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Impact of constitutional TET2 haploinsufficiency on molecular and clinical phenotype in humans

Eevi Kaasinen et al.#

Clonal hematopoiesis driven by somatic heterozygous TET2 loss is linked to malignant degeneration via consequent aberrant DNA methylation, and possibly to cardiovascular disease via increased cytokine and chemokine expression as reported in mice. Here, we discover a germline TET2 mutation in a lymphoma family. We observe neither unusual predisposition to atherosclerosis nor abnormal pro-inflammatory cytokine or chemokine expression. The latter finding is confirmed in cells from three additional unrelated TET2 germline mutation carriers. The TET2 defect elevates blood DNA methylation levels, especially at active enhancers and cell-type specific regulatory regions with binding sequences of master transcription factors involved in hematopoiesis. The regions display reduced methylation relative to all open chromatin regions in four DNMT3A germline mutation carriers, potentially due to TET2-mediated oxidation. Our findings provide insight into the interplay between epigenetic modulators and transcription factor activity in hematological neoplasia, but do not confirm the putative role of TET2 in atherosclerosis.
Clonal hematopoiesis (CH) is common in aged individuals and bears implications to health through risk of malignant degeneration of cells and possible risk of cardiovascular disease (CVD)\(^2\). Heterozygous tet methylcytosine dioxygenase 2 (TET2) and DNA methyltransferase 3A (DNMT3A) mutations are the two most frequent drivers of CH\(^3\), and mechanistic studies connecting CH and CVD revolve around Tet2-deficient models. Heterozygous and homozygous Tet2 loss in mice accelerates atherosclerosis, possibly via enhanced macrophage-driven inflammation\(^4,5\). Acceleration of heart failure has also been suggested\(^6\). In particular, two macrophage-mediated mechanisms have been proposed: exacerbated expression and inflammasome-mediated secretion of interleukin (IL)-1\(\beta\), as well as aberrant chemokine expression signature\(^7,8\). These findings have promoted hopes for population level prevention of CVD through detection of individuals with TET2-mutation-positive clones and subsequent use of cholesterol-lowering medications or compounds targeting IL-1\(\beta\) and other inflammatory pathways\(^9,10\). Thus, understanding whether heterozygous TET2 loss is associated with CVD in humans, and if yes through what mechanism, is of utmost importance.

DNA methylation is a key regulator of cell development and differentiation, and its aberrations are an essential factor in hematological neoplasia\(^7\). DNA methylation is mediated by DNA methyltransferase enzymes that transfer a methyl group to carbon atom 5 of cytosine nucleotide at CpG dinucleotides or CxG context at gene bodies, x standing for bases T, A, or C\(^8\). In DNA demethylation, TET protein family of dioxygenases catalyze the oxidation of 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC), 5-formylcytosine, and 5-carboxylicytosine, acting as an initiator of DNA demethylation cascade subsequently resulting in an unmodified cytosine\(^8\).

In addition to CH, somatic frameshift, nonsense, and missense TET2 mutations are commonly seen for example in myelodysplastic syndrome (6–26% prevalence), acute myeloid leukemia (AML; 12–27% in adult de novo AML), chronic myelomonocytic leukemia (20–58%), and angioimmunoblastic T-cell lymphoma (33–83%)\(^8\). Although a key event, TET2 loss alone is not sufficient to trigger malignancy\(^7\). Careful examination of individuals with a germline mutation could provide valuable insight into the effects of TET2 loss in humans. In this study, we observed the effects of constitutional heterozygous TET2 loss in a unique pedigree of seven carriers segregating a truncating TET2 germline mutation, as well as one case of de novo TET2 germline mutation. For these individuals, extensive clinical documentation was available. Methylation analysis of four individuals with a DNMT3A germline mutation as well as analysis of inflammatory response in two additional TET2 germline mutation carriers reported earlier by Schaub et al.\(^9\) provided further context to the results.

**Results**

**Study subjects.** The Finnish family segregating a TET2 germline mutation is presented in Fig. 1a. Ly1 was diagnosed with nodular lymphocyte predominant Hodgkin lymphoma (NLPHL) at age 46 (Supplementary Table 1, Supplementary Fig. 1), and Ly2 at age 45. At age 52, Ly2 experienced a relapse diagnosed as T-cell-rich B-cell lymphoma. Ly3 was diagnosed with NLPHL at age 39. Relapse at age 41 was diagnosed as mixed-cellular Hodgkin lymphoma. Clinical bone marrow examination was done twice (with 7 years’ time interval) for Ly1 and Ly2 after lymphoma diagnosis. As the only finding of note, Ly2 had slightly hyperplastic bone marrow in the second examination. Whole-genome (Ly1) and exome (Ly2 and Ly3) sequencing analysis revealed a heterozygous one-base deletion NM_001127208.2:c.4500delA in TET2 (Fig. 1b, Supplementary Table 2). The mother of Ly1, Ly2, and Ly3 was found to be a carrier of the deletion based on analysis of archival tissue DNA, and three further carriers (Ly9, Ly11, and Ly14) were found in the next generations. The deletion is similar to those often seen in somatic form in hematological neoplasia and causes a frameshift at lysine 1500 residue, resulting in a premature stop codon 70 residues later (NP_001120680.1:p. Lys1500AsnfsTer71) (Fig. 1c). An AML patient has been found with the same mutation previously\(^10\). Multiple amino-acid residues critical for the structural integrity of TET2\(^11\) are lost due to the deletion (Fig. 1d–f), and although both alleles are expressed at the mRNA level (Supplementary Fig. 2), the truncation leads to heterozygous loss of TET2 at the protein level (Fig. 1g, Supplementary Fig. 3). TET2 c.4500delA (hereafter TET2delA) was absent in 5197 in-house controls, the Exome Aggregation Consortium database (version 0.2; http://exac.broadinstitute.org/), and in 80,000 individuals in the Genome Aggregation Database (version 2.0; http://gnomad.broadinstitute.org/).

