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ARTICLE

An intronic *FTO* variant rs16952570 confers protection against thiopurine-induced myelotoxicities in multiethnic Asian IBD patients

Sylvia Chen[1] · Wei Zhi Tan[1] · Natalia Sutiman[2] · Cindy Lim[3] · Sze Sing Lee[1] · Wai Fook Leong[2] · Madeline Tjai[2] · Chunyan Wang[2] · Chris San Choon Kong[4,5] · Sai Wei Chuah[4] · Brian John Schwender[4,6] · Webber Chan[4] · Hang Hock Shim[4] · Wee Chian Lim[7] · Chiea Chuen Khor[8,9,10] · Khoon Lin Ling[4] · Balram Chowbay[1,2,11,12]

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Abstract

Thiopurines are used in the treatment of inflammatory bowel disease (IBD) but remain clinically challenging to manage due to wide interpatient variability in clinical outcomes and adverse events. Apart from genetic variants in thiopurine S-methyltransferase (TPMT) and nudix hydrolase 15 (NUDT15) genes, polymorphisms in FTO alpha-ketoglutarate dependent dioxygenase (FTO) were found predictive of thiopurine-induced leukopenia, albeit with conflicting results. To clarify the role of *FTO* variants in a multiethnic Asian IBD cohort, we recruited 149 patients on thiopurine-based therapy and genotyped two *FTO* variants p.Ala134Thr (rs79206939) and rs16952570 T > C using Sanger sequencing. *FTO* p.Ala134Thr (rs79206939) was non-polymorphic and absent whereas intronic rs16952570 T > C was equally prevalent in Chinese (22%) and Indians (18%) and higher in Malays (28%). Higher nadir white blood cell (WBC) and absolute neutrophil count (ANC) levels were observed in patients harboring *FTO* rs16952570 CC genotypes compared with TT carriers at 4, 8, and 12 weeks after start of thiopurine therapy ($P < 0.05$). A similar trend was observed in patients carrying the previously well-characterized *NUDT15* rs116855232 wild-type CC genotypes. Further in silico analysis suggests that *FTO* variants linked to rs16952570, particularly rs74018601, may play a regulatory role in altering the *FTO* expression. The findings from this study indicate a novel protective association with the *FTO* variant rs16952570 CC genotype and hematological parameters.

Introduction

Thiopurines such as azathioprine (AZA) and 6-mercaptopurine (6-MP) are widely used immunosuppressants in the treatment of autoimmune disorders and inflammatory bowel disease (IBD), particularly Crohn's disease (CD) and ulcerative colitis (UC). In spite of its

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✉ Balram Chowbay
ctebal@nccs.com.sg

1 Clinical Pharmacology Laboratory, National Cancer Centre, Singapore, Singapore

2 Clinical Pharmacology, SingHealth, Singapore, Singapore

3 Biostatistics Unit, Clinical Trials & Epidemiology Sciences, National Cancer Centre, Singapore, Singapore

4 Department of Gastroenterology & Hepatology, Singapore General Hospital, Singapore, Singapore

5 Department of General Medicine, Gastroenterology and Hepatology Service, Sengkang General Hospital, Singapore, Singapore

6 Tucker Medical, Singapore, Singapore

7 Department of Gastroenterology, Tan Tock Seng Hospital, Singapore, Singapore

8 Singapore Eye Research Institute, Singapore National Eye Centre, Singapore, Singapore

9 Department of Ophthalmology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Singapore

10 Division of Human Genetics, Genome Institute of Singapore, Singapore, Singapore

11 Centre for Clinician-Scientist Development, Duke-NUS Medical School, Singapore, Singapore

12 Present address: Division of Cellular & Molecular Research, Humphrey Oei Institute of Cancer Research, National Cancer Centre, 11 Hospital Drive, Singapore 169610, Singapore

demonstrated clinical benefit of inducing and maintaining clinical remission even in steroid-dependent Asian IBD patients, over 25% of patients discontinue or experience dose interruptions in thiopurine treatment as a result of thiopurine-induced myelosuppression and hepatotoxicities. Particularly, leukopenia occurs more frequently in Asians compared with their counterparts of European descent [1–3].

Thiopurines are known to undergo a highly complex metabolism pathway whereby thiopurine-S-methyltransferase (TPMT) converts AZA and 6-MP to form its major metabolite, 6-thioguanine nucleotide (6-TGN) through a series of biotransformations [4]. While genetic mutations (*TPMT*2, TMPT*3A, TPMT*3B*, and *TPMT*3C)* in *TPMT* may be clinically relevant in predicting for leukopenia risk, their utility remains limited in Asians given that <5% harbor at least one defective allele and homozygous carriers have been minimally detected [5, 6]. On a contrary, genetic variants in the gene encoding for nudix hydrolase 15 *(NUDT15)* have been associated with an increased risk in thiopurine-induced myelotoxicities amongst East Asians [7–12]. A recent meta-analysis by Zhang et al. also reported that the *NUDT15* rs116855232 TT genotype was associated with a 6-times increase in leukocytopenia risk compared with wild-type CC carriers and heterozygotes [12].

