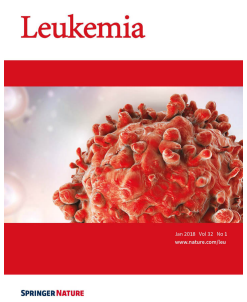

GATA2 Monoallelic Expression Underlies Reduced Penetrance in Inherited *GATA2*-mutated MDS/AML

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GATA2 Monoallelic Expression Underlies Reduced Penetrance in Inherited *GATA2*-mutated MDS/AML

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Running Title: Reduced Penetrance in *GATA2* Deficiency.

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Whilst the majority of myelodysplasia and acute myeloid leukemia (MDS/AML) cases are sporadic, rare familial predisposition syndromes have been delineated and now represent a separate disease entity in the revised World Health Organization (WHO) classification of myeloid neoplasms[1]. Germline mutations in ~14 disease genes have been uncovered thus far, with *GATA2* representing one of the key transcriptional regulators commonly mutated in inherited MDS/AML[2]. Increasing evidence suggests aberrations in *GATA2* impair its transcription and promoter activation leading to a loss-of-function, supporting a mechanism of *GATA2* haploinsufficiency[3-5]. Reduced penetrance, the observation that family members carry an identical germline mutation yet display variable clinical manifestations, is common and poses a clinical challenge in the diagnosis and management of familial leukemias, particularly when identifying 'silent' mutation carriers for genetic screening and exclusion as potential stem cell transplant donors[6, 7]. Indeed, we have noted that reduced penetrance is a feature amongst certain *GATA2*-mutated MDS/AML families[8], especially those harboring missense germline mutations such as c.1061C>T (p.Thr354Met) (Table S1) although the precise molecular explanation of such occurrence has not been investigated.

Analysis of five MDS/AML families harboring p.Thr354Met *GATA2* mutations displayed significant intra- and inter-familial variations in disease latency, phenotype and penetrance (Figure S1). These observations suggest that individuals require additional co-operating events for the development of overt malignancy within the context of a shared germline mutation. To investigate this hypothesis further, we examined an extensive five-generation pedigree[9] (Figure 1A) where two first-degree cousins (IV.1 and IV.6) developed high-risk MDS/AML with monosomy 7 while a third cousin (IV.10) presented with recurrent minor infections and significant monocytopenia [$0.1x10^9$/L] and neutropenia [$0.8x10^9$/L] in year (yr.) 1-3 which subsequently stabilized (monocyte count, neutrophils [$>1x10^9$/L]) 3 years after presentation (Figure 1B). This contrasted with the parental generation (III.1, III.5 and III.7) where mutation carriers remain symptom-free with no evidence of hematopoietic abnormality over 60 years of age.

We therefore started with targeted deep sequencing of 33 genes frequently mutated in MDS/AML to define the landscape of secondary genetic mutations across mutation carriers. Notably, while no acquired mutations were detected in asymptomatic family members, all affected cousins analyzed shared an identical somatic *ASXL1* mutation (p.Gly646TrpfsTer12) (Figure 1C). The variant allele frequency (VAF) however was lower (12%) in IV.10 and remained stable (range 12-6%) over a 6-year monitoring period. While the co-occurrence of *ASXL1* and *GATA2* mutations has been proposed as one mechanism for driving the onset and severity of disease symptoms[9-11], the low VAF of *ASXL1* mutation and stable improvement in hematopoiesis at IV.10 later follow-up suggested that a combination of *GATA2-ASXL1* mutation alone is insufficient to promote clonal expansion and leukemic transformation, as this secondary somatic hit may not represent disease progression or identify when treatment is indicated. Intriguingly, apart from the *ASXL1* mutation, no other acquired mutations were detected in the 33-myeloid genes assessed in the affected individuals. Moreover, on the basis of our observations and in agreement with previous studies[12,13] it seems that monosomy 7 in IV.1 and IV.6 is acquired following acquisition of *ASXL1* mutations, hence contributing to the malignancy but not initiating symptoms.