Through Northern Finland Intellectual Disability (NFID) Cohort, we could identify a carrier of de novo nonsense mutation in TET2 (NM_001127208.2:c.1471C>T, NP_001120680.1:p. Gln491Ter; hereafter individual Id1 with TET2X mutation). His developmental milestones had been normal except for linguistic skills, and he received speech therapy until age 7. Comprehensive etiological examinations including copy number analysis did not reveal the cause of developmental delay\(^12\). He was diagnosed with mild mental retardation at age 7 and had features of attention deficit hyperactivity disorder. According to the latest examination by Wechsler Intelligence Scale For Children–IV at age 16, he had reached normal cognitive skills apart from verbal comprehension. His older sister had experienced a delay in speech development but managed in standard school education. The parents were confirmed as TET2X-negative by Sanger sequencing, and the patient’s germline status was confirmed from saliva-derived DNA.

To provide context to molecular findings in the TET2 mutation carriers, DNA from non-carrier relatives, unrelated controls, as well as from four DNMT3A germline mutation carriers from NFID Cohort displaying features of Tatton-Brown-Rahman syndrome\(^13\) were available for methylation analyses (Supplementary Table 3). In addition, we had the opportunity to study immune responses in blood mononcytes isolated from two carriers of a heterozygous 4 base pair (bp) deletion in TET2 (hereafter TET2delA) reported earlier by Schaub et al.\(^9\) (Fig. 1c).

**Blood DNA methylation.** Since deficient DNA demethylation is the key consequence of TET2 mutations in hematological neoplasia\(^14,15\), we compared blood DNA methylation by targeted and whole-genome bisulfite sequencing (WGBS) in TET2 mutation carriers and non-carriers (Supplementary Fig. 4, Supplementary Tables 3, and 4). For comparisons between TET2delA carriers and mutation-free controls, targeted data from 5 carriers and 10 controls were utilized, as the higher number of samples reduces the likelihood that changes are caused by disproportions in cell types between the two groups. WGBS data, created from the three lymphoma-free TET2delA carriers and three age-matched controls, were used for genomic enrichment analyses due to the higher number of data points and because the chromatin annotation of affected regions after TET2 loss is likely identical in different cell types. TET2delA carriers displayed a significantly higher degree of overall hypermethylation (two-sided Wilcoxon rank sum test, \(p\)-value 0.003) and decreased hypomethylation (two-sided Wilcoxon rank sum test, \(p\)-value 0.01), compatible with demethylation deficiency (Fig. 2a, Supplementary Table 5).
Altogether 644/660 (97.6%) of differentially methylated regions (DMRs) between TET2delA carriers and controls were hypermethylated (Supplementary Data 1). To confirm that similar changes are not found in carriers of common TET2 single-nucleotide polymorphisms (SNPs), we compared carriers of heterozygous TET2 p.Leu1721Trp and p.Pro363Leu (\(n = 4\); rs34402524 and rs17253672 found in the same haplotype) to non-carriers (\(n = 11\)), derived from two Finnish families. Blood DNA methylation of all 15 individuals was measured by targeted bisulfite sequencing similar to TET2delA carriers and...
controls. No difference in the number of hypermethylated (two-sided Wilcoxon rank sum test, $p$-value 0.5714) nor hypomethylated (two-sided Wilcoxon rank sum test, $p$-value 0.1773) CpG sites was observed between the groups. Lymphoma patients (Ly1 and Ly2) and cancer-free TET2delA carriers (Ly9, Ly11, and Ly14) were also compared and no skewing toward hypermethylation was observed, although the results should be interpreted with caution due to low number of individuals in this comparison (Supplementary Data 2). The demethylation deficiency caused by heterozygous TET2 loss was further confirmed by WGBS. Hypermethylation was more prominent than hypomethylation when WGBS data of three cancer-free

TET2delA carriers and TET2X carriers were compared to age-matched non-carriers (Fig. 2b–f). While repetitive regions of the genome cannot be evaluated with short reads, the detected hypermethylated CpG sites located mostly at CpG islands of the genome (Supplementary Fig. 5a).

Next, we examined whether the hypermethylation in TET2X and TET2delA carriers was enriched in regions with regulatory function. We analyzed the overlap of the differentially methylated CpG (DMC) sites from the WGBS data with respect to regions displaying known chromatin marks in various primary human blood cell types derived from the Roadmap Epigenomics Project. Hypermethylation showed strongest enrichment at normally H3K4me1-marked chromatin across the different blood cell types (Fig. 3a). Individual comparisons of TET2X and TET2delA mutation carriers to their age-matched controls showed similar enrichment (Supplementary Fig. 6–9). The regions with enriched hypermethylation at H3K4me1 displayed H3K27ac but not H3K27me3 chromatin mark in the same blood cell types, implying that methylation was increased in normally active enhancer regions (Supplementary Fig. 5b). Hypermethylation, on the other hand, showed strongest enrichment at normally H3K9me3-marked, transcriptionally repressed chromatin in all primary human blood cells characterized in the Roadmap Epigenomics Project (Fig. 3b).

As the enhancer regions with increased methylation are likely to have lower activity, we studied chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-seq) of H3K27ac from immortalized lymphoblastoid cell lines. ChIP-seq enrichment analysis revealed reduced histone acetylation at CpG islands as a function of methylation increase in TET2delA carriers as compared to non-carriers (Pearson’s correlation; $r = -0.06$, 95% confidence interval $-0.08$ to $-0.03$, and $p$-value $2.192 \times 10^{-08}$) (Fig. 3c).

Overall DNA methylation level of the hypomethylated CpGs in partially methylated domains (PMDs) has been proposed to represent a biomarker for cellular aging.16 We extracted methylation values from the CpGs in the PMD regions and did not observe methylation difference between the three cancer-free TET2delA carriers and their age-matched non-carrier family members in the WGBS data (Supplementary Fig. 10).