In addition to the well-described *NUDT15* and *TPMT* polymorphisms, a recent GWAS study by Kim et al. has reported the effect of *FTO* variants with leukopenia susceptibility in Korean IBD patients. In a discovery cohort of 267 Korean IBD patients, a loci containing intronic *FTO* rs16952570 was found to be significantly associated with thiopurine-induced leukopenia (odds ratio (OR) = 2.5, 95% CI: 1.7–3.7, $P = 1.3 \times 10^{-6}$) [13]. This single nucleotide polymorphism (SNP) was found to be in tight linkage disequilibrium (LD) ($r^2 = 0.06$, $D' = 1$) with a non-synonymous *FTO* variant rs79206939 (p.Ala134Thr, c.400G > A) and validation in replication cohorts demonstrated an increased risk in leukopenia in patients carrying a single risk allele of rs79206939. Notably, although *FTO* rs79206939 occurs in only 2.3% of Korean IBD patients (approximately six patients), functional studies indicate a 65% reduction in FTO demethylase activity in the presence of this SNP, thereby suggesting its potential role in predicting thiopurine-induced myelotoxicities. Moreover, the authors also reported that *FTO*-deficient mice were more susceptible to myelosuppression [13], implying a novel function of FTO as a modulator of hematological toxicities. In contrast, Sato et al. reported a minor allelic frequency (MAF) of 3.0% for rs79206939 and failed to observe the same adverse association with leukopenia in an ethnically similar East-Asian cohort comprised of 160 Japanese IBD patients receiving thiopurines [11]. Similar to our previous findings, this latter study also confirmed that *NUDT15* rs116855232 is strongly predictive of early and late leukopenia [7, 11]. Given the conflicting results reported in two East-Asian cohorts of Japanese and Koreans, we sought to clarify these associations in a multiethnic Asian population of IBD patients.

Methods and materials

Study population and thiopurine treatment

Patients diagnosed with either CD or UC and had received thiopurine-based outpatient treatment at Singapore General Hospital, were recruited into the study. Details on thiopurine dose administration have been previously reported [7]. Written informed consent was provided by all patients and the study was approved by the Institutional Review Board of Singapore Health Services (CIRB/2012/193/E).

Clinical characteristics and myelotoxicity assessment

Patient demographics, clinical information including thiopurine regimens, concomitant medications, and nadir full blood counts including white blood cell (WBC), absolute neutrophil count (ANC), and platelets at the following time points: 4, 8, 12 weeks, and 6 months following the initiation of thiopurine treatment were retrospectively retrieved from the electronic medical records system. Patients with incomplete clinical information were excluded from the analysis. 6-MP doses were converted into AZA-equivalent doses using a conversion factor of 2.08.

Pharmacogenetic analysis and quantification of 6-TGN and 6-MMP levels

Genomic DNA was extracted from 6 mL of peripheral whole blood samples collected from patients using DNeasy Blood & Tissue kit according to the manufacturer's protocol (Qiagen, Hilden, Germany). Genotyping of FTO variants (rs79206939 G > A, A134T and rs16952570 T > C) was performed using direct Sanger sequencing. Briefly, target regions were amplified by PCR and visually verified via agarose gel electrophoresis prior to enzymatic cleanup by ExoSAP. Sanger sequencing was carried out using the BigDye Terminator v3.1 Cycle Sequencing Kit (ThermoFisher Scientific, MA, USA). PCR and sequencing primers used were as follows: rs79206939, fwd 5′-AAGCCAGC AGTGTATCTG-3′, rev 5′-CTCAGAAAATGCCATAC CTG-3′; rs16952570, fwd 5′-TGCCCTCATCAAGCTC ACAT-3′, rev 5′-AGCAACACCAGAAACAGGAG-3′. *NUDT15* rs116855232 (p.Arg139Cys c.415C > T) genotyping data were obtained previously using pyrosequencing methods [7]. Genotyping was visually inspected using the Applied Biosystems Sequencing Analysis 5.2 software and verified by two independent persons.

Steady-state concentrations of 6-TGN and 6-methylmercaptopurine (6-MMP) metabolites in erythrocytes were quantified using a validated liquid chromatography–tandem mass spectrometry method described previously [7].

Statistical analysis

Chi-square test was used to check for conformity to Hardy–Weinberg equilibrium. Boxplots showing the median and interquartile range of nadir WBC and ANC levels by *FTO* rs16952570 genotype group at baseline, 4, 8, 12 weeks, and 6 months were plotted. The nonparametric Mann–Whitney U test was used to compare nadir WBC and ANC levels between two *FTO* genotype groups, whilst the Kruskal–Wallis test was used to compare all the three genotype groups. For each timepoint, nadir WBC and ANC levels were compared between *FTO* rs16952570 genotype groups using the multiple linear regression. *NUDT15* rs116855232 genotype was adjusted for in the model, with the wild-type CC genotype as the reference group. Mean differences in nadir WBC and ANC levels for *FTO* rs16952570 TC and CC genotypes, relative to the wild-type TT genotype, were estimated from the model with corresponding 95% confidence intervals and *P* values. Metabolite concentrations measured at first follow-up were correlated with WBC levels taken within 30 days, using the Spearman's correlation. All *P* values were two-tailed and values lower than 0.05 were considered to be statistically significant. No adjustments were made for multiple comparisons. All statistical analyses were performed on Graphpad Prism version 6.0 (GraphPad, La Jolla, CA, USA) or STATA version 15 (StataCorp, College Station, Texas, USA).

In silico analysis

To further investigate the putative regulatory role of *FTO* rs16952570, we performed an in silico analysis using online tools and databases. Identification of SNPs in high LD ($r^2 > 0.8$) and putative transcription factor binding motifs was conducted on HaploReg v4.1 web-based tool (https://pubs.broadinstitute.org/mammals/haploreg/haploreg.php) (Broad Institute, Cambridge, MA, USA) [14]. Regulatory motifs reported in HaploReg were prioritised based on position weight matrices and converted into log-odds (LOD) scores [14]. The difference in LOD scores were calculated using the following formula: LOD(altered allele) − LOD(reference allele). Correlations between gene expression and SNP were performed using GTex data obtained from Ensembl release 92 (http://www.ensembl.org) by entering SNP ids. Expression data of *FTO* mRNA was available from 10294 healthy samples and categorized into 28 unique tissue types. The GTex data used in the analyses described here was obtained from: dbGaP accession number phs000424.v7.p2 on 19/06/2018 [15]. Integrated regulation information tracks from ENCODE data comprised of DNAse1 hypersensitivity regions (ENCODE v3), transcription factor binding motifs (ENCODE 3 Nov 2018), and histone modifications overlayed within the *FTO* loci were visualized using UCSC Genome Browser with assembly version GRCh37/hg19 (http://genome.ucsc.edu).

