=	-
54.01	CN-LOH CN-LOH	6pterp21.31 6pterp21.1	~10% ~10%	none	1 6	248903467 31323108	C/T A/−	20 341	40% 16%	Upstream Frameshift Del	*LYPD8* *HLA-B*	n/a 4	n/a p.Leu294.fs	M n/a
58.01	None			18–25%	X	15349337	C/T	42	36%	Splicing	*PIGA*	2	?	n/a
					1	100908529	G/A	130	22%	Nonsyn SNV	*CDC14A*	7	p.Val166Met	M
					7	25162020	C/T	354	18%	UTR3	*CYCS*	n/a	n/a	M
					X	71427503	G/A	261	18%	Stopgain	*ERCC6L*	2	p.Arg372*	M
312.01	CN-LOH CN-LOH	15q21.1qter 15q12qter	~5–8% ~5–8%	17–35%[1]	X X	134022913 134022943	A/C A/G	67 55	9% 11%	UTR3 UTR3	*MOSPD1* *MOSPD1*	n/a n/a	n/a n/a	- -
252.01	None			90%	18	43432246	AC/−	59	17%	UTR3	*EPG5*	n/a	n/a	-

Patient Number	Acquired Structural Abnormalities			PNH Clone	Acquired Mutations									
	Type	Chr	Clone		Chr	Position	Ref/Alt	Depth	Freq	Classification	Gene	Exon	Protein	Lineage
					X	15343166	CACGATCGC CATGCAGAA TG/−	230	13%	Frameshift Del	*PIGA*	3	pAla313fs	-
					X	39932819	C/T	104	13%	Nonsyn SNV	*BCOR*	4	p.Val594Ile	-
					8	10286083	A/T	154	12%	UTR3	*MSRA*	n/a	n/a	-
					19	56701720	C/A	33	55%	Stopgain	*ZSCAN5B*	4	p.Glu322*	M
					6	137465187	C/T	530	30%	UTR3	*IL22RA2*	n/a	n/a	M
145.01	None			9%	X	15344108	A/−	89	21%	Frameshift Del	*PIGA*	3	p.Phe259fs	M
					19	990233	G/A	271	16%	Nonsyn SNV	*WDR18*	4	p.Ala156Thr	-
					17	65850376	G/C	358	13%	Nonsyn SNV	*BPTF*	2	p.Asp312His	-
					10	75601956	G/A	162	12%	Nonsyn SNV	*CAMK2G*	12	p.Thr306Met	-
					6	165741064	T/−	140	12%	UTR3	*PDE10A*	n/a	n/a	-
20.01	CN-LOH	5q15qter	∼15–20%	16%[1]	12	40153078	G/T	213	8%	UTR3	*SLC2A13*	n/a	n/a	-
					X	15349789	T/A	74	22%	Nonsyn SNV	*PIGA*	2	p.Lys88Asn	-
					12	55615758	G/A	103	12%	Synonymous	*OR10A7*	1	p.=	-
					18	28911768	A/G	74	9%	Nonsyn SNV	*DSG1*	6	p.Met208Val	-
					5	154194771	T/G	497	9%	UTR3	*LARP1*	n/a	n/a	-
385.01	None			n/a	10	119305251	C/T	57	9%	Nonsyn SNV	*EMX2*	2	p.Ala172Val	-
434.01	None			none	7	150929476	T/G	19	58%	Upstream	*CHPF2*	n/a	n/a	Both
Adult-Onset aAA														
					17	40359729	T/G	60	23%	Nonsyn SNV	*STAT5B*	16	p.Asn642His	M
					3	125247758	C/T	122	20%	UTR3	*OSBPL11*	n/a	n/a	M
					11	31447832	AGA/−	134	17%	Splicing	*DNAJC24*	4	?	M
					7	116438157	A/G	328	16%	UTR3	*MET*	n/a	n/a	M
					20	43753010	C/A	186	16%	Nonsyn SNV	*WFDC12*	1	p.Gly27Cys	M
					20	36146442	G/A	363	16%	UTR3	*BLCAP*	n/a	n/a	n/a
					6	76425706	A/G	144	16%	UTR3	*SENP6*	n/a	n/a	M
					X	49861453	G/T	227	15%	UTR3	*CLCN5*	n/a	n/a	M
364.01	None			none	9	14085693	T/A	378	15%	UTR3	*NFIB*	n/a	n/a	n/a