We further compared enrichment of hyper- and hypomethylation at the chromatin marks in blood DNA samples collected at 10 years’ time intervals, available from two carriers (Ly9 and Ly11) and two non-carriers (Ly8 and Ly10). Age-related methylation changes showed a similar pattern of enrichment across the different chromatin marks between TET2delA and wild-type individuals (Supplementary Fig. 11).

We next examined whether methylation changes in TET2delA carriers had occurred in hematopoietic cell lineage-specific open chromatin regions that we derived from public single-cell ATAC-seq data.17 We could observe significantly increased methylation in the targeted bisulfite sequencing data of TET2delA carriers as compared to 10 controls in some of the lineage-specific regions, in particular those found in monocytes, granulocyte/macrophage progenitors, common lymphoid progenitors, B cells, and megakaryocyte/erythroid progenitors (two-sided Wilcoxon rank sum test, false discovery rate (FDR) < 0.05) (Fig. 4a). Because the emergence of hematopoietic lineages depends on activity of transcription factors (TFs) such as PU.1, RUNX1/2, CEBPα/β, GATA1/2, PAX5, and TRX2,18 we scrutinized the methylation statuses of the consensus binding sequences of these TFs within the lineage-specific open chromatin regions (Supplementary Fig. 12). We found that many cell-type-specific open chromatin regions at RUNX2 (binding specificity similar to RUNX1 and RUNX3), GATA1 (binding specificity similar to GATA2 and GATA3), and PU.1 binding sequences were significantly enriched for methylation in the TET2delA carriers (two-tailed permutation $p$-values corrected for multiple testing via FDR < 0.05) (Fig. 4b, Supplementary Table 6).

To scrutinize further the methylation changes related to gene defects commonly detected in CH and hematological malignancies,19 and to provide additional context to findings in TET2 mutation carriers, we derived targeted bisulfite sequencing data from blood DNA of four DNMT3A germline mutation carriers and their age-matched controls from the NFID Cohort (Supplementary Table 3). DNMT3A mutation carriers displayed increased overall hypomethylation (Fig. 4c) and decreased methylation at open chromatin regions of all blood cell types (Supplementary Fig. 13a). Comparison of the effects of TET2 and DNMT3A loss in mouse hematopoietic stem cells has been reported.19 By enrichment analysis of DMC sites from our WGBS data, we could observe that hypermethylation in TET2 mutation carriers was most prominent in regions reported to be sites of DNMT3A and TET2 competition (Supplementary Fig. 13b). Thus, we next examined whether the methylation changes caused by human DNMT3A germline mutation could be seen in the same regulatory regions as in TET2delA carriers. Indeed, the TF-binding sites at lineage-specific open chromatin regions affected in the TET2delA carriers were affected also in DNMT3A mutation carriers, albeit to the opposite direction, showing decreased methylation (Pearson’s correlation of relative ratios; $r = 0.31$, 95% confidence interval 0.03–0.54, and $p$-value 0.03) (Fig. 4d).

Blood immune cell phenotyping and single-cell analysis. Leukocyte counts of TET2X and TET2delA carriers were grossly normal (Supplementary Table 7), whereas further T- and B-lymphocyte subtyping analyses revealed marked changes (Supplementary Table 8). The two tested TET2delA carriers previously treated for lymphoma (Supplementary Table 1) displayed impaired B-cell maturation, compatible with chemotherapy. The young, approximately 20 years of age TET2delA and TET2X carriers (Ly14 and Id1) had increased percentages of recent thymic emigrant and naive subsets of CD3+CD4+ T cells. The most notable overall changes in the TET2 mutation carriers were the high percentages of activated (CD38lowCD21low) CD19+B cells and low percentages of CD3+CD4/8+CCR7−CD45RA− T effector memory cells. In addition, the elderly mutation carriers (Ly1, Ly2, Ly9, and Ly11, aged 36–64 years) exhibited skewing of CD3+CD8+ T cells toward increased proportions of the
CD3^+CD8^+CCR7^-CD45RA^+ terminally differentiated effector memory cells re-expressing CD45RA (TEMRA) phenotype, which in the absence of chronic viremia or lymphopenia suggests immune exhaustion.

