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Clonal Evolution and Clinical Significance of Copy Number Neutral Loss of Heterozygosity of Chromosome Arm 6p in Acquired Aplastic Anemia

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Author Contributions

M.B., D.B., J.R., P.M., J.B., C.L., D.M.G.P., M. Bessler, and T.O. participated in the design and/or execution of this study, as well as interpretation of data; T.O. and M. Betensky performed clinical record review; D.B., J.R., J.B. and T.B. analyzed SNP-A; C.L., A.P. and D.M. analyzed HLA data; Y.L. performed statistical analysis; M. Betensky and T.O. prepared the manuscript. All authors approved the manuscript.

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Supplemental Material

Supplemental Table 1. Timing of Immunosuppressive Therapy and SNP-A Sample Collection in 6p CN-LOH patients

Supplemental Table 2. HLA Allele Frequency in AA Cohort

Abstract

Acquired aplastic anemia (aAA) results from the T cell-mediated autoimmune destruction of hematopoietic stem cells. Factors predicting response to immune suppression therapy (IST) or development of myelodysplastic syndrome (MDS) are beginning to be elucidated. Our recent data suggest most patients with aAA treated with IST develop clonal somatic genetic alterations in hematopoietic cells. One frequent acquired abnormality is copy-number neutral loss of heterozygosity on chromosome 6p (6p CN-LOH) involving the human leukocyte antigen (HLA) locus. We hypothesized that because 6p CN-LOH clones may arise from selective pressure to escape immune surveillance through deletion of HLA alleles, the development of 6p CN-LOH may affect response to IST. We used single nucleotide polymorphism array genotyping and targeted next-generation sequencing of HLA alleles to assess frequency of 6p CN-LOH, identity of HLA alleles lost through 6p CN-LOH, and impact of 6p CN-LOH on response to IST. 6p CN-LOH clones were present in 11.3% of patients, remained stable over time, and were not associated with development of MDS-defining cytogenetic abnormalities. Notably, no patient with 6p CN-LOH treated with IST achieved a complete response. In summary, clonal 6p CN-LOH in aAA defines a unique subgroup of patients that may provide insights into hematopoietic clonal evolution.

Keywords

aplastic anemia; clonal evolution; single nucleotide polymorphism array

Introduction

Acquired aplastic anemia (aAA) is a bone marrow failure disorder characterized by peripheral blood pancytopenia and a hypocellular bone marrow in the absence of neoplastic cell infiltration, dysplasia or fibrosis.[1] Although the precise mechanism of pathogenesis remains unknown, aAA is believed to arise from the immune-mediated destruction of hematopoietic stem cells (HSCs). HSC destruction is thought to be caused by auto-reactive, cytotoxic T lymphocytes (CTLs), which target specific, but as yet unknown auto-antigens, presented in the context of patient-specific human leukocyte antigens (HLA).[2,3]

In addition to immune-mediated bone marrow aplasia, aAA is characterized by frequent development of clonal cytogenetic abnormalities that can lead to transformation into MDS and acute leukemia in 10-15% of patients with aAA.[4] Our group has recently shown that the majority of patients with aAA (>70%) have evidence of clonal hematopoiesis with somatic mutations detectable by whole exome sequencing early in the course of their disease.[5] An autoimmune pathogenesis is likely implicated in the evolution of the clonal hematopoiesis seen in these patients.[6] Recent studies have identified acquired copy number neutral loss of heterozygosity on the short arm of chromosome 6 (6p CN-LOH) as a recurrent clonal abnormality uniquely present in aAA but interestingly not in inherited bone marrow failure conditions.[5-9] The HLA loci map to chromosome 6p, and deletions or LOH in this region result in the loss of alleles from one parental HLA haplotype within the affected clone, suggesting that 6p CN-LOH may emerge as the result of selective pressure to escape the auto-reactive immune response in aAA.[6]

We hypothesized that the development of 6p CN-LOH hematopoietic clones defines a unique subgroup of patients with aAA who may have a distinct response profile to IST and are likely to exhibit other features of immune-driven clonal evolution. To investigate this hypothesis, we evaluated the prevalence of 6p CN-LOH, the identity of specific HLA alleles lost through 6p CN-LOH, and the impact of 6p CN-LOH on clinical response to IST in our institutional cohort of 75 adult and pediatric patients diagnosed with aAA.

