Published in final edited form as:

Exp Hematol. 2019 December ; 80: 36–41.e3. doi:10.1016/j.exphem.2019.11.008.

Inflammatory Cytokines Promote Clonal Hematopoiesis with Specific Mutations in Ulcerative Colitis Patients

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Keywords

Hematopoietic stem cell; clonal hematopoiesis; inflammation

Introduction

Clonal hematopoiesis of indeterminate potential (CHIP) is a pre-malignant condition associated with expansion of blood cell populations driven by age-dependent acquisition of somatic mutations in hematopoietic stem cells (HSCs) [1–7]. Although CHIP mutations are typically present at low variant allele fraction (VAF), they are cancer-drivers and frequently involve genes mutated in myeloid malignancies [1–3, 8]. Despite the overall prevalence of CHIP, few of these individuals progress to hematopoietic malignancy [1, 2, 5, 7, 8]. In addition to somatic mutations, environmental factors likely contribute to clonal evolution of CHIP. Accumulating evidence suggests inflammation might serve as a selective pressure that contributes to malignant progression of CHIP clones. Individuals with autoimmune diseases [9] or a history of chronic infection [10] have increased lifetime risk of myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML). TET2 is a negative regulator of the

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DISCLOSURE OF CONFLICTS OF INTEREST

The authors declare no competing interests.

inflammatory response in myeloid cells [11], and *TET2*-mutant CHIP is associated with overproduction of IL6 which promotes mutant HSC expansion [12] and accelerates atherosclerosis [13]. As chronic inflammation compromises HSC function, inflammation could shape hematopoiesis by selecting for HSCs with mutations that increase stress tolerance. However, direct evidence demonstrating this is lacking. A recent study suggested patients with rheumatoid arthritis, a chronic inflammatory autoimmune disease, had no increased incidence of CHIP [14]. But as this patient cohort was relatively small, we sequenced 187 patients with ulcerative colitis (UC), an inflammatory bowel disease characterized by infiltration of T-cells in the colon and overproduction of pro-inflammatory cytokines such as TNF α and IFN γ , to evaluate the role of inflammation in CHIP.

Materials and Methods

Sequencing and Analysis

Peripheral blood mononuclear cell DNA from ulcerative colitis patients (>50-years old) without prior history of hematologic disease was obtained from the Digestive Diseases Research Core Center (DDRCC) under Washington University IRB protocol 20111078. Samples were sequenced using a panel targeting 40 genes and hotspots recurrently mutated in CHIP, MDS, and AML (Washington University MyeloSeq; Supplementary Table 1). HaloplexHS (Agilent) amplicon sequencing provides coverage of each target with multiple, distinct amplicons that incorporate unique molecular indexes (UMIs) into sequencing reads for error-correction. Libraries were generated using 500ng DNA, then sequenced on the NovaSeq (Illumina) platform to obtain 16 million reads per sample (10,000X raw sequence coverage). Sequence data were aligned to build 37 human reference with BWA-MEM, creating 'read families' from multiple reads with the same UMI sequence, which were used as input for variant identification using VarScan2, platypus, and pindel. Variant filtering was performed on the union of identified variants from these callers using only read families with at least 3 supporting reads, a VAF >0.5%, at least 2 supporting HaloplexHS amplicons, >8 error-corrected reads that support the alternative allele, and at least 3 supporting read families on each strand. Variants with a population allele frequency >0.1% in the ExAC database or that possessed a VAF >35% were excluded as likely germline variants. Comparisons of the frequency of mutated genes in ulcerative colitis patients was compared to the aggregate incidence across indicated control cohorts.

Serum Cytokine Analysis

Serum TNF α and IFN γ levels were examined using a cytokine-specific ProQuantum Immunoassay kit (Invitrogen, USA). Patients groups were matched for age and gender.

Statistics

Comparisons of CHIP prevalence in this study to published datasets were performed using a chi-squared test for each age range and gene independently followed by Bonferroni correction for multiple hypothesis testing. One-way ANOVA with Bonferroni multiple test correction was used for analysis of serum cytokine levels. Generalized linear models for multivariate analysis were established using R (version 3.5.3).

Results and Discussion

We sequenced 40 CHIP-associated genes in 187 UC patients (Table 1) to identify genetic variants in their blood compartment. As germline DNA was unavailable, variants with VAF >35% that were identified in the ExAC database were excluded as likely germline variants. To compare these data to the incidence of CHIP in the general population, we used the following definition of CHIP (CHIP-classical or “CHIP-c”) as outlined by a prior study with similar limitations [15]. CHIP-c was defined here by;

- VAF >2%
- Variant reported in COSMIC in “hematopoietic and lymphoid” category OR;
 - Damaging variants (frameshift and stop_gain mutations) in *DNMT3A* gene within exons 7–23.
 - Any damaging variants in the genes *ASXL1*, *TET2*, *PPM1D*, *TP53*.
 - CALR exon 9 indels.