Next, we performed single-cell RNA-sequencing analysis of peripheral blood of the five living TET2delA carriers as well as three non-carrier family members. The main cell types were identified through cluster-specific expression patterns and were present in similar fractions in both groups although individual variation was observed (Fig. 5a, Supplementary Fig. 14a). Compatible with the most prominent effect of TET2 loss on methylation in monocytes (Fig. 4a), TET2 expression was highest in this cell type and mutation carriers did not display compensation through increased TET1 or TET3 expression (Fig. 5b, Supplementary Fig. 14b). A slight decrease in DNMT3A expression was observed in CD4^+ T cells of TET2delA carriers (Supplementary Fig. 14b). We then scrutinized the differentially expressed genes between cancer-free TET2delA carriers and age-matched wild-type individuals (Supplementary Tables 9-12). The most prominent difference was increased CXCR4 expression, especially in natural killer (NK) and CD4^+/CD8^+ T cells (Fig. 5c). We also detected significantly elevated expression of
TSC22D3 in NK cells, CD8+ T cells, and monocytes of cancer-free TET2delA carriers (Fig. 5d). TSC22D3 is the gene with the highest positive correlation to CXCR4 expression at whole transcriptome level in public expression data from AML (n = 162, The Cancer Genome Atlas data20 in cBioPortal) (Supplementary Fig. 15). Furthermore, we detected reduced expression of JCHAIN (IgJ), IGLL5, and AL928768.3, a human monoclonal IgA1-IgA2 λ hybrid molecule from IgH locus, especially in B cells of TET2 mutation carriers (Supplementary Table 11). Compatible with expression changes of immunoglobulin genes, we detected significantly elevated methylation in a B-cell-specific open chromatin region at the promoter of the IGHJ6 gene in TET2delA carriers (Supplementary Fig. 16, Supplementary Data 1). Analysis of regulon activity by looking at TFs and their co-expressed cis target genes revealed 26 TFs with significantly increased and 12 TFs with significantly decreased activity in cancer-free TET2delA carriers as compared to wild-type individuals, including TBX21 and EOMES that displayed lower activity especially in
Methylation is significantly increased in TET2delA carriers at lineage-specific open chromatin regions of monocytes, granulocyte/macrophage progenitors (GMPs), common lymphoid progenitors (CLPs), B cells, and megakaryocyte/erythroid progenitors (MEPs). Percentages represent average methylation differences at the respective open chromatin regions between mutation carriers (Ly1, Ly2, Ly9, Ly11, and Ly14) and 10 controls (Ly8, Ly10, Ly12, Ly13, HLRCC_N7, and controls 1–5) from targeted bisulfite sequencing. False discovery rate (FDR)-adjusted p-values are from two-sided Wilcoxon rank sum test. b Schematic of the human hematopoietic cell lineage hierarchy showing the 10 cell types analyzed in a. Black arrows depict emergence of lineages that showed significant enrichment of methylation at indicated TFBSs at lineage-specific open chromatin regions in the TET2delA carriers. c Count of hypermethylated (dark red bars) and hypomethylated (light gray bars) cytosines per million tested CpGs in DNMT3A mutation carriers (+/−) and age-matched controls (+/+) from the Northern Finland Intellectual Disability Cohort. Each sample was compared to the same set of five baseline controls as in Fig. 2a. d Magnitudes of the methylation changes at master TFBSs located in cell-specific open chromatin regions correlate in TET2 and DNMT3A mutation carriers, albeit these methylation changes occur toward different directions. X- and Y-axis represent average methylation change in mutation carriers as compared to controls at open chromatin regions with master TFBS relative to all open chromatin regions in each of the 10 cell types. Each dot of a particular color represents one of the 10 cell types, respectively. Pearson’s product-moment correlation coefficient (r) and p-value (p) are calculated for the relative ratios. Methylation values from TET2delA carriers were compared to the 10 controls as in a, and those from DNMT3A mutation carriers to the four controls as in c. RUNX and GATA represent binding scores of RUNX1/2/3 and GATA1/2/3, respectively.
Fig. 5 Single-cell RNA-sequencing analysis from peripheral blood. a The main cell types were present in similar numbers between cancer-free TET2delA carriers (Ly9, Ly11, and Ly14; right) and wild-type individuals (Ly8, Ly10, and Ly13; left). Colors represent blood cells with similar expression profiles in K-means (K = 10) clustering. Each point represents a cell in the coordinates specified by the two t-SNE (t-distributed stochastic neighbor embedding) components. b TET2 expression in each of identified cell types represented with unique molecular identifier (UMI) counts. Boxplot shows the mean ± standard deviation. TET2delA carriers are marked with +/- and wild-type individuals with +/-+. c CXCR4 was the most significantly increased transcript in natural killer (NK) cells and CD8+ T cells [log2 fold change (FC) 2.06], CD4+ T cells (FC 1.12), and all cell types compiled (FC 0.92), in cancer-free mutation carriers (right) as compared to non-carriers (left). d TSC22D3 expression was significantly increased in NK cells and CD8+ T cells (FC 1.14) and monocytes (FC 1.1) of mutation carriers.
TET2 knockdown by siRNA in human blood monocyte-derived macrophages did not affect IL-6 expression (Supplementary Fig. 19f).

Clinical evaluation of atherosclerosis. Detailed clinical information was available from the eight Finnish TET2 mutation carriers. The mother of Ly1, Ly2, and Ly3, who was a carrier of TET2delA, died at age 74 years due to pneumonia, with meningioma as a contributing condition according to her death certificate. Her mutation-free spouse had died of a presumed myocardial infarction at the age of 45. Ly1, Ly2, and Ly3 all had received radiotherapy as well as chemotherapy on one to three different occasions (Supplementary Table 1). Ly1 has a history of celiac disease and hypercholesterolemia (∼8.5 mmol/l prior to medication) and coronary disease was diagnosed at age 62. Ly2...
has been diagnosed with rheumatoid arthritis, hypercholesterolemia (total ∼5 mmol/l, high-density lipoprotein ∼ 0.7 mmol/l, low-density lipoprotein ∼ 3.5 mmol/l prior to medication) and hypertension. He had smoked 10 pack years but at the age of 63 remains free of clinically manifest atherosclerotic disease. Ly3 died of septic 
Neisseria meningitidis meningitis free of CVD at the age of 50. Ly4 is a 41-year-old female ex-smoker, who carries TET2delA and has remained in good health. Ly11 is a 35-year-old female TET2delA carrier, diagnosed with mitochondrial encephalomyopathy, lactic acidosis, and stroke-like syndrome, inherited from her mother. She has remained free of atherosclerotic disease. Ly14 is a 19-year-old healthy female smoker carrying TET2delA mutation. Id1 is a 21-year-old male who carries de novo TET2X mutation, free of CVD.

We then re-evaluated the existing radiographic data of the Finnish TET2 mutation carriers. Computed tomography (CT) scans of chest and abdomen revealed calcified plaques in Ly1 and Ly2 at age 47 and 48, respectively, and thereafter, whereas Ly3 was lesion-free in five (abdominal, body, and three occasions chest) CTs between 46 and 48 years of age. Next, we performed carotid intimal ultrasound to all six living Finnish TET2 mutation carriers. Measured mean and maximum far wall carotid intima-media thickness values from the right and left common carotid artery were evaluated in light of reference values derived from 740 Northern Finland Birth Cohort 1966 study subjects free from vascular disease or cardiovascular risk factors (K.K., personal communication). Non-obstructive atherosclerotic plaques as well as increased intima thickness in carotid arteries were detected in two subjects (Ly1 and Ly2, 65 and 63 years of age, respectively). In the four younger subjects (Ly9, Ly11, Ly14, and Id1), carotid ultrasound was normal.