Methods

Patients

Consecutive adult and pediatric patients diagnosed with moderate or severe aAA, paroxysmal nocturnal hemoglobinuria (PNH) or hypocellular MDS, [10-12] enrolled in the Bone Marrow Failure Syndrome Repository Study at the University of Pennsylvania (PENN) and The Children's Hospital of Philadelphia (CHOP) from 2010 - 2014 were eligible to participate. Patients with isolated PNH and hypocellular MDS were included as these disorders share significant clinical and histological similarities with aAA and their clinical presentations overlap. Informed consent was obtained in accordance with the Declaration of Helsinki from all study participants or their legal guardians before participation; all studies were approved by the Institutional Review Boards of CHOP and PENN. Bone marrow (BM) or peripheral blood (PB) samples were collected for genome-wide single nucleotide polymorphism arrays (SNP-A) either as part of standard clinical evaluation or on a research basis. High-resolution HLA typing information was obtained from the clinical chart or performed on a research basis from PB and/or skin fibroblast samples. Clinical outcome data were extracted from the patient's charts. Clinical response was assessed in a cross-sectional fashion at the time of analysis. Response was defined based on the parameters used at our institution: a complete response (CR) after IST was defined as an absolute neutrophil count (ANC) greater than $1 \times 10^9/\text{L}$; hemoglobin greater than 11 g/dL; and a platelet count greater than $100 \times 10^9/\text{L}$. A partial response (PR) was defined as transfusion independence with an ANC $> 0.5 \times 10^9/\text{L}$, though with blood counts not meeting criteria for CR. Patients with treatment failure or relapse were those who remained transfusion dependent after the first round of IST and/or required additional therapy (second course of IST or bone marrow transplant). Adult patients were those 21 years of age or older at the time of study enrollment. Ethnicity was self-reported. Twenty-one patients were previously included in genetic characterization studies.[5,8]

SNP-A analysis

PB or BM DNA was extracted using Qiagen Gentra Puregene Tissue Kit (Qiagen, Valencia, CA) according to manufacturer's protocol. SNP-A genotyping was performed using the Illumina HumanOMNI1-Quad, InfiniumCytoSNP 850K or Human610-Quad BeadChip arrays at the CHOP Center for Applied Genomics as previously described.[13] The array chip used was determined based on the platform that was available within our institution at the time of sample processing. Samples were analyzed for the presence of copy number abnormalities and LOH using GenomeStudio (Illumina), which allows direct visualization of the LogR ratio (LRR) and B allele frequency (BAF) plots. The minimum length cutoff for defining a region of LOH was 5 Mb. The percentage of mosaicism for 6p CN-LOH was

estimated visually using the BAF plot.[14] For patients with confirmed 6p CN-LOH, repeat SNP-A analyses were performed at intervals determined by clinical follow-up schedule.

HLA Typing

High resolution HLA typing of PB, buccal swabs and/or skin fibroblast samples was performed either by conventional Sanger sequencing methods, or the Holotype HLA kit by Omixon (Budapest, Hungary), a paired-end next generation sequencing (NGS) approach using the Illumina MiSeq platform. For patients with confirmed 6p CN-LOH, quantitative relative parental HLA allele frequencies were determined to identify the parental allele(s) lost within 6p CN-LOH clones using PB, BM and buccal (control) samples sequenced at a depth of $>10,000\times$. [15] Sequence alignment and HLA allele assignment were performed using Target™ (Omixon; Budapest, Hungary) and NGSengine™ (GenDx; Utrecht, Netherlands) software. HLA allele frequency in our cohort was compared to that of published control populations from the National Marrow Donor Program freely available at <https://bioinformatics.bethematchclinical.org/HLA-Resources/>. [16]

Statistical Analysis

Frequencies between patient groups were compared using Fisher's exact test with a significance p -value < 0.05 (two-tailed). Comparison of HLA frequency data in our cohort to ethnically-matched population controls was performed using the Chi-square test, with a two-tailed significant p -value of < 0.05 . Significance was set at $p < 0.005$ based on Bonferroni method for multiple comparison adjustment (adjusted p value = 0.05 divided by 10 alleles compared for each gene).

Results

Patient Characteristics

A total of 75 consecutively enrolled patients were included, of whom 57 (76%) were pediatric, and 18 (24%) were adults (Table 1). The majority had a diagnosis of moderate or severe aAA ($n=67$), four of whom had a concurrent detectable PNH clone ($>5\%$ CD55-negative, CD59-negative white or red blood cells); the remainder had isolated PNH ($n=3$), drug-induced aplastic anemia ($n=3$), and hypoplastic MDS ($n=2$). Fifty-four patients received immunosuppressive therapy, including anti-thymocyte globulin (ATG) plus cyclosporine (CsA, $n=51$) or CsA alone ($n=3$). The three patients that received CsA alone had a diagnosis of non-severe aAA and none of them developed a 6p CN-LOH clone by the time of analysis. Ninety-five percent of patients ($n=71$) had SNP-A data available. Of these, 50% had their first SNP-A performed within 6 months from diagnosis. High resolution HLA typing was available or performed for 85% ($n=64$) of patients.