Analysis of selected *FTO* variants in LD was performed by using Ferret version 2.1.1 (http://limousophie35.github.io/Ferret/) to extract SNP data of three ethnic populations, European-Caucasian, East Asians, and South Asians, from the 1000 Genomes Project, followed by calculation and visualization of r^2 and D' values with Haploview 4.2 (https://www.broadinstitute.org/haploview/haploview).

Results

Patient demographics and clinical characteristics

A total of 149 patients were recruited into the study, of which, 28 patients were excluded from analyses due to unavailability of DNA samples ($N = 22$), non-Asians ($N = 3$) and thiopurines being prescribed for indications other than IBD ($N = 3$). The demographics and clinical characteristics of patients ($N = 121$) are listed in Table 1. The cohort comprised of patients with a median age of 40 years old, predominately males ($N = 77$, 64%) who were of Chinese descent ($N = 81$, 66.9%) and were receiving thiopurine-based therapy for the treatment of CD ($N = 83$, 68.6%). Majority of patients (98.3%) were on AZA whereas only two patients were on 6-MP (1.7%). Sixty percent of the patients received concomitant 5-aminosalicylates while only 19% were on corticosteroids (budesonide or prednisone).

Observed frequencies of the *FTO* variants are shown in Table 2. *FTO* rs79206939 was absent and non-polymorphic in our study cohort whereas the overall MAF of *FTO* rs16952570 was 21% and similar amongst Chinese (22%) and Indians (18%). The MAF of *FTO* rs16952570 in Malays was higher at 28% compared with the two other ethnicities; most likely due to the low sample size in the cohort ($N = 9$) (Table 2). No deviation from Hardy–Weinberg equilibrium were observed ($P = 0.999$) in all three ethnic groups.

Effect of intronic *FTO* variant rs16952570 genotype on nadir WBC, ANC and platelet counts

Comparison of baseline WBC, ANC, and platelet counts in patients harboring *FTO* rs16952570 genotypes reveal no significant difference (Fig. 1), though CC carriers had

Table 1 Demographics and clinical characteristics of Asian IBD patients ($N = 121$).

	N	%
Age		
Mean (±SD)	41 ± 14.6	
Median (range)	40 (17–81)	
Gender		
Male	77	64
Female	44	36
Ethnicity		
Chinese	81	66.9
Indian	31	25.6
Malay	9	7.5
Clinical indications of thiopurine therapy		
Crohn's disease	83	68.6
Ulcerative colitis	38	31.4
Thiopurine drug received		
Azathioprine	119	98.3
6-mercaptopurine	2	1.7
Concomitant medications at thiopurine initiation		
Corticosteroids	23	19
5-aminosalicylates	73	60
Azathioprine-equivalent daily dose		
Mean (± SD) in mg	109.2 ± 46.4	
Median (range) in mg	100 (25–208)	

Table 2 Genotypic and allelic frequencies of *FTO* variants rs79206939 and rs16952570 in Asian IBD patients ($N = 121$).

FTO rs79206939 (G > A)	*N*	Genotypic frequency, *N* (%)			Allelic frequency (%)	
		GG	GA	AA	G	A
Chinese	81	81 (100)	0 (0)	0 (0)	100	0
Indians	31	31 (100)	0 (0)	0 (0)	100	0
Malays	9	9 (100)	0 (0)	0 (0)	100	0
All patients	121	121 (100)	0 (0)	0 (0)	100	0
FTO rs16952570 (T > C)		TT	TC	CC	T	C
Chinese	81	49 (60)	28 (35)	4 (5)	78	22
Indians	31	21 (68)	9 (29)	1 (3)	72	28
Malays	9	5 (56)	3 (33)	1 (11)	82	18
All patients	121	75 (62)	40 (33)	6 (5)	79	21

slightly higher baseline WBC and ANC levels compared with other patients. Heterozygous patients with TC genotype had significantly lower nadir WBC counts compared with wild-type TT carriers at 4, 8, and 12 weeks after thiopurine initiation whereas homozygous CC carriers exhibited higher nadir WBC counts at across all time-points (Fig. 1a). Higher nadir ANC levels were also detected in CC carriers, with significant differences from TT and TC carriers across all time-points of 4, 8, 12 weeks, and 6 months (Fig. 1b). No significant difference in nadir platelet counts at all time-points was observed, although there was a trend of higher platelet counts in CC patients, similar to that of WBC and ANC levels (Fig. 1c),

Owing to the strong association of well-studied *NUDT15* rs116855232 in several Asian cohorts, we performed a subgroup analysis to account for the contribution of *FTO* rs16952570 in the absence of *NUDT15* rs116855232 T risk allele. A similar trend was observed whereby heterozygous TC patients displayed significantly lower median WBC counts, compared with wild-type TT carriers (Fig. 2a). Patients carrying *FTO* rs16952570 CC genotype continued to exhibit higher nadir WBC counts over those with either TT or TC genotypes (Fig. 2a). Nadir ANC levels were also elevated in CC carriers compared with the other genotype groups (Fig. 2b). Likewise, CC patients had the tendency to exhibit higher nadir platelet counts at 4 weeks and 6 months compared with their TT counterparts (Fig. 2c).

Further linear regression analysis, controlling for *NUDT15* rs116855232 genotype, revealed that patients harboring *FTO* rs16952570 TC genotype had lower WBC counts (ranging from -0.5 to -1.9×10^9/L) across studied timepoints of 4, 8, 12 weeks, and 6 months, compared with wild-type carriers (Table 3). This relationship was most significant at 4 weeks where TC patients had lowest absolute WBC (WBC: -1.94×10^9/L relative to wild-type, $P = 0.002$). Conversely, an inverse relationship between CC genotype and nadir WBCs was noted in patients harboring the CC genotype. Compared with their wild-type counterparts, significantly higher WBCs (ranging from $+2.3$ to $+3.1 \times 10^9$/L) was observed in the CC group at 4, 8, 12 weeks, and 6 months ($P \le 0.024$).