Discussion

In this study, we identified a family with heterozygous TET2 loss in seven individuals. Three of these individuals had been diagnosed with NLPHL. In addition, an individual with a de novo TET2 mutation was identified from NFID Cohort data. In our genome-wide bisulfite sequencing analyses, significant global hypermethylation was seen in blood of TET2 mutation carriers as compared to TET2 wild-type cases. Hypermethylation was elevated especially at active enhancer regions. Our findings establish TET2 as a gene predisposing to familial DNA demethylation deficiency and hematological aberrations when constitutionally mutated. We propose that the perturbation in hematopoiesis caused by reduced TET2 function appears to relate to aberrations in lineages that require synergistic actions of TET2 and master TFs involved in hematopoiesis.

Unlike many other lineage-specifying TFs, e.g CEBPα/β and PAX5, RUNX1/2/3 and PU.1 belong to TFs that have binding preference to unmethylated DNA28. Interestingly, TET2 has been shown to directly interact with RUNX29 and PU.130,31. Our data provided evidence that TET2-mediated DNA demethylation is required at binding sequences of methyl-sensitive TFs involved in hematopoiesis, such as RUNX1/2/3 and PU.1, and that loss of one allele has a significant effect on lineage-specific methylation levels. When cellular TET2 activity is reduced, the concerted action of methyl-sensitive TFs and TET2 may become compromised, leading to increased methylation in key regions for lineage development. It is important to note that even though we measured DNA methylation in circulating blood cells, the methylation differences that had emerged during differentiation remained in the progeny, and thus could be observed.

Based on regulon-level analysis of single-cell RNA-sequencing data, we found significantly lowered activity of the T-box TFs T-box 21 (TBX21) and eomesodermin (EOMES) in circulating CD8+ T cells and NK cells of the cancer-free TET2delA carriers. These TFs play crucial and partly overlapping roles in the development, activation, cytotoxic effector functions, and exhaustion of CD8+ T cells and NK cells, as well as in the development and maintenance of the CD8+ T memory cell pool32–36. These findings may imply dysregulation of cytotoxic lymphocyte maturation and function, and are compatible with the changes observed in detailed T-cell phenotyping by flow cytometry, where reduced CD8+ T effector memory cells and increased CD8+ exhausted TEMRA phenotype were detected in the TET2delA carriers. Interestingly, TBX21 has been shown to recruit TET2 to the lineage-specific signature gene IFNG during CD4+ T helper 1 cell differentiation, resulting in increased 5-
5-methylcytosine (5mC) deposition at IFNG promoter and (−6) enhancer region37. We could show significantly increased methylation in TET2delA carriers at binding sequences of TBX21 in NK cells. Thus, reduced TBX21- and EOMES-mediated transcription in TET2 mutation carriers could conceivably stem from inadequate recruitment of TET2 to gene regulatory regions by these TFs, although this remains to be studied in detail.

We observed decreased methylation relative to all open chromatin regions in the four DNM73A germline mutation carriers in the binding regions of master TFs involved in hematopoiesis, suggesting that these regions are targets of the joint activity of TET2 and DNM73A. When DNM73A action is reduced, oxidation activity of TET2 leads to reduced levels of 5-mC, and emergence of 5-hmC and other TET2 oxidation products (toward unmethylated cytosine), as compared with normal state. Because this oxidation is a multistep cascade, the clearance of methylation in these TF-binding regions is likely strongest in hematological malignancies with concomitant mutations in TET2 and DNM73A that indeed are known synergistic drivers of neoplasia38.

Recently, it was proposed that by studying DNA methylation in late-replicating domains, accumulation of cell divisions and cellular aging could be measured16. We did not observe a difference in methylation of these domains between TET2 mutation carriers and their age-matched wild-type controls, suggesting similar history of cell divisions in both groups. Thus, both the clinical as well as molecular phenotype of the study subjects are compatible with low neoplastic potential of an individual TET2+/− cell. Absence of second hits imply little additional selective value for loss of both TET2 alleles and, consequently, rare emergence of TET2-null cells in humans. This is compatible with findings on
CVD is the most common cause of death in the Finnish population (Supplementary Data 7), radio- and chemotherapy promote atherosclerosis, and also other risk factors such as positive family history from the paternal side, hypercholesterolemia, and smoking were present in the TET2delA family. Considering these, CVD burden in the Finnish mutation carriers was unremarkable, and we were unable to derive significant clinical evidence for a role of TET2 germline mutation positivity in predisposition to atherosclerosis. This result needs to be interpreted with appropriate caution as the number of individuals examined was small. CH is associated with CVD34, smoking39,40 as well as age, the latter two associating with inflammation and CVD. In two studies, association between CH and atherosclerosis-related conditions remained after adjusting for smoking54. In two other studies, Genovese et al46 found that excess of mortality in CH patients was explained by a combined effect of hematological malignancy and smoking, and Ziaei et al39 found no significant association between CH and smoking-associated disease after adjusting for smoking. A more recent study41 found no evidence of association of TET2- and DNMT3A-driven CH to CVD in a female cohort. CH and CVD are both conditions that conceivably associate with quantity of smoking exposure, not accounted for in the publications thus far, and strongly with age. An individual’s aging process is not directly reflected by years, but also nutrition, lifestyle, environment, and genetics play a role42. It is difficult to perfectly adjust for these factors, and thus separating two age-related traits in association analyses is challenging.