6p CN-LOH Prevalence and Clone Characteristics

At least one 6p CN-LOH clone was identified in eight of the 71 patients with available SNP-A data, defining a cohort prevalence of 11.3%. All patients with 6p CN-LOH had a diagnosis of aAA (Supplemental Table 1). 6p CN-LOH clones were detected in both BM and PB samples but clones tended to be of smaller size in PB compared to BM. In six of the eight patients, 6p CN-LOH clones were identified 6 months after diagnosis (Table 2),

suggesting the possibility that this abnormality may arise during hematopoietic recovery or that the low myeloid cellularity in patients with aAA within the first 3 months of diagnosis may preclude detection at diagnosis in some patients. The prevalence of 6p CN-LOH clones was slightly higher in adults compared to pediatric patients (17.6% vs 9.3% respectively) but this difference was not statistically significant ($p = 0.38$). Six of the patients with 6p CN-LOH had multiple breakpoints within the regions where CNLOH was detected (Figure 1A), indicating the presence of several clones with distinct percentages of cells affected by LOH. One patient displayed a clone with 6p CN-LOH involving 15-20% of cells that extended throughout the entire chromosome 6 (Patient 7, Table 3). This clone did not represent constitutional mosaicism, as it was not detectable in a prior SNP-A (data not shown). Patient 7 was the only 6p CN-LOH patient who had a normal SNP-A prior to the detection of a 6p CN-LOH clone. The initial normal SNP-A was performed two months after diagnosis. The 6p CN-LOH clone was not detected until six months after diagnosis in a follow up SNP-A (Supplemental Table 1).

Among the patients with evidence of multiple clones, clone size ranged from relatively small, estimated at 5-10%, to estimates as high as 80-85% of BM or PB cells (Table 3). In all cases, the commonly shared telomeric region included at least a portion of the HLA locus (Figure 1B). The universal involvement of the HLA locus, including the HLA alleles encoding the MHC, suggests that the HLA locus is the genetic target of the selective pressure leading to 6p CN-LOH evolution.

Clinical Outcomes and Clone Evolution in patients with aAA and 6p CN-LOH

Of the eight patients with 6p CN-LOH, six received IST as first line therapy and one received an allogenic bone marrow transplant from a matched sibling donor (Table 3). Interestingly, the remaining patient with 6p CN-LOH achieved a spontaneous remission over the course of 9-12 months after being treated with supportive care alone (no IST). All six patients treated with IST received both ATG and CsA and the majority of them were treated within two months from diagnosis (Supplemental Table 1). Notably, none of the patients with 6p CN-LOH who received IST achieved a CR with this therapy: three failed IST altogether, while the other three achieved only a PR. Of the three patients with a PR, two remain calcineurin-inhibitor dependent more than two years after initial IST. In contrast, 42% of patients without 6p CN-LOH ($n=19$) achieved a CR after IST (Table 2) (two-tailed p value = 0.07).

Six patients with 6p CN-LOH had repeat SNP-A genotyping performed in a schedule determined by clinical follow up care. In these patients, the 6p CN-LOH clones remained mostly stable in terms of size and distribution. One patient, Patient 3, demonstrated a decrease in length of the most centromeric clonal region (6p21.33p21.2 from 30,716,991 – 56,625,123 to 30,716,991 – 39,564,811) on follow-up SNP-A genotyping. The only new cytogenetic abnormality observed on follow up SNP-A genotyping developed in Patient 7, an interstitial deletion of 13q12-14 seen on a repeat SNP-A six months after diagnosis. Chromosome 13q deletions have been previously described as a recurrent clonal abnormality in aAA, though whether the development of this aberration has pathogenic consequences is unclear.[17] None of the patients with 6p CN-LOH developed bone marrow

dysmorphology or classic cytogenetic changes (i.e. Monosomy 7, Trisomy 8) known to be associated with transformation to MDS.

Identification of HLA alleles lost through 6p CN-LOH

In order to identify the HLA alleles deleted within the individual 6p CN-LOH, we used deep NGS of the HLA region to determine the quantitative majority and minority allele frequencies in patients' PB or BM samples. In patients with a large percentage of cells affected by 6p CN-LOH (Patients 1-6), minority (deleted) alleles were clearly detectable by comparing the percentage of reads for the two parental alleles, with the percentage of reads for the minority allele being inversely proportional to the number of clonal cells with 6p CN-LOH spanning each HLA gene (Table 4). For Patients 3 and 5, where the differences in minority/majority allele percentage were small, the identity of the minority alleles were validated by comparing these allele frequencies obtained from blood cell samples with allele frequencies in constitutional (skin fibroblast) DNA for each patient, and by comparison with common HLA haplotypes (data not shown). In Patient 7 and Patient 8, minority HLA alleles could not be determined, likely due to the small percentage of cells with 6p CN-LOH in each patient. Interestingly, alleles *HLA-B*14:02*, *HLA-C*08:02*, *HLA-DRB1*07:01* and *HLA-DQB1*02:02* were found to be affected in multiple patients (Table 4).