Similarly, nadir ANCs remained low in TC carriers at 4, 8, 12 weeks, and elevated in CC genotype group across all studied timepoints, relative to the wild-type group (Table 3).

Correlation of *FTO* rs16952570 and WBC counts with 6-TGN concentrations

An inverse correlation between 6-TGN concentrations and corresponding WBC counts was observed during the first follow-up (Spearman $r = -0.413$, $P < 0.0001$) (Supplementary Fig. S3a). A weaker yet significant correlation was found between 6-TGN levels and change in WBC counts from baseline (ΔWBC) (Spearman $r = -0.295$, $P = 0.0025$) (Supplementary Fig. S3b). Correlations with weight-dose normalized 6-TGN levels remained statistically significant (data not shown). Median 6-TGN concentrations were similar across the three *FTO* rs16952570 genotype groups (Supplementary Fig. S3c).

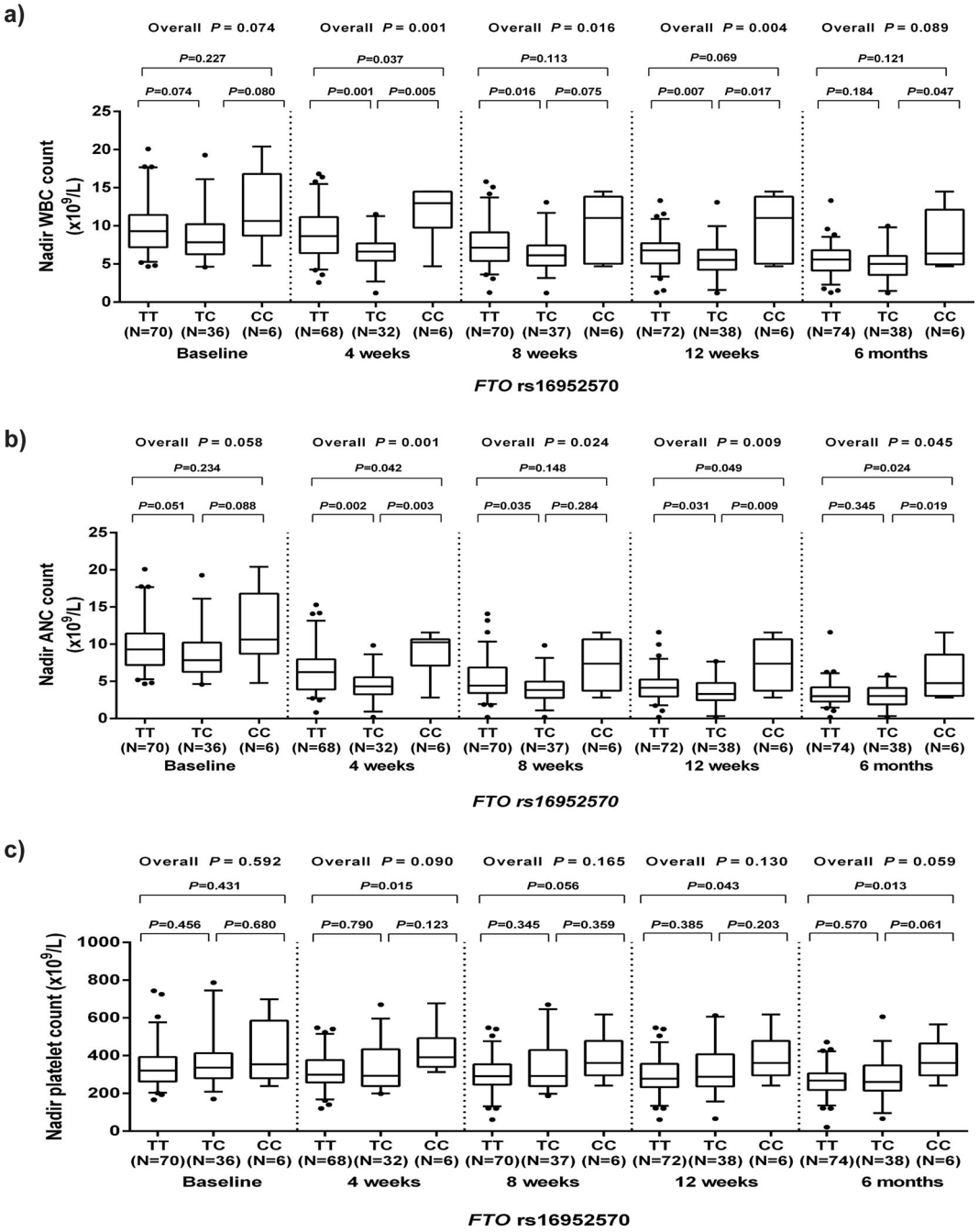

Fig. 1 Nadir WBC counts (a), ANC (b) and platelet counts (c) prior to and after 4, 8, 12 weeks, and 6 months of thiopurine therapy in *FTO* rs16952570 genotype carriers.

In silico analysis

The *FTO* rs16952570 overlaps the intronic regions of 12 out of 23 *FTO* transcripts; seven transcripts are protein-coding, four transcripts resulting in nonsense-mediated RNA decay and another as a non-protein coding processed transcript. No significant expression quantitative trait locus was identified in all tissue types upon filtering with Q-value threshold. Subgroup analysis stratified with *FTO* (gene id: ENSG00000140718) depict the effect sizes of rs16952570 on *FTO* mRNA expression within the range of ±0.3 in all tissues evaluated, with the exception of adipose visceral tissue, where the effect size was larger and significant (Effect size: -0.57, $P = 1.91 \times 10^{-4}$) (Supplementary Fig. S1).