Mechanistically, exacerbated IL-1β secretion by macrophages was proposed to be essential for accelerated atherosclerosis in context of CH due to TET2 loss in mice5. However, a more recent study could not show increased IL-1β secretion in TET2−/− mouse macrophages34. Instead, increased CXCR chemokine secretion was proposed as the key factor for accelerated atherosclerosis4, with increased IL-8/CXCL8 secretion suggested as the possible effector in humans. We did not find evidence supporting either mechanism in the six TET2 germline mutation carriers examined, representing three different truncating mutations. Our results as such are not discrepant with the mouse work, as the clinical and molecular consequences of TET2 loss in mice do not need to be identical to the human phenotype. The effects of TET2 loss are mediated through hypermethylation within a genome, and the two genomes were separated tens of millions of years ago. For example, mice are devoid of TEMRA cells43, which we found to be elevated in the elderly TET2 mutation carriers and may influence the human phenotype.

The single-cell RNA-sequencing data revealed significantly elevated expression of CXCR4 in all blood cells of the TET2delA individuals, especially in T and NK cells. This chemokine receptor mediates leucocyte trafficking in response to its ligand CXCL12 and has crucial roles e.g. in B-cell development44. Notably, CXCR4 knockdown/knockout in bone marrow or in vascular wall cells aggravates the progression of atherosclerosis in mouse models35,46. Moreover, a common human CXCR4 variant, C-allele at rs2322864, is significantly associated with increased risk of coronary heart disease and with reduced CXCR4 expression in whole blood and in carotid atherosclerotic plaques45. Thus, the elevated CXCR4 expression in blood cells of TET2delA carriers exemplifies one possible atheroprotective effect by TET2 loss that may help to counterbalance possible proatherogenic effects in humans. We could also detect reduced expression of immunoglobulin genes such as Igf in B cells of cancer-free TET2delA carriers. It has been shown that IGF, Igl, and VDJ rearranged IgH co-localize to the same transcription factories47. Furthermore, the expression of Igf and Igl is PU.1 regulated and Igx is silenced in mouse early B cells with Tet2/Tet3 loss31,48,49. Altogether, these data suggest that TET2 has a role in regulating immunoglobulin genes in human B cells, possibly through cooperation of TET2 and lineage-specific TFs, and/or through high CXCR4 expression30.

It is important to note that the exposure of the germline mutation carriers to effects of TET2 loss is life-long and extreme, as compared to the typical CH setting of a minor somatic heterozygous TET2-mutant subclone—2% or higher variant allele frequency39—emerging late in life. Our data raise the possibility, that circulating TET2-mutant cells may be of limited importance as an atherosclerosis risk factor, at least in the context of germline mutation where all cell types carry the mutation. While this finding does not support a major role of TET2 loss in atherosclerosis, it is equally important to note that molecular characters of CH cells that have accumulated additional changes during somatic selection may be more aggressive and atherogenic. If so, the detailed mechanisms need to be determined.

Vitamin C boosts TET2 function51. Thus, adequate vitamin C intake in the context of hereditary heterozygous TET2 loss-of-function mutations might reduce risk of neoplasia, a subject that needs further study. At present, it appears appropriate to at least advise such individuals to ensure that the daily dose recommended for the general population is reached. In the context of TET2-mutation-positive CH, prophylactic treatment for atherosclerosis using cholesterol-lowering medications or compounds targeting IL-1β and other inflammatory pathways as proposed24.5 would, if effective, be a significant advance. If ineffective, such an intervention would cause cost and side effects. Thus, it is important to more thoroughly scrutinize the proposed association between CH and CVD, and the possible mechanisms underlying it.

Methods

Study approval. The samples and patient information were obtained with approval from the ethics committee of the Hospital District of Helsinki and Uusimaa, the Northern Ostrobothnia Hospital District, the Ethik Kommission Beider Basel as well as Finnish National Supervisory Authority for Welfare and Health. Informed consent was obtained for all participants. This study was conducted in accordance with all relevant rules and regulations, including the Declaration of Helsinki.

Immunohistochemistry. Histopathological analysis of NLPHL samples was performed by an experienced hematopathologist. Formalin-fixed paraffin-embedded (FFPE) samples were sectioned to 5 μm thickness, and stained with hematoxylin-eosin, and with antibodies against CD3, CD15, CD20, and CD30 according to standard procedures at Helsinki University Hospital Pathology Lab.

Detection of genomic regions segregating with lymphoma. Genomic DNA was extracted from blood samples of three family members, Ly1, Ly2, and Ly8. Blood sample from Ly3 could not be obtained, since the person is deceased, so a FFPE tissue was used for DNA extraction. Whole-genome SNP genotypes were obtained using HumanOmniExpress-FFPE BeadChip (Illumina, Inc., CA, USA) of 693,543 markers. All procedures were performed according to the manufacturer’s instructions at Finnish Institute for Molecular Medicine (FIMM) Genome and Technology Centre. Genotype calling and quality control of the data were done with GenomeStudio Genotyping module version 1.9.4 (Illumina). A parametric linkage analysis with dominant inheritance model (50% penetrance) was performed using MERLIN52 similar to Saarinen et al53. Ly8 was considered as unaffected. Further germline variant analyses were focused on linked regions i.e. the segregating genomic regions with a positive logarithm of odds score (>0) and a length of at least 5 cm.