We next considered whether disease symptoms are modulated by endogenous levels of *GATA2.* Quantitative real-time PCR (qRT-PCR) of bone marrow material demonstrated total *GATA2* expression to be significantly lower in the symptomatic (IV.10-yr.1) compared with an asymptomatic carrier (III.7) (Figure 1D). Significantly, Sanger sequencing of cDNA template revealed striking allele-specific expression (ASE) favoring the mutant (T) allele with absence of the wildtype (WT) (C) allele expression in the symptomatic patient (IV.10) contrasting with biallelic expression in asymptomatic members (III.5 and III.7) (Figure 1E). This observation was validated by cDNA cloning of III.7 and IV.10 bone marrow samples and subsequent Sanger sequencing of individual clones (Figure S2). As this suggested that an allelic imbalance in WT:mutant *GATA2* expression ratio may account for the variable disease penetrance in this pedigree, we assessed *GATA2* expression in IV.10 over a 6-year disease period at 4 time-points (yr.1, 3, 4 and 6) demonstrating increased *GATA2* expression at later time-

78 points (yr.4 and 6) (Figure 1D) coinciding with reactivation of the WT (C) allele expression (Figure 1F) and an
79 improvement in hematological parameters, in the absence of any clinical intervention (Figure 1B).
80
81 To test whether monoalleic *GATA2* expression has an impact on the transcriptome driving the onset of disease
82 symptoms, we performed RNA-seq with a view of examining downstream biological features distinctive of
83 *GATA2* monoallelic (IV.10-yr.1 and 3) vs. biallelic (IV.10-yr.4 and III.7) groups. Unsupervised analysis revealed a
84 clear separation between *GATA2* monoallelic and biallelic samples (Figure 1G, S3 and Table S2). It was
85 noteworthy that certain canonical pathways and gene sets related to tumorigenesis (e.g. DNA replication and
86 cell cycle) were enriched in *GATA2* monoallelic vs. biallelic groups (Figure S4) potentially reflecting the clinical
87 and phenotypic switch between these two groups. We also noted a significant overexpression of genes with
88 *GATA2* co-factor *PU.1* motifs in their regulatory regions (p-value NES = 2.06) in *GATA2* biallelic vs. monoallelic
89 samples, in support of a recent finding[14] that p.Thr354Met mutants bind and interact with PU.1 more tightly
90 than WT thus leading to sequestration of PU.1 from its normal cellular functions. Consequently, the
91 transcriptional activation triggered by PU.1 will be diminished in our *GATA2* monoallelic samples.
92
93 The differences observed in these gene expression profiles prompted us to explore the molecular mechanisms
94 underlying monoallelic *GATA2* expression. We hypothesized that these allele-specific changes in *GATA2*
95 expression are driven by transient epigenetic mechanisms that include changes in DNA methylation and
96 chromatin mark deposition. A CpG single nucleotide polymorphism (CpG-SNP) (rs1806462) [C/A] located
97 within the promoter and 5'UTR of *GATA2* overlapping a CpG island offered a marker to distinguish between
98 mutant and WT alleles where this SNP creates/abolishes a CpG dinucleotide within the *GATA2* promoter
99 region (Figure 2A). More specifically, cDNA sequencing of 5'UTR allowed us to define haplotypes, where the
100 promoter SNP allele (A) resides on the germline mutant *GATA2* allele (T) (Figure 2Aii). Apart from IV.10, no
101 other family members and only 2/12 individuals from pedigrees presented in (Figure S1) were heterozygous

for this SNP (one of whom is an asymptomatic carrier). Therefore, we do not infer that this haplotype would contribute to the progression of symptoms. Instead, we used this SNP to determine whether allele-specific differences in DNA methylation could explain the silencing of WT *GATA2* allele expression observed in earlier time-points of IV.10. As illustrated in Figure 2B and S5, bisulfite sequencing of a 200bp region encompassing rs1806462 demonstrated a significant increase in promoter methylation in the WT allele of IV.10 in yr.1 and yr.3 following diagnosis, in contrast with the absence of allele-specific differences in methylation at a later time-point.