Using HaploReg and ENCODE ChIP-seq data, we analysed the allele-specific changes of transcription factor binding of rs16952570. Only NF-1 was altered in the

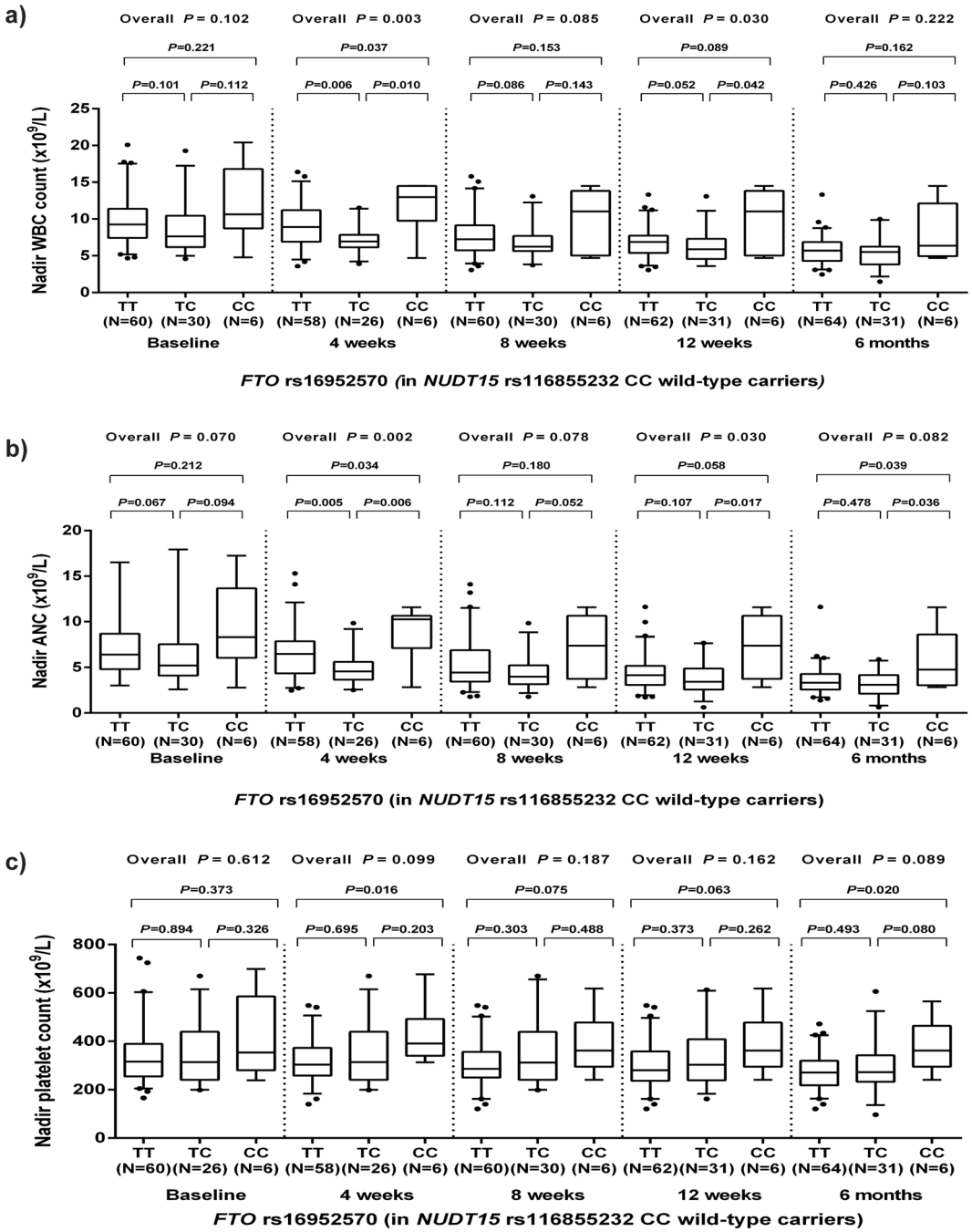

Fig. 2 Nadir WBC counts (a), ANC (b) and platelet counts (c) prior to and after 4, 8, 12 weeks, and 6 months of thiopurine therapy in *FTO* rs16952570 genotype and *NUDT15* rs116855232 wild-type CC carriers.

presence of the C allele, resulting in a change in LOD score of 11 (Table 4, Supplementary Table S1). No further discernible regulatory information could be derived.

To ascertain whether rs16952570 may be in LD with a causal SNP, we further identified and annotated four SNPs in tight LD with rs16952570 ($r^2 \geq 0.8$) (Table 4, Fig. 3). The LD of these SNPs in three 1000 Genome Project populations of European-Caucasians, East Asian and South Asians were also derived and plotted (Supplementary Fig. S2). All four SNPs (rs74018601, rs10521308, rs74021303, and rs141642152) are located in introns 2, 3, and 4 of canonical *FTO* transcript (ENSTG0000471389) (Fig. 3). The triallelic SNP rs74018601 (C/G/T) is also located ~2500 bp upstream of the last exon of a non-protein coding *FTO* transcript (ENST00000570395). Regulatory histone marks H3K27Ac were detected at 1000 bp upstream of rs74018601 in HSMM, HUVEC, and K562 cells and a strong DNAse I hypersensitivity cluster was observed within this 300-bp

Table 3 Multiple linear regression of hematological parameters, nadir WBC and ANC levels, on *FTO* rs16952570 genotype, adjusted for *NUDT15* rs116855232 genotype.