Germline variant analysis. Ly1 genomic DNA library was prepared and sequenced according to Complete Genomics (Complete Genomics Inc., Mountain View, CA, USA) paired-end sequencing service protocols. Complete Genomics’ service was conducted with standard coverage: 40x average coverage and 90% callable diploid loci on the human reference genome. Both read alignment and variant calling were included in the service.
Whole-exome sequencing was performed for patients Ly2 and Ly3 by capturing exonic regions using SureSelect Human All Exon Kit v.1.0 (Agilent Technologies, Inc., Santa Clara, USA) using the EZ Human Exome 12 Library (Roche NGS Solutions, Branford, USA, WI, USA), respectively, using the standard protocols. Paired-end sequencing was performed using Illumina Genome Analyzer II at the Finnish Institute of Molecular Medicine (Ly2) and Illumina HiSeq2000 at Karolinska Institutet (Sweden; Ly3). The read lengths were 82 bp (Ly2) and 76 bp (Ly3). The paired-end reads were quality-controlled, demultiplexed, and QC’d using BCBio-Tools (version v0.14.4)

Next, a functional effect prediction was done by two different algorithms (Provean and SIFT). Single-nucleotide or multiple single-nucleotide variants that were predicted damaging or deleterious by both algorithms, and indels and stop codon producing variants constitute the set of preliminary shared candidate variants (Supplementary Table 2). Finally, we filtered the shared variant list further using by the Exome Aggregation Consortium (version 0.2) dataset consisting of 60,706 unrelated individuals’ exomes by allowing no hits in the control set. We further limited the false positive calls down by using the strict genomic regions accessible to accurate analysis from the 1000 Genomes Project (phase 1). The remaining variants on the list were validated by Sanger sequencing. The deletion c.455_462delACAGCCAC in ABRa was found to be homozygous in the healthy mother of the patients Ly1, Ly2, and Ly3, excluding it from the final candidate variants (Supplementary Table 2). Furthermore, the TET2 c.490delA mutation status of the mother was examined from archival tissue derived DNA, and she was found to carry the mutation. All primer sequences used in the validation of the candidate variants are available data were used (UK10K, www.uk10k.org, and Sequencing Initiative 100 Genomes Project (human_g1k_v37.fasta) using an in-house pipeline consisting of publicly available tools (bwa (version 0.5.9) for alignment, Genome Analysis Toolkit (GATK) (version 1.0.500.35575) for variant calling, and samtools for sorting and indexing).

Analysis of targeted bisulfite sequencing data. Detection of DMCs and DMRs was done with the DSS63 R package (version 2.14.0). Only cytosines with minimal depth of coverage of six were included. Methylation values were smoothed in 300 bp windows before DMC detection. DMCs were detected on autosomes with Wald test requiring methylation difference >0.2 and posterior probability >0.99999 in comparison of each sample against the five baseline control samples (controls 1–5). For the comparison of number of hyper- or hypomethylated CpG sites between five TET2 mutation carriers and five non-carriers, two-tailed Wilcoxon rank sum tests were performed. DMCs were detected by comparing (i) TET2delA carriers (Ly1, Ly2, Ly9, Ly11, and Ly14) to wild-type and control samples (Ly8, Ly10, Ly12, Ly13, HLRCC_N7, and controls 1–5) as well as by comparing (ii) NLPHL cases to cancer-free TET2delA carriers (Ly1, Ly2, and Ly3) –5 as well as by comparing (ii) NLPHL cases to cancer-free TET2delA carriers (Ly1, Ly2, and Ly3) –5 as well as by comparing

Bisulfite sequencing library preparation and data processing. The SureSelect target enrichment system covering 845.5 Mb (Agilent Technologies, Inc., CA, USA) was used to prepare bisulfite sequencing libraries from blood DNA of patients (Ly1 and Ly2), healthy family members (Ly8, Ly9, Ly10, Ly11, Ly12, Ly13, and Ly14), baseline controls (controls 1–5), and DNM3A mutation carriers and their age-matched controls (Supplementary Table 3). In addition, blood DNA of a patient (HLRCC N7) with germline fumarate hydratase (FH) mutation (c.1027C>T, p.R343X) was included in the study, because FH-null paragangliomas have been described in patients with DNM3A/FH mutations carriers (Ly1, Ly2, Ly9, Ly11, and Ly14) were compared to 10 wild-type and control samples (Ly8, Ly10, Ly12, Ly13, HLRCC_N7, and controls 1–5). Pearson’s correlation displayed in Fig. 4e was calculated for methylation

Analysis of WGBS data. Detection of DMCs was done with the DSS63 R package (version 2.14.0) at cytosines with minimal depth of coverage of 2. Methylation values were smoothed in 300 bp windows before DMC detection, DMCs were detected on autosomes with Wald test requiring methylation difference >0.1 and posterior probability >0.99999. Changes in mutation carriers as compared to controls at open chromatin regions are shown in Fig. 4c.

Immunophenotyping of cancer cells. Lymphocyte, B-, and T-cell phenotyping were performed as previously published by Zhou et al.

Whole-exome sequencing was performed for patients Ly2 and Ly3 by capturing exonic regions using SureSelect Human All Exon Kit v.1.0 (Agilent Technologies, Inc., CA, USA) using the EZ Human Exome 12 Library (Roche NGS Solutions, Branford, USA, WI, USA), respectively, using the standard protocols. Paired-end sequencing was performed using Illumina Genome Analyzer II at the Finnish Institute of Molecular Medicine (Ly2) and Illumina HiSeq2000 at Karolinska Institutet (Sweden; Ly3). The read lengths were 82 bp (Ly2) and 76 bp (Ly3). The paired-end reads were quality-controlled, demultiplexed, and QC’d using BCBio-Tools (version v0.14.4)

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T-lymphocyte immunophenotyping. Four- or 10-color flow cytometry panel with monoclonal antibodies (mAbs) against the surface antigens IgM, IgD, CD3, CD4, CD8, CD66s, CD19, CD21, CD27, CD33, CD34, CD38, CD45, CD56, CD133, HLA-DR, CD62L, CD45RA, and CD45RO (BD Biosciences, San Jose, CA)18. The memory status of T cells was studied with the antibody panel including anti-CD45, -CD3, -CD4, -CD8-CD45RA, and -CCR7 (R&D Systems, Minneapolis, MN, USA)19.20.