Due to the sequencing depth and error-correction, the limit of detection of 0.5% VAF facilitated a more complete view of clonal hematopoiesis in these subjects. Such variants were classified as CHIP-high sensitivity (CHIP-hs) mutations by satisfying the following criteria;

- VAF >0.5%
- Variant reported in COSMIC in “hematopoietic and lymphoid” category >2 times.

CHIP-c was identified in 12.8% of patients in this cohort, with 22.5% of patients being positive for CHIP-hs. 26 CHIP-c variants were identified in 24 patients (Fig. 1A), with an additional 31 CHIP-hs variants harbored in 18 patients (Fig. 1B; Supplementary Table 2). C>T transitions were the most prevalent single-nucleotide variants, consistent with an age-dependent mutational signature (Fig. 1A,B). The most recurrently mutated gene in UC patients with CHIP-c (Fig. 1C) and CHIP-hs (Fig. 1D) was *DNMT3A*, followed by *PPM1D*. As a comparable control cohort was not available for this study, to gain a sense of the prevalence of CHIP in UC patients compared to the general population, we compared our data to epidemiological sequencing cohorts using the quantitative definition of CHIP outlined above across all datasets. The frequency of CHIP-c in UC patients trended higher after the sixth decade, although sample size restrictions in older individuals in the UC cohort limits this comparison (Fig. 1E). There was an age-dependent increase in CHIP-hs in UC patients without gender bias (Fig. 1F). The frequency of *DNMT3A*⁺ and *PPM1D*⁺ CHIP was higher in UC patients (Fig. 1G) compared to the aggregate incidence across the other studies [1–7]. *TET2* is typically the second most mutated gene (after *DNMT3A*) in CHIP sequencing studies of healthy individuals [1, 2, 7, 8], but was less frequent in UC patients (Fig. 1C,D). This was intriguing because certain inflammatory cytokines (e.g. IL6) enhance the competitive fitness of *Tet2*-mutant HSCs [12]. It is possible the inflammatory milieu of UC is not conducive for the clonal expansion of *TET2*-mutant clones. The relative incidence of *TET2*⁺ CHIP was lower in UC because certain mutations were positively selected,

notably *PPM1D* (Fig. 1G). *PPM1D*-mutant clones undergo strong positive selections in response to stress such as chemotherapy. The stress of chronic inflammation in UC patients may provide another environment that promotes growth of *PPM1D*-mutant clones.

The incidence of CHIP-hs in UC patients was not related to colectomy, a disease flare at time of blood collection (diarrhea, blood in stool, abdominal pain), current therapy or treatment history (Fig. 2A). CHIP-hs⁺ patients in this cohort were associated with increased age (Fig. 2B), but not time since UC diagnosis (Fig. 2C). No difference in blood counts were noted between CHIP-hs⁺ and CHIP⁻ individuals (Fig. 2D). The VAF of most variants was <5%, however there were several cases where clones dominated the blood, particularly for *ASXL1* mutations and *DNMT3A*^{R882H} (Fig. 2E). The distribution of CHIP-hs⁺ mutations in UC patients was not clustered with age or gender for any given gene (Fig. 2F). The mutational spectrum of *DNMT3A* in UC patients reflected the typical pattern for this gene in CHIP, with mutations clustered in functional domains without enrichment for the R882 hotspot (Fig. 2G). *PPM1D* mutations were concentrated in the C-terminus (Fig. 2G), mirroring those in therapy-related myeloid neoplasms [16, 17]. We sought to determine if inflammatory signals selected for specific clones in UC patients. UC patients were stratified into three groups matched for age and gender; CHIP⁻ (*n*=21), CHIP-hs⁺ without *DNMT3A* mutations (CHIP⁺*DNMT3A*⁻; *n*=17), and CHIP-hs⁺ with *DNMT3A* mutations (CHIP⁺*DNMT3A*⁺; *n*=19). We focused on TNFα and IFNγ since these cytokines have described roles in the symptoms of UC, and treatment can involve targeting these molecules. Serum TNFα levels were not different amongst the three patient groups (Fig. 2H). However, CHIP⁺*DNMT3A*⁺ patients was associated with significantly higher levels of serum IFNγ (Fig. 2H). Average clone size did not contribute to the observed variances (Fig. 2H), although *DNMT3A* VAF was a significant contributor to increased IFNγ levels in multivariate analysis using a generalized linear model (*p*=0.026). These data implicate increased IFNγ may select for clones with *DNMT3A* mutations. Focused mechanistic studies will be required to show this relationship is causative, and not correlative. Cumulatively, these data show that UC patients may harbor slightly higher levels of CHIP than the general population, and that the inflammatory environment of UC potentially selects for the growth of HSC clones with specific mutations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

The Washington University Digestive Diseases Research Cores Center (DDRCC) BioBank Core is supported by NIH P30 DK052574. This work was supported by the Washington University McDonnell Genome Institute and the National Institutes of Health (R01 DK102428 to G.A.C.). E.L.O. was supported by NIH F31DK114951. Philanthropic support comes from the Lawrence C. Pakula IBD Research Innovations Fund (M.A.C.). G.A.C. is a scholar of the Leukemia and Lymphoma Society.