	FTO rs16952570 Estimated mean difference in nadir WBC/ANC relative to TT genotype (95% CI)			All Patients ($N = 121$) *P* value	
	TT	TC	CC	TT vs TC	TT vs CC
Nadir white blood cell ($\times 10^9$/L)					
Baseline ($N = 112$)	Ref	−1.03 (−2.42, 0.36)	2.38 (−0.52, 5.27)	0.146	0.107
4 weeks ($N = 105$)	Ref	−1.94 (−3.14, −0.74)	2.79 (0.41, 5.17)	**0.002**	**0.022**
8 weeks ($N = 111$)	Ref	−0.96 (−1.96, 0.04)	2.41 (0.33, 4.50)	0.061	**0.024**
12 weeks ($N = 115$)	Ref	−0.98 (−1.87, −0.09)	3.09 (1.21, 4.97)	**0.032**	**0.002**
6 months ($N = 117$)	Ref	−0.45 (−1.25, 0.35)	2.30 (0.61, 3.98)	0.263	**0.008**
Nadir absolute neutrophil counts ($\times 10^9$/L)					
Baseline ($N = 112$)	Ref	−1.02 (−2.29, 0.24)	2.36 (−0.27, 4.99)	0.111	0.078
4 weeks ($N = 105$)	Ref	−1.82 (−2.97, −0.67)	2.40 (0.11, 4.69)	**0.002**	**0.040**
8 weeks ($N = 111$)	Ref	−0.97 (−1.93, −0.01)	2.00 (0.01, 3.99)	**0.047**	**0.049**
12 weeks ($N = 115$)	Ref	−0.88 (−1.65, −0.10)	2.72 (1.08, 4.35)	**0.027**	**0.001**
6 months ($N = 117$)	Ref	−0.41 (−1.05, 0.24)	2.23 (0.87, 3.59)	0.212	**0.002**

95% CI 95% confidence interval, *Ref* reference group

$P < 0.05$ indicated in bold

SNP-flanking region in 38 out of the 125 cell types assayed (Fig. 3). Allele-specific changes in transcription factor binding scores reveal that binding of Foxo 3/4 motifs may be increased in the presence of the T allele on the positive strand (ΔLOD: 11.9) whereas RFX5 motif is likely to be altered in the similar magnitude on the negative strand (ΔLOD: 12) (Supplementary Table S1).

The latter two SNPs rs10521308 and rs74021303 are located in closer proximity with rs16952570, at intron 3, with ~604 bp and ~508 bp distance away respectively. Although no histone marks or DNAse I hypersensitivity footprints were noted in this region, transcription factor binding analyses indicate that these two SNPs may be regulating binding affinity of transcription factors. The presence of rs10521308 A allele resulted in a reduction in binding of Homez and NRSF motifs (ΔLOD: −2.6 and −11.9, respectively), whereas the A allele of rs74021303 could potentially increase binding of BCL, GATA-associated motifs, HDAC2, HMGN3, and Lmo2-complex to the promoter region (Supplementary Table S1).

Lastly, rs141642152 situated in intron 4, is ~250 bp downstream of exon 3. Similar to the three SNPs located in intron 3, no histone or chromatin modifications were observed from the databases used. Large differences in LOD scores (>10) of commonly known transcription factors such as AP-1, NF-Y, Pbx-3, RFX5, SP-1, and TATA, however, were observed in the presence of the variant G allele; implying that these motifs are likely to bind to this region (Supplementary Table S1).

Discussion

The gene *FTO* encodes for the fat-mass and obesity associated protein and has been identified as a novel genetic marker for obesity risk, with several SNPs conferring a greater risk of overweight and higher body mass index [16, 17]. In addition, FTO acts as an alpha-ketoglutarate-dependent dioxygenase to catalyse demethylation reactions of N^6-methyladenosine (m^6A), its first known major substrate in the nucleus [18] and other cytoplasmic nucleotides substrates such as 3-methylthymidine and 3-methyluracil [18–21]. Modification of m^6A is highly regulated and demethylases such as FTO play critical roles in RNA splicing, translation, and degradation, amongst many other cellular processes, to modulate protein–RNA interactions and dynamically alter RNA nucleotide pools [21, 22].

The influence of FTO in other disorders, including autoimmune, psychiatric, and cardiovascular diseases have also been reported [13, 23, 24]. To date, two studies have evaluated the influence of *FTO* variants in leukopenia susceptibility of IBD patients receiving thiopurine treatment [11, 13]. These discrepant results warranted further clarification in a multiethnic cohort comprised of Chinese, Malays, and Indians. In this present study, we failed to observe any association between *FTO* rs79206939 and thiopurine-induced myelotoxicities due to its non-polymorphic nature in our patient population. Interestingly, rs79206939 is also absent in several 1000 Genome Project populations, namely European-Caucasians, Africans, and South Asians and low in East Asians (2.2%). Given that our multiethnic cohort is representative of both East and South Asians populations, it is indeed puzzling that rs79206939 was not detected. Evidently, its low allelic frequencies in other populations may be impractical as a biomarker for leukopenia risks, despite its strong functional effect. On the contrary, the overall MAF of intronic *FTO* variant rs16952570 was 21% in our cohort; similar to that reported in Koreans of 29.6% [13]. Due to the absence of *FTO* rs79206939 in our population and its posited linkage

Table 4 SNPs in linkage disequilibrium with *FTO* rs16952570 and effect on predicted regulatory elements.

Gene	rs#	Chr. position[a]	Strand	Allele	LD relative to rs16952570 (r^2, D')	Location within gene locus	Promoter histone marks	Enhancer histone marks	DNAse	Transcription factor motifs altered by variant allele
FTO	rs16952570	Chr16:53865730	Positive	T/C	–	Intron	–	Enhanced H3K4me1 in ESC, mesenchymal adipocyte cells, skin melanocyte and Breast myoepithelial primary cells		NF-1
	rs74018601	Chr16:53857113	Positive	C/T	1, 1	Intron	Whole blood	20 tissues[b]	10 tissues[c]	Foxo, RFX5
	rs10521308	Chr16:53865126	Negative	G/A	1, 1	Intron	–	–	–	Homez, NRSF
	rs74021303	Chr16:53865222	Positive	G/A	1, 1	Intron	–	–	–	6 altered motifs[d]
	rs141642152	Chr16:53878450	Positive	C/G	0.83, 1	Intron	–	in 6 tissues[e]	–	8 altered motifs[f]

SNPs and putative transcription factor binding motifs were identified from HaploReg v4.1 web-based tool