Patient Number	Acquired Structural Abnormalities			PNH Clone	Acquired Mutations									
	Type	Chr	Clone		Chr	Position	Ref/Alt	Depth	Freq	Classification	Gene	Exon	Protein	Lineage
					16	30544011	G/T	86	14%	UTR3	*ZNF747*	n/a	n/a	M
					11	62748488	G/A	89	11%	Nonsyn SNV	*SLC22A6*	6	p.Arg336Cys	M
					20	35624870	G/A	113	30%	UTR3	*RBL1*	n/a	n/a	-
					X	15349874	-/A	118	28%	Frameshift Ins	*PIGA*	2	p.Ile61fs	-
					X	71425778	G/T	157	27%	Nonsyn SNV	*ERCC6L*	2	p.Pro947Thr	-
					11	111784673	G/T	114	26%	UTR3	*HSPB2*	n/a	n/a	-
					12	49220845	C/T	58	24%	Nonsyn SNV	*CACNB3*	12	p.Thr347Met	-
					20	44685832	T/G	81	19%	Nonsyn SNV	*SLC12A5*	25	p.Met1073Arg	-
					1	20825987	T/A	48	17%	UTR3	*MUL1*	n/a	n/a	-
					11	118036457	C/T	52	14%	UTR3	*SCN2B*	n/a	n/a	-
76.01	None			80–90%	4	145629462	A/G	146	9%	Nonsyn SNV	*HHIP*	7	p.Arg434Gly	-
					6	137322940	C/T	184	49%	Nonsyn SNV	*IL20RA*	7	p.Glu473Lys	Both
					14	75746727	G/A	175	43%	Nonsyn SNV	*FOS*	2	p.Gly97Arg	Both
					20	31022573	GT/−	121	40%	Frameshift Del	*ASXL1*	12	p.Cys687fs	Both
					4	119952055	G/A	218	39%	Nonsyn SNV	*SYNPO2*	4	p.Glu709Lys	Both
					20	25459761	G/A	177	37%	Nonsyn SNV	*NINL*	16	p.Arg667Cys	Both
					22	42383706	G/A	54	34%	Nonsyn SNV	*SETP3*	5	p.Arg165His	Both
					7	97736216	C/T	46	27%	UTR5	*LMTK2*	n/a	n/a	Both
356.01	None			none	X	152848098	A/G	121	18%	UTR3	*ATP2B3*	n/a	n/a	M
	CN-LOH	6WC	∼5%											
390.01	del	13q13.3q21.1	10%	6%	X	15339766	GAGGAA/−	260	1%	Del	*PIGA*	6	P.F438del.L439del	-

Chr, chromosome. PNH Clone, percentage of CD55, CD59-negative granulocytes as measured by flow cytometry of peripheral blood. Ref/Alt, reference sequence/mutated sequence. Depth, read depth of WES at a given location. Freq, mutant allele frequency in bone marrow, calculated as proportion of mutant allele reads at a given position. Lineage, lineage-specificity of somatic mutation (myeloid (M), lymphoid (L), or both).

[1] In agreement with published studies of *PIGA* gene sequencing in PNH [1, 2], three patients had flow-cytometric evidence of PNH for which we were unable to identify a mutation; these were presumed to be caused by multiple independent mutations in *PIGA* below the level of detection of WES and Sanger sequencing [3].

Table 4

Gene Ontology (GO) pathway analysis of genes disrupted by Tier 1 mutations in aAA

GO Category	Category Name	GO ID	R	raw P	adj P
Biological Process					
	antigen processing and presentation of exogenous peptide antigen via MHC class I, TAP-independent	GO:0002480	125	1E-04	**0.0229**
	interferon-gamma-mediated signaling pathway	GO:0060333	22.51	3E-04	**0.0229**
	response to bacterium	GO:0009617	8.06	3E-04	**0.0229**
	innate immune response	GO:0045087	6.26	3E-04	**0.0229**
	detection of bacterium	GO:0016045	62.52	5E-04	**0.0254**
	cellular response to interferon-gamma	GO:0071346	18.76	5E-04	**0.0254**
	response to interferon-gamma	GO:0034341	15.49	9E-04	**0.0381**
	detection of biotic stimulus	GO:0009595	43.28	0.001	**0.0381**
	response to cytokine stimulus	GO:0034097	5.56	0.002	0.0576
	immune response	GO:0006955	3.68	0.002	0.064
Molecular Function					
	MHC class I receptor activity	GO:0032393	61.88	5E-04	**0.036**
	transcription regulatory region DNA binding	GO:0044212	7.17	0.002	**0.0414**
	regulatory region DNA binding	GO:0000975	7	0.002	**0.0414**
	regulatory region nucleic acid binding	GO:0001067	7	0.002	**0.0414**
	heat shock protein binding	GO:0031072	13.06	0.01	0.1469
	transcription regulatory region sequence-specific DNA binding	GO:0000976	8.22	0.024	0.194
	secondary active transmembrane transporter activity	GO:0015291	6.12	0.042	0.194
	structure-specific DNA binding	GO:0043566	6.03	0.043	0.194
	sequence-specific DNA binding	GO:0043565	3.46	0.027	0.194
	DNA binding	GO:0003677	2.04	0.033	0.194

R, Ratio of enrichment; raw p, p value from hypergeometric test, adj P, p value adjusted by the multiple test adjustment (Benjamini-Hochberg).