Overrepresentation of HLA alleles targeted by 6p CN-LOH in the overall aAA cohort

Examination of the allele frequency in our entire aAA cohort demonstrated that the minority HLA alleles lost by 6p CN-LOH represented both frequent as well as uncommon alleles (Supplemental Table 2). Given that several HLA alleles were recurrently deleted in 6p CN-LOH clones, we postulated that these minority alleles might represent "risk" alleles to which T cell responses in aAA are commonly restricted even in patients without 6p CN LOH. We thus asked if these risk alleles were found more frequently in patients who develop aAA compared to the general population.

We compared the frequencies of HLA allele which were targeted by 6p CN-LOH within our aAA cohort to the previously published HLA allele frequencies obtained from the National Marrow Donor Program stratified by ethnicity.[16] In patients reporting Caucasian ethnicity (n =47 patients, representing 94 alleles for each HLA gene), several HLA alleles were significantly over-represented in our aAA cohort compared to ethnicity-matched controls (Table 5). Of these, *HLA-A*33:01*, *HLA-B*14:02* and *HLA-C*8:02* were found to be lost within 6p CN-LOH clones in our cohort, with *HLA-B*14:02* and *HLA-C*8:02* each lost in two patients with 6p CN-LOH.

Discussion

The development of clonal hematopoiesis is a well-described complication in aAA, as manifested by a 10-15% risk of evolution to MDS and the detection of PNH clones in up to half of patients with aAA.[2, 18-20] However, with the emergence of high-throughput and whole genome-based analytic platforms for detecting somatic genetic alterations, many forms of clonal hematopoiesis are now being detected in patients with aAA for which clinical correlations regarding outcomes have not been elucidated.[21] Indeed, our group

recently demonstrated early emergence of clonal hematopoiesis in a striking majority of patients (72.7%) within our combined pediatric and adult cohort, even after exclusion of PNH clones which occurred in 45% of these patients, indicating that clonal hematopoiesis in aAA extends beyond the known association with *PIGA* mutations.[5]

The discovery of these frequent patterns of clonal hematopoiesis in aAA further blurs the dividing line between the diagnoses of hypocellular MDS and aAA, a distinction that is already difficult to make on morphologic grounds given the paucity of bone marrow elements present at diagnosis in both conditions.[22,23] For patients with poor-risk cytogenetic lesions associated with MDS or the development of acute myelogenous leukemia,[24] our center takes a therapeutic approach similar to that used in other forms of MDS. However, for patients with clonal cytogenetic abnormalities of unknown clinical significance, determining how these abnormalities may influence disease course with respect to IST efficacy or evolution to MDS is critical to therapy decisions such as when or if to employ unrelated donor stem cell transplantation strategies.

As 6p CN-LOH is a particularly common structural chromosomal aberration arising during the course of aAA, epidemiologic studies of how the development of 6p CN-LOH correlates with clinical outcome are critical. In one of the first reports of 6p CN-LOH in aAA, Katagiri et al (2011) showed that 13% of patients in a Japanese aAA cohort developed clonal 6p CN-LOH.[7] More recently, Yoshizato et al (2015) also reported a prevalence of clonal 6p CN-LOH of 13% in a multi-institutional aAA cohort that included patients from the prior published Japanese study.[9] However, a correlation of clinical outcomes with the presence of 6p CN-LOH in patients receiving IST was not reported.

In the present study, we have confirmed similar prevalence rates of clonal 6p CN-LOH (11.3%) in an aAA cohort that is demographically distinct, in terms of ethnicity and race, from those previously published. Our North American cohort was comprised mostly of Caucasian and pediatric patients. In our cohort, there was a trend towards a higher prevalence of 6p CN-LOH clones in adult patients, consistent with our previous findings that clonal hematopoiesis is more commonly identified in older patients with aAA.[5] However, it is possible that the observed trend might be related to the greater proportion of adult versus pediatric patients undergoing SNP-A more than six months from diagnosis (70.5% vs. 42.6% respectively; Table 1). In the majority of our patients, 6p CN-LOH clones were identified 6 months after the diagnosis of aAA suggesting that clones with 6p CN-LOH may arise during hematopoietic recovery. However, one limitation of our study is that DNA samples from the initial time of diagnosis were not available for SNP-A genotyping for all patients; thus determining the true incidence and kinetics of 6p CN-LOH onset will require future prospective studies.