[a]based on hg19 genome assembly

[b]ESDR (embroyonic stem cell-derived), ESC (embryonic stem cells), LNG (lung), IPSC (induced pluripotent stem cells), FAT(adipose-derived mesenchymal stem cells), STRM (bone marrow derived cultured mesenchymal stem cells), BRST (breast), BLD (Blood), MUS (muscle), SKIN, BRN (brain), GI (gastrointestinal), THYM (thymus), HRT (heart), PANC (pancreas), PLCNT (placenta), SPLN (spleen), CRVX (cervix), VAS (vascular), BONE

[c]Primary T cells from peripheral blood, primary T cells from cord blood, primary hematopoietic stem cells, SKIN, THYM, Lymphoblastoid cells, CRVX, MUS (Skeletal Muscle Myoblasts), MUS (Skeletal Muscle Myotubes cells), BLD

[d]BCL, GATA, Gfi1b, HDAC2, HMGN3, Lmo2-complex

[e]ESDR, BRST, SKIN, BRN, THYM, BLD

[f]AP-1, NF-Y, Pbx3, RFX5, RXRA, Rad21, SP1, TATA

disequilibrium with rs16952570, we further evaluated the influence of *FTO* rs16952570 with hematological parameters and myelotoxicity risks.

We observed patients harboring *FTO* rs16952570 CC genotype were associated with increased nadir WBC counts and ANCs at 4, 8, 12 weeks, and 6 months after the initiation of thiopurine treatment, compared with wild-type TT carriers. Further subgroup analysis in noncarriers of *NUDT15* rs116855232 variant allele maintained these observations of CC carriers having higher WBC and ANC counts over their counterparts carrying either *FTO* rs16952570 TT or TC genotypes; particularly a two-to-three-fold increase in WBC and ANCs over the reference TT genotype group. No discernible difference in platelet counts between the genotype groups was observed.

Although a similar detrimental effect of a single *FTO* rs16952570 C allele was first reported by Kim et al. [13], our data seems to suggest that the homozygous CC genotype confers a protective effect against lower WBC and ANC counts compared with other genotype groups. Notwithstanding the fact that our cohort comprised only a limited number of patients with leukopenia ($N = 9$) (defined as WBC $< 3.0 \times 10^9$/L) and neutropenia ($N = 10$) (defined as ANC $< 1.5 \times 10^9$/L), none of the *FTO* rs16952570 CC carriers had WBC nor ANC levels below these threshold values. Thus we were unable to fully ascertain its relative risks on myelosuppression. Coincidentally, patients with *FTO* rs16952570 CC genotype were also wild-type CC carriers of *NUDT15* rs116855232 and noncarriers of *TPMT*2, *3A, *3B,* and **6* variants. Of these six patients who had a mean nadir WBC count of 10×10^9/L after 4 weeks of thiopurine initiation, only one subject was on concomitant corticosteroids (hydrocortisone and prednisolone). Moreover, median 6-TGN levels at first follow-up of 248 pmol/8×10^8 RBCs suggested adequate detoxification of active thiopurine metabolites which was well within the therapeutic range of 235–400 pmol/8×10^8 RBCs. Preferential shunting towards 6-MMP production was also ruled out due to an average 6-MMP/6-TGN ratio of 12.1 and median 6-MMP levels of 2127 pmol/8×10^8 RBCs (Reference threshold for hepatotoxicity 6-MMP > 5700 pmol/8×10^8 RBCs and 6-MMP/6-TGN ≥ 20 [25]). Unlike the *NUDT15* rs116855232 variant, which has been widely demonstrated as a quintessential predictor of thiopurine-induced leukopenia and neutropenia risk in Asians [26], little is known about the role of *FTO* variants in thiopurine metabolism. Our findings here point toward a potential protective effect of *FTO* rs16952570 CC genotype on hematological parameters, such that it may be acting independently from *NUDT15* rs116855232.

Thus far, FTO has not been known to play a direct role in the thiopurine metabolism. Indeed, we also observed a lack of association between *FTO* rs16952570 and steady-state 6-