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Figure 1: Clonal hematopoiesis in UC patients.

(A) Number of CHIP-c variants identified in UC patients (left), and categories of those SNVs (right). (B) Number of CHIP-hs variants identified in UC patients (left), and categories of those SNVs (right). (C) Number of CHIP-c mutations identified per gene. (D) Number of CHIP-hs mutations identified per gene. (E) CHIP incidence by age in referenced studies. Error bars represent the 95% binomial confidence interval of the point estimate in UC patients. The confidence interval bands for referenced studies represent the 95% confidence interval around the point estimate. (F) CHIP-hs incidence by gender in UC patients over age brackets. Grey bars denote the CHIP-hs incidence in given age bracket. (G) Incidence of CHIP-c mutations by gene across indicated studies. Significance is indicated for incidence of CHIP-c mutations in given gene in UC patients compared to aggregate incidence across other reference studies.

Figure 2: CHIP Dynamics in UC Patients.

(A) Correlation of clonal hematopoiesis in UC patients with clinical variables and treatment history. (B) Tukey plot showing ages of CH- and CH+ UC patients. * $p < 0.05$, two-tailed t-test. (C) Tukey plot showing time since diagnosis of CH- and CH+ UC patients. (D) Tukey plots showing blood count parameters of CH- and CH+ UC patients. (E) VAF of CH variants in UC patients. Each dot represents a single mutation, line represents mean VAF. (F) Mutational profile of CH variants in UC patients. (G) Mutational spectrum of somatic variants identified in *DNMT3A* and *PPM1D* in UC patients. (H) TNF α and IFN γ levels in serum of UC samples, and clone size (by VAF) in patients surveyed in this experiment. Each dot indicates the levels of a given cytokine in the serum of one patient. Graphs represent mean $\pm$ S.E.M. * $p < 0.05$ one-way ANOVA with Bonferroni multiple test correction.

Table 1:

Demographic information of UC patient cohort.

Parameter	CH−	CH+	n(%) of patients without information available
Male (% of patients)	72.7%	27.3%	0 (0)
Female (% of patients)	83.0%	17.0%	0 (0)
Age (years)	63.1 ± 0.68 (50 – 84)	66.6 ± 1.18 (55 – 81)	0 (0)
Time from diagnosis to blood draw (years)	11.9 ± 1.1 (0.087 – 48.4)	12.8 ± 1.67 (0.70 – 39.1)	47 (25.1)
Colectomy (% of patients)	55.9%	52.0%	69 (36.9)
Flare at time of blood collection (% of patients)	64.7%	61.3%	59 (31.6)
Blood counts			
WBC (1000/cc)	9.6 ± 0.47 (3.7 – 27.3)	9.1 ± 1.1 (3.7 – 34.5)	60 (32.1)
Neutrophils (1000/cc)	7.7 ± 0.49 (1.4 – 25.3)	7.3 ± 1.1 (2.2 – 32.1)	66 (35.3)
Lymphocytes (1000/cc)	1.2 ± 0.070 (0.10 – 3.4)	1.3 ± 0.12 (0.30 – 3.0)	66 (35.3)
Hgb (g/dL)	11.6 ± 0.25 (6.6 – 16.0)	11.6 ± 0.38 (8.2 – 15.8)	61 (32.6)
HCT (%)	34.5 ± 0.69 (19.0 – 47.1)	36.8 ± 2.2 (23.0 – 44.6)	61 (32.6)
MCV (fL)	90.6 ± 0.78 (70.8 – 107.7)	89.8 ± 2.7 (33.4 – 113.4)	62 (33.2)
Platelets (1000/cc)	301.1 ± 12.9 (97.0 – 706.0)	308.1 ± 24.2 (98.0 – 580.0)	61 (32.6)
Treatments			
Current Anti-TNF (% of patients)	27.9%	23.1%	75 (40.1)
Previous Anti-TNF (% of patients)	24.4%	31.2%	77 (41.2)
Current Immunomodulator (% of patients)	27.9%	23.3%	76 (40.6)
Previous Immunomodulator (% of patients)	29.4%	23.7%	77 (41.2)
Current Steroids (% of patients)	30.3%	19.6%	75 (40.1)
Previous Steroids (% of patients)	29.0%	23.8%	76 (40.6)