Our study provides detailed information on the clinical outcomes of patients with 6p CNLOH and on the stability of 6p CN-LOH clones over time. A striking finding was that none of the six patients with aAA and 6p CN-LOH clones who received IST achieved a long term CR, with three individuals experiencing treatment failure/relapse. This finding appears to stand in contrast to the previously mentioned Japanese cohort, which had a combined CR + PR rate of 100% in response to IST in all evaluable patients with 6p CNLOH clones.[7]

The reason for this discrepancy in outcome and the mechanism(s) resulting in the poor response to IST among patients with 6p CN-LOH in our cohort are unclear, and confirms the need for future multi-institutional studies. One potential hypothesis is that since 6p CN-LOH clones have already provided the potential for escaping the autoimmunity that drives aAA by functionally deleting expression of specific HLA antigens targeted by CTLs, the use of IST may not provide additional benefit in this subgroup of patients. Therefore, in patients who develop 6p CN-LOH, hematopoietic recovery may be primarily limited by the size of the surviving hematopoietic stem cell pool, rather than by ongoing autoimmunity.

However, other potential explanations exist that could explain the discrepancy in outcome seen in aAA patients with 6p CN-LOH in our cohort versus those previously published. For example, specific demographic factors in our cohort, such as ethnicity and younger age (median age of our patients with 6p CN-LOH was 17 years) may have impacted therapy response. Our results may also be influenced by a referral bias, given that our cohort is derived from our regional Comprehensive Bone Marrow Failure Center, in which we provide care for a significant proportion of high risk and treatment refractory patients with aAA.

Importantly, none of the seven patients with 6p CN-LOH clones, who had follow-up cytogenetic studies, developed additional high risk, MDS-associated cytogenetic abnormalities at the time of their last follow-up (median follow-up 9 months, range 3-17 months from first +6p CN-LOH SNP-A). One patient with 6p CN-LOH developed an acquired chromosome 13q deletion without morphologic evidence of myelodysplastic syndrome on bone marrow histopathology. Chromosome 13q deletions have been previously described in aAA and their pathogenic significance remains incompletely defined, though recent studies suggest these patients have a favorable outcome with good response to IST. [17,25] Thus 6p CN-LOH did not appear to be a marker of transformation to MDS in our cohort.

All cases of 6p CN-LOH in our cohort involved the HLA locus. Further, two of our patients with 6p CN-LOH clones were found to also carry somatic loss-of-function mutations targeting the same HLA allele as the 6p CN-LOH clone (reported elsewhere).[5] These findings strongly suggest that in contrast to other forms of clonal hematopoiesis driven by mutations that convey a proliferative advantage, the HLA genes may be the genetic targets of the 6p CN-LOH rearrangement. Importantly, the loss of specific HLA alleles may provide a mechanism to restore hematopoiesis via immune escape, and implicates the HLA molecules derived from these specific alleles in presenting the antigens that foster the pathogenic T cell-mediated autoimmune response driving aAA.[6,7] Our study identified several candidate alleles that were deleted more than once among our patients with 6p CN-LOH (*HLA-B*14:02*, *HLA C*08:02*, *HLA-DRB1*07:01* and *HLA-DQB1*02:02*). Of these, *HLA-B*14:02* and *HLA C*08:02* were significantly over-represented in an ethnicity-matched (Caucasian) comparison of our aAA cohort compared with normal population controls. These alleles are different from the ones found to be recurrently lost through 6p CN-LOH in the previously mentioned Japanese cohort (*HLA-A*31:01*, *HLA-B*40:02*, *HLA-A*02:01*, *HLA-A*02:06*) and are likely unique to the ethnic group reported in our study.[7] While the specific identity of alleles targeted by 6p CN-LOH may vary according to ethnicity, the

findings of our study and others underscore the important roles that these targeted alleles may play in the initial development of pathogenic CTL-mediated autoimmunity in aAA, and the deletion of these alleles through multiple genetic mechanisms may provide a mechanism to restore hematopoiesis via immune escape.

Finally, our study to our knowledge is the first to report the use of NGS as a sensitive technique capable of identifying specific HLA alleles lost through 6p CN-LOH. NGS provides a high-resolution, high throughput method for quantitative HLA allele detection in contrast to the more time and resource intensive flow cytometry methods previously reported.[7]

In conclusion, we have found in our large, mostly pediatric, North American cohort of patients with aAA, that 6p CN-LOH is a common recurrent clonal abnormality in aAA, occurring at a frequency of ~11%, and that patients who develop 6p CN-LOH may represent a potentially unique subset of patients with poor response to IST. We have identified HLA alleles specifically lost through 6p CN-LOH that may represent candidate high-risk alleles implicated in the immune pathogenesis of aAA. Prospective longitudinal studies are needed to further explore the prognostic significance of 6p CNLOH, and to elucidate the interplay between immune pathogenesis and clonal evolution in aAA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ANC	Absolute neutrophil count
aAA	Acquired aplastic anemia
BM	Bone marrow
CR	Complete response
CN-LOH	Copy neutral loss of heterozygosity
CTLs	Cytotoxic T lymphocytes
HSC	Hematopoietic stem cells
HLA	Human leukocyte antigen