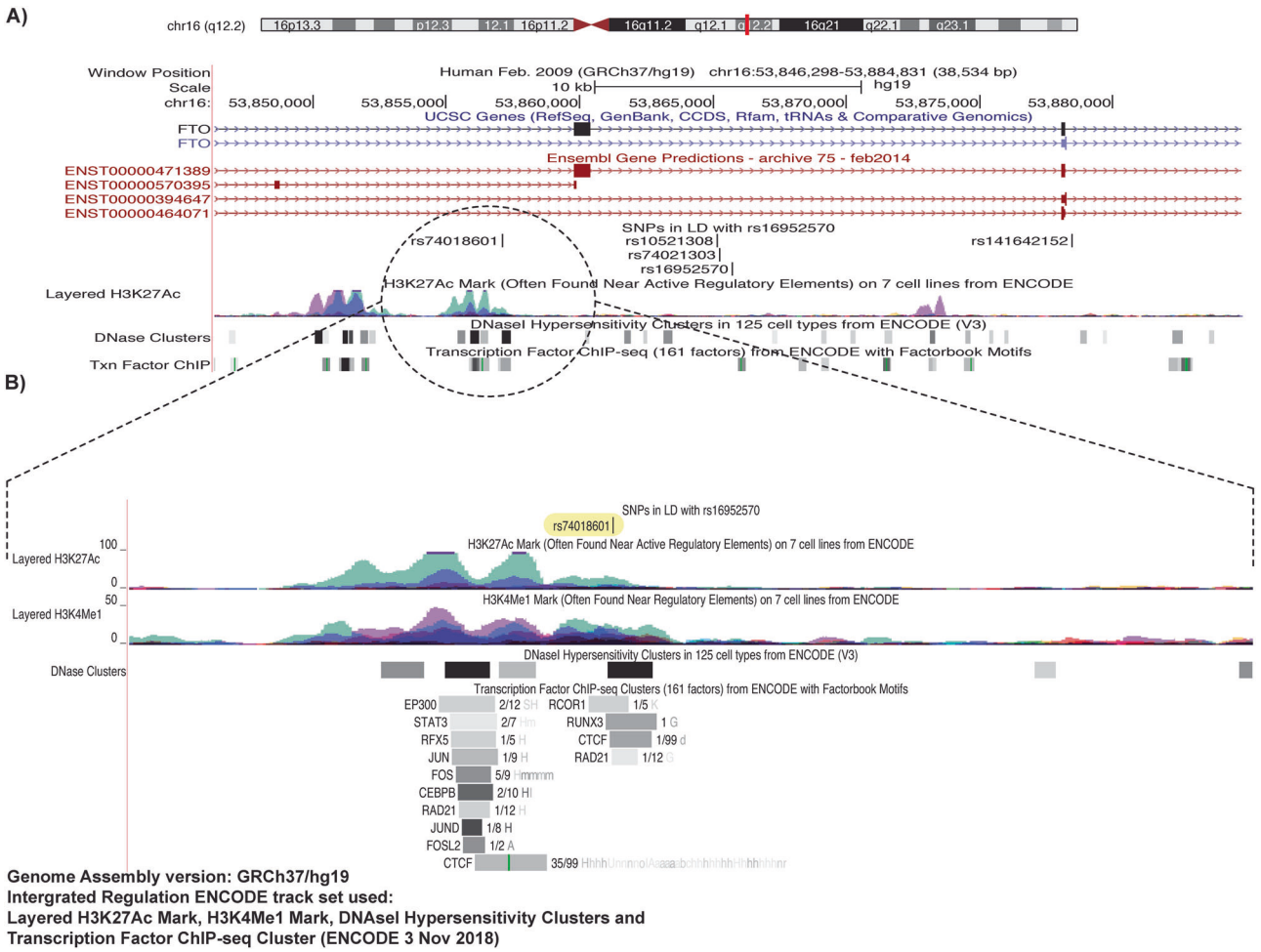

Fig. 3 Canonical *FTO* transcript and other spliced variants visualised on UCSC Genome Browser with version GRCh37/hg19. (**a**) Custom track depicting rs16952570 and its linked SNPs. (**b**) Zoomed-in region of rs74018601 depicting tracks for histone marks H3K27Ac, DNase I Hypersensitivity clusters and transcription factors binding motifs from ENCODE. High H3K27Ac signal was observed in the region of rs74018601, indicative of histone modifications. This regulatory event is supported by the strong DNAse I hypersensitivity cluster (depicted in black shaded boxes) in 38 out of 125 cell types. This region also overlaps with transcription factor binding sites, identified from ChIP-sequencing. The color intensity of the shaded boxes is proportionate to the presence of the binding site in any cell line, where black represents maximum signal strength.

TGN concentrations in our cohort ($P > 0.05$; Supplementary Fig. S3c). We next asked if rs16952570 could be linked to functionally-relevant *FTO* variants. Four intronic SNPs were found to be in strong LD with rs16952570. Of these, rs74018601 is located in close proximity to regulatory regions of the *FTO* gene where histone modifications and DNAse I hypersensitivity clusters have been identified by genome-wide CHIP-seq and DNAse-seq methods and transcription factors are postulated to have strong DNA-binding affinity in this region. Given that this regulatory region is ~2500 bp upstream of the 3′ end of a processed non-protein coding *FTO* transcript, it is plausible that the presence of rs74018601 may favor increased transcription of this non-functional transcript over that of the canonical *FTO* transcript to alter *FTO* mRNA levels. Further mechanistic studies are necessary to confirm these postulations.

Taken together, we postulate that the observed protective effect against thiopurine-induced hematological toxicities associated with rs16952570 CC, and by proxy rs74018601 TT, could be resulting from a reduction in *FTO* mRNA expression which in turn translates into a decrease in FTO-mediated demethylation of RNA substrates, including those present in the thiopurine metabolic pathway. While m^6A was recently identified as the most preferred substrate of FTO [21, 27], FTO may act to catalyse the demethylation of methyl-containing substrates such as 6-MMP and 6-methylthioinosinate amongst many others. Alternatively, FTO may also be acting to regulate the nucleotide pools for DNA and RNA synthesis, in the presence of thiopurine-mediated inhibition of de novo purine synthesis. Consequently, the precise role of FTO in the thiopurine metabolic pathway remains unclarified and deserves further

investigation. Interestingly, *Tpmt* expression was one of the four genes in the methylation pathway that was upregulated in mice gastrocnemius muscle overexpressing *Fto* gene [28].

In addition to *NUDT15* polymorphisms, *TPMT* and *ITPA* (inosine triphosphate pyrophosphatase) variants have been implicated in modulating thiopurine metabolism and toxicity in largely European-Caucasian cohorts [29–31]. Accordingly, we have previously demonstrated the absence of *TMPT*2*, *3A, and *3B* in our patient population and low minor allelic frequencies of *TPMT*3C* and *6 of <2% [7] are unlikely candidates for predicting hematological toxicities. The pharmacodynamics influence of *ITPA* 94C > A has also been investigated, albeit in limited number of IBD studies and the results have been very controversial.

Conclusion

In summary, this study demonstrates the protective effect of *FTO* variant rs16952570 CC genotype on WBC and ANC counts at 4, 8, 12 weeks, and 6 months. This effect was preserved in patients harboring only the reference *NUDT15* rs116855232 genotype. Our study suggests that the influence of *FTO* rs16952570 on thiopurine-induced leukopenia and neutropenia in IBD patients may be distinct from *NUDT15* rs116855232. While preemptive genotyping of *NUDT15* rs116855232 may be able to help identify high-risk patients of thiopurine-toxicities, it is possible that other genetic factors such as *FTO* rs16952570 may play a minor yet important role in ameliorating these adverse events. Validation of these findings in other independent cohorts, as well as functional investigation of this variant and/or its linked SNPs may provide deeper insights into the role of FTO in regulating hematological events.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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