**Single-cell transcriptome sequencing and analysis.** Whole blood was collected in EDTA-treated collection tubes. Red blood cells were lysed by adding a volume of ACK lysis buffer (Gibco, New York, NY, US) corresponding to 10–20 times the volume of the blood sample and incubating at room temperature (RT) for 3 min. White blood cells were collected by centrifugation at 300 × g for 5 min at RT. The cells were resuspended in cold phosphate buffered saline (PBS) corresponding to five times the volume of the whole-blood sample and collected by centrifugation at 4 °C. After resuspending in cold 0.4% bovine serum albumin/PBS, the cells were filtered with FlowMiTM Tip strainer of 40 μm porosity (SP Scienceware, Bel-Art Products, Wayne, NJ, US). Finally, the cells were stained with Trypan Blue (Invitrogen, Waltham, MA, USA), counted with Countess Automated Cell Counter (ThermoFisher Scientific), and adjusted to the final concentration of 1 × 106 cells/ml.

Single cells were captured into 10x barcoded gel beads and RNA-sequencing library preparation was done using Chromium Single Cell 3’ v2 chemistry (10x Genomics, Pleasanton, CA, USA) at FIMM Single Cell Analytics core facility (Finland). Sequencing was performed as recommended with 98 bp length of read 2 using HiSeq4000 sequencer (Karolinska Institutet, Sweden). Capture and sequence data were generated in Supplementary Table 14. Cell Ranger (v2.0.0, 10x Genomics) was used for data processing with default parameters using prebuild human hg19 genome reference provided by 10x Genomics. Cell types were identified from K-mean (K = 10) clusters utilizing CD3D, NKGT, IL7R, CD79a, CST3, ST04A9, and L1Z marker genes (Supplementary Fig. 14). Data were separated according to gender from all eight samples and from six cancer-free family members with cellranger aggr pipeline. Statistical tests between cancer-free and for large counts, the fast asymptotic beta test. Resulting genes were filtered, crystallized at RT, dried, and ground to a size range of 1–10 μm using a sterile mortar and a pestle. Uric acid (Sigma-Aldrich) at 5 g/l in 20× deionized water was heated to 60 °C, sterile-filtered, crystallized at RT, dried, and ground to a size range of 1–10 μm using a sterile mortar and a pestle. Uric acid (Sigma-Aldrich) at 5 g/l in deionized water was heated to 60 °C, adjusted to pH 8.9 with 0.5 N NaOH, and stored at RT. The MSU crystals were recorded by centrifugation, washed, dried, and sterilized at 180 °C. Endotoxin levels of the cholesterol and MSU crystals were below the detection limit (<0.03 EU/ml) of Pyrogen gel clot LAL assay (Lonza, Switzerland). Monocyte immunofluorescence response to prolonged LPS exposure was studied by 24 h stimulation with 1 μg/ml LPS from E. coli O111:B4 (Invitrogen) for 5 h. Cholesterol (Sigma-Aldrich) at 12.5 μl/g in 95% ethanol was heated to 60 °C, sterile-filtered, crystallized at RT, dried, and ground to a size range of 1–10 μm using a sterile mortar and a pestle. Uric acid (Sigma-Aldrich) at 5 g/l in deionized water was heated to 60 °C, adjusted to pH 8.9 with 0.5 N NaOH, and stored at RT. The MSU crystals were recorded by centrifugation, washed, dried, and sterilized at 180 °C. Endotoxin levels of the cholesterol and MSU crystals were below the detection limit (<0.03 EU/ml) of Pyrogen gel clot LAL assay (Lonza, Switzerland). Monocyte immunofluorescence response to prolonged LPS exposure was studied by 24 h stimulation with 1 μg/ml LPS from E. coli O111:B4 (Invitrogen) for 5 h. Cholesterol (Sigma-Aldrich) at 12.5 μg/l in 95% ethanol was heated to 60 °C, sterile-filtered, crystallized at RT, dried, and ground to a size range of 1–10 μm using a sterile mortar and a pestle. Uric acid (Sigma-Aldrich) at 5 g/l in deionized water was heated to 60 °C, adjusted to pH 8.9 with 0.5 N NaOH, and stored at RT. The MSU crystals were recorded by centrifugation, washed, dried, and sterilized at 180 °C. Endotoxin levels of the cholesterol and MSU crystals were below the detection limit (<0.03 EU/ml) of Pyrogen gel clot LAL assay (Lonza, Switzerland). Monocyte immunofluorescence response to prolonged LPS exposure was studied by 24 h stimulation with 1 μg/ml LPS from E. coli O111:B4 (Invitrogen) for 5 h. Cholesterol (Sigma-Aldrich) at 12.5 μg/l in 95% ethanol was heated to 60 °C, sterile-filtered, crystallized at RT, dried, and ground to a size range of 1–10 μm using a sterile mortar and a pestle. Uric acid (Sigma-Aldrich) at 5 g/l in deionized water was heated to 60 °C, adjusted to pH 8.9 with 0.5 N NaOH, and stored at RT. The MSU crystals were recorded by centrifugation, washed, dried, and sterilized at 180 °C. Endotoxin levels of the cholesterol and MSU crystals were below the detection limit (<0.03 EU/ml) of Pyrogen gel clot LAL assay (Lonza, Switzerland).
Material availability = value archival. Data that potentially allow identification of individuals must be protected, and thus, an access committee has been established. ATAC-seq open chromatin regions and fragment counts for hematopoietic cell types were downloaded from GEO accession GSE74912. Roadmap Epigenetics data provided in the LOLA extended databases (version 170206) were downloaded from [http://cloud.databio.org/regiondb/]. The human-specific databases for RcisTarget were downloaded from [https://resources.aertslab.org/cistarget/databases/homo_sapiens/hg19/refsqcg_r45/mc9n/gene_based/hg19-500bp-upstream-7species.mc9n.5mer.th] and [https://resources.aertslab.org/cistarget/databases/homo_sapiens/hg19/refsqcg_r45/mc9n/gene_based/hg19-1ss-centered-10kb-7species.mc9n.5mer.th] with R version 3.5.0.

References


Additional information
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