IST	Immune suppression therapy
MDS	Myelodysplastic syndrome
NGS	Next generation sequencing
PNH	Paroxysmal nocturnal hemoglobinuria
PR	Partial response
PB	Peripheral blood
SNP-A	Single nucleotide polymorphism array

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Figure 1. 6p CN-LOH in Patients with aAA Always Includes the HLA Locus

A. Diagram of SNP-A of the chromosome arm 6p region in the eight patients with 6p CNLOH. The top plot of each pair shows the B-allele frequency (BAF) on the Y-axis with genotypes for BB (BAF=1), AB (BAF=0.5) and AA (BAF=0). The lower plot shows the log *R* ratio (LRR), a measure of copy number for each SNP, on the Y-axis. The X-axis represents the location on chromosome arm 6p in both plots. A shift away from the heterozygous AB line (BAL plot) in the absence of an alteration in copy number (LRR plot) is indicative of CN-LOH, and the degree of this deviation corresponds to the percentage of cells with CN-LOH. Patient 2 SNP shows a representation of the presence of multiple 6p CN-LOH clones within one individual, each with different breakpoints, -75%, -60%, -25% clone size. **B.** Diagram of 6p CN-LOH within the HLA Locus. The different breakpoints of 6p CN-LOH found in our 8 patients are shown, mapped to a coordinate line for chromosome 6p (bottom) as well as to the HLA locus (top). Different colors are used to represent distinct percentages of cells containing the clone for the mapped regions found in each patient.

*Indicates a likely 6p22.1 heterozygous deletion, a germ-line alteration of unknown significance. WC: whole chromosome.

TABLE 1**Patient Characteristics**

	Children (n=57)	Adults (n=18)
Median age (range)	11 (0.5 – 20)	39.5 (21 – 67)
Gender male/female no.	29/28	7/11
Ethnicity no. (%) of patients		
African American	12 (21.1)	3 (16.6)
Caucasian	38 (66.6)	13 (72.2)
Hispanic	2 (3.5)	1 (5.6)
Other [^]	5 (8.8)	1 (5.6)
Diagnosis no. (%) of patients		
iAA	48 (84.2)	15 (83.2)
dAA	2 (3.5)	1 (5.6)
AA / PNH	3 (5.3)	1 (5.6)
PNH only	2 (3.5)	1 (5.6)
Hypocellular MDS	2 (3.5)	----
Therapy no. (%) of patients		
IST [*]	39 (68.4)	15 (83.2)
Eculizumab	4 (7.0)	1 (5.6)
URD-BMT	6 (10.5)	1 (5.6)
MSD-BMT	9 (15.8)	2 (11.1)
Observation	7 (12.3)	----
SNP-A no. (%)	54 (94.7)	17 (94.4)
Timing of initial SNP-A from diagnosis		
<6 months	31 (57.4)	5 (29.4)
6 months	23 (42.6)	12 (70.5)
HLA typing no. (%)	55 (96.4)	9 (50)

[^] Other: Mixed Ethnicity, Middle Eastern, Asian; iAA: idiopathic AA; dAA: drug-induced AA; PNH: paroxysmal nocturnal hemoglobinuria; MDS: myelodysplastic syndrome;

^{*} IST: immunosuppressive therapy including ATG+CsA (n=51) or CsA alone (n=3); CsA: cyclosporine A; BMT: bone marrow transplant; URD: unrelated donor; MSD: matched sibling donor; SNP-A: single nucleotide polymorphism array.

TABLE 2

Prevalence of 6p CN-LOH and Treatment Outcomes After IST

6p CN-LOH				
		Yes	No	
Subjects with SNP-A	n	# (%)	# (%)	p value
Total	71	8 (11)	63 (89)	
Children	54	5 (9)	49 (91)	0.387 [*]
Adult	17	3 (18)	14 (82)	
Timing of Initial SNP-A from time of diagnosis				
< 6 months	36	2 (6)	34 (94)	0.15 [^]
6 months	35	6 (17)	29 (83)	
Response to IST				
Total Treated with IST	51	6	45	
CR off CsA	16	0	16 (36)	0.073 [#]
CR on CsA	3	0	3 (7)	
PR	19	3 (50)	16 (36)	
Failed/Relapse	13	3 (50)	10 (22)	

SNP-A: single nucleotide polymorphism array; CR: complete response; PR: partial response; IST: immunosuppressive therapy (ATG+CsA, CsA alone); CsA: cyclosporine; Failed/Relapse: patients requiring additional therapy and/or demonstrating progression to MDS.

^{*} Comparison of 6p CN-LOH frequency in adults versus children.

[^] Comparison of 6p CN-LOH frequency based on time from diagnosis when initial SNP-A was performed.

[#] Comparison of CR frequency in patients with and without 6p CN-LOH.

6pLOH Clone Description, Clone Evolution and Patient Outcomes

TABLE 3

ID	Ethnicity Age (years)	First SNP-A with 6pLOH			Follow up SNP-A					Medical Therapy	Outcome
		Sample Type	6p CN-LOH region	Genomic Coordinates 6p CN-LOH ⁺ (hg19)	Clone size (%)	Number of follow up SNP-A	Sample Type (Time from 1st SNP-A)	Clone size (%)	Other New Clones		
1	Caucasian 14	BM	6pterp21.31	165,391-35,477,032	80-85	2	BM (4 & 7 months)	~85-90	None	Failed IST (relapse)	Died of Sepsis Post-BMT (2011)
			6p21.3p21.1	35,477,303-43,063,516	65			~65-75			
			6p21.1p12.1	43,065,913-56,973,902	50-55			~55-65			
			6p12.1p11.2	56,984,698-57,299,092	~15-20			~10-20			
2	Caucasian 16	BM	6pterp21.32	165,632-33,231,060	70-75	0	----	----	N/A	Failed IST, HD Cy, & Eltrombopag	MUD-BMT (2014) 100% donor
			6p21.32p21.31	33,232,055-33,885,802	55-60						
			6p21.31p21.2	33,886,682-40,416,800	20-25						
			6p21.2p12.1	40,417,196-56,398,637	~10						
3	Indian 5	BM	6pterp22.1	89,571-29,023,214	45-50	1	PB (11 months)	~30	None	Supportive Care	CR (Plt >100K)
			6p22.1	29,144,127-29,859,135	45-50			~30			
			6p22.1p21.33	29,899,389-30,716,719	45-50			~30			
			6p21.33p12.1	30,716,991-56,625,123	~20			~10 [^]			
4	Caucasian 46	BM	6pterp21.32	89,571-33,025,700	45-50	3	BM (3, 7, & 19 months)	50-55	None	IST	PR: low Hgb, low Plt, CNI-dep
			6p21.32p11.1	33,025,953-58,779,628	~20			~15-20			
5	Caucasian 18	PB	6pterp21.31	89,571-34,236,429	~15-20	1	BM (7 months)	~15	None	IST	PR: low Plt, low ANC
			6p21.31p21.1	34,238,514-43,109,751	~10			~5-10			
6	AA 35	BM	6pterp21.2	89,571-37,529,834	~20	1	BM (17 months)	25-30	None	Failed IST	Receiving Eltrombopag
7	Caucasian 61	BM	6 Whole Chromosome	Whole Chromosome	15-20	1	BM (3 months)	~5-10	13q del	IST	PR: low Hgb, PNH clone, CNI-dep
8	Caucasian 8	BM	6pterp21.31	165,632-35,675,696	~10	0	----	----	N/A	None (MSD-BMT)	MSD-BMT (2014) 100% donor
			6p21.31p12.1	35,677,971-53,989,526	~5						

All patients had a diagnosis of aAA.

AA: African American; BM: bone marrow; PB: peripheral blood; Plt: platelets; Hgb: hemoglobin; ANC: absolute neutrophil count; IST: immunosuppressive therapy; HD-CY: high dose cyclophosphamide; CNI: calcineurin inhibitor; MUD: matched unrelated donor; BMT: bone marrow transplant; MSD: matched sibling donor

⁺ Genomic coordinates of 6p CN-LOH were mapped to the human genome version 19 (hg19) build.

[^] Patient #3 demonstrated a decrease in length of the smallest clone (6p21.33p21.2 30,716,991 - 39,564,811) on follow-up SNP analysis.

TABLE 4

Identification of Specific HLA Alleles Lost Through 6p CN-LOH

P	Allele	HLA-A	HLA-B	HLA-C	HLA-DRB1	HLA-DQB1	
1	#1	A*03:01	B*07:02	81.80%	DRB1*03:01	DQB1*02:01	78.10%
	#2	Homozygous	B*14:02	18.20%	DRB1*04:04	DQB1*03:02	21.90%
2	#1	A*01:01	B*18:01	86.60%	DRB1*04:01	DQB1*03:02	75.90%
	#2	A*11:01	B*35:01	13.40%	DRB1*14:54	DQB1*05:03	24.10%
3	#1	A*24:02 [^]	B*52:01	53.50%	DRB1*13:01	DQB1*06:03	66.90%
	#2	A*33:03	B*44:03	46.50%	DRB1*07:01	DQB1*02:02	33.10%
4	#1	A*26:01	B*18:01	68.90%	DRB1*13:02	DQB1*06:04	66.20%
	#2	A*02:01	B*35:03	31.10%	DRB1*04:08	DQB1*03:04	33.80%
5	#1	A*24:02	B*44:03	57.50%	DRB1*07:01	DQB1*02:02	54.00%
	#2	A*33:01	B*14:02	42.50%	DRB1*11:01	DQB1*03:01	46.00%
6	#1	A*02:05	B*51:01	61.90%	DRB1*13:01	DQB1*06:09	69.50%
	#2	A*29:02	B*13:02	38.10%	DRB1*07:01	DQB1*02:02	30.50%
7	#1	A*68:01	B*51:01	51.50%	DRB1*15:01	DQB1*03:01	52.50%
	#2	A*32:01	B*14:02	48.50%	DRB1*11:04	DQB1*06:02	47.50%
8	#1	A*29:02	B*14:02	51.60%	DRB1*13:02	DQB1*06:02	50.30%
	#2	A*68:01	B*07:02	48.40%	DRB1*15:01	DQB1*06:09	49.70%

Numbers in red represent minority HLA alleles deleted by 6p CN-LOH in each patient as determined by relative allele frequency (%) obtained from Next Generation Sequencing of blood cell samples.

Alleles in blue were deleted more than once among patients with 6p CN-LOH. In Patients #7 and #8, minority HLA alleles could not be determined, likely due to the small size of the 6p CN-LOH clones.

[^] Majority allele for Patient #3 was a unique A*24 allele not previously defined, but most closely homologous to A*24:02.

HLA Allele Frequency Comparison in aAA Cohort Compared to Published Frequency data, European Ethnicity Only

TABLE 5

HLA - A	Allele Frequency			HLA - B	Allele Frequency			HLA - C		Allele Frequency			HLA - DRB1		Allele Frequency			HLA - DQB1		Allele Frequency		
	Cohort n=94	Control n=15738	p		Allele	Cohort n=94	Control n=15736	p	Allele	Cohort n=94	Control n=15740	p	Allele	Cohort n=94	Control n=15740	p	Allele	Cohort n=94	Control n=15740	p		
2:01	24.5	29.6	n.s.	8:01	12.8	12.5	n.s.	7:01	16	16.7	n.s.	3:01	14.9	12.9	n.s.	02:01/02	20.2	23	n.s.			
1:01	21.2	17.2	n.s.	14:02	9.6	3.1	0.001	8:02	10.6	3.9	0.002	1:01	10.6	9.1	n.s.	3:01	17	18.5	n.s.			
3:01	16	14.3	n.s.	7:02	8.5	14	n.s.	5:01	8.5	9.1	n.s.	15:01	10.6	14.4	n.s.	5:01	13.8	12.3	n.s.			
24:02	4.3	8.7	n.s.	18:01	7.5	4.6	n.s.	6:02	8.5	9.3	n.s.	4:01	8.5	9.1	n.s.	6:02	9.6	14.3	n.s.			
32:01	4.3	3.1	n.s.	44:02	7.5	9	n.s.	7:02	8.5	15	n.s.	13:02	8.5	4	n.s.	3:02	8.5	9.5	n.s.			
33:01	4.3	1	0.008	13:02	4.3	2.6	n.s.	4:01	6.4	10.5	n.s.	7:01	7.4	13.8	n.s.	6:09	5.3	0.8	<0.001			
68:01	3.2	2.5	n.s.	15:01	4.3	6.7	n.s.	12:03	5.3	4.9	n.s.	11:04	6.4	3.2	n.s.	3:03	4.3	4.5	n.s.			
11:01	2.1	5.6	n.s.	51:01	4.3	4.5	n.s.	3:03	4.3	5.5	n.s.	11:01	4.3	5.7	n.s.	6:03	4.3	6.5	n.s.			
25:01	2.1	1.9	n.s.	40:02	3.2	1	n.s.	1:02	3.2	2.9	n.s.	4:04	3.2	3.6	n.s.	4:02	3.2	2.5	n.s.			
26:01	2.1	2.9	n.s.	58:01	3.2	0.47	n.s.	2:02	3.2	3.7	n.s.	8:01	3.2	2.4	n.s.	6:04	3.2	3.2	n.s.			

Population frequency data from <https://bioinformatics.bethematchclinical.org/HLA-Resources>, as reported in Maiers M (2007).
Gray-shaded = minority alleles in 6p CN-LOH patients. Statistically significant differences are shown in bold.