We next sought to establish whether these allele-specific changes in *GATA2* methylation and expression are accompanied by changes in chromatin structure at the promoter. H3K4me3 and H3K27me3 define poised or closed chromatin, respectively, rendering them more or less accessible for transcription factors thereby regulating gene expression[15]. The deposition of these bivalent marks was assessed in IV.10 by allele-specific chromatin immunoprecipitation (ChIP) followed by Sanger sequencing within *GATA2* promoter region encompassing the SNP rs1806462 [C/A]. While there were no apparent allele-specific differences in H3K27me3 deposition across the different time-points of IV.10, an enrichment in the deposition of H3K4me3 on the promoter of the mutant allele (A) relative to the WT allele (C) was noted in IV.10 monoallelic samples (yr.1 and 3) (Figure 2C, S6 and S7). In contrast, and consistent with the pattern observed with DNA methylation, there was no demonstrable difference in H3K4me3 deposition in the IV.10 biallelic sample (yr.4), coinciding with reactivation of the WT allele expression and an overall improvement in clinical parameters. We believe that these observations are in keeping with the notion that H3K4me3 occupancy inhibits *de novo* DNA methylation[16] which was borne out by subsequent bisulfite sequencing of H3K4me3-enriched DNA from our ChIP experiments demonstrating that DNA methylation and H3K4me3 deposition are mutually exclusive in our IV.10 samples (Figure S8).

Collectively, our findings provide a step forward in understanding the molecular mechanisms underlying reduced penetrance in *GATA2*-mutated MDS/AML pedigrees, which may be governed by the acquisition of additional co-operating mutations (e.g. *ASXL1*) combined with dynamic epigenetic reprogramming and subsequent allele-specific expression of *GATA2* mutant allele, adding another level of complexity to the (epi)genetic basis of familial MDS/AML.

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Authorship Contributions

J.F., A.F.A. and A.R-M. designed the study; A.F.A. and A.R-M. performed experiments; A.F.A., A.R-M., K.T. and J.F. analyzed the data and wrote the manuscript; K.T., H.S., C.H., T.V., I.D., M.S. and J.C. collated familial clinical information; S.I. provided patient material from tissue bank; S.B., N.L. and D.M. performed targeted deep sequencing; J.W. and A.N. carried out RNA-seq analysis; J.A.H. provided technical ChIP expertise; E.J.K., M.W.W. and C.M.N. provided familial samples; T.B. provided patient blood films; C.B., A.E., S.R.C., H.T., T.V. and I.D. assisted with data analysis and contributed to the study with fruitful discussions. All authors read, reviewed and approved the final manuscript.

Supplementary information is available at Leukemia's website.

(http://www.nature.com/leu/index.html)

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Figure Legends

Figure 1: Investigating the molecular mechanisms underlying the reduced penetrance of germline p.Thr354Met mutations observed in a *GATA2*-mutated MDS/AML family. (A) Genogram of the *GATA2*-mutated pedigree. Squares denote males and circles denote females. This 5-generation MDS/AML family presented to Barts Health hospital in London with identical germline *GATA2* mutations (p.Thr354Met; c.1061C>T) and variable clinical manifestations. Two first-degree cousins (IV.1 and IV.6) presented at 23 and 18 years of age, respectively, with high-grade MDS transforming to AML and monosomy 7. Both cousins died post allogeneic hematopoietic stem cell transplant (HSCT) due to transplant-related complications (IV.1 from graft vs. host disease (GvHD) and IV.6 from relapsed MDS/AML). Ten years later, their first cousin (IV.10) developed symptoms at 31 years including recurrent minor infections and significant leukopenia (monocytopenia [$0.1x10^9$/L] and neutropenia [$0.8x10^9$/L]) with mild macrocytosis and normal hemoglobin and platelet counts. She remains under close surveillance where her blood counts are routinely monitored. All four of her children have inherited her WT *GATA2* allele. Similarly, members (IV.7, IV.8 and IV.9) were screened for the mutation and all have a WT *GATA2* configuration. The paternal grandmother (II.2) of IV.10 as well as her paternal great-uncle (II.3) and great-grandmother (I.2) all were reported to have died of AML (ages of disease onset were 53, 24 and 53 years old respectively). Not only did *GATA2* mutations correlate with early age of disease onset in the 4^{th} generation (IV.1/23yr, IV.6/18yr and IV.10/31yr), but the parental 3^{rd} generation carriers (III.1, III.5 and III.7) remain hematologically normal and symptom-free into their mid-late 60's. No material was available from other family members. **(B)** A clinical time-line of IV.10 showing the change in clinical parameters over the course of disease presentation. Photographs of peripheral blood smears from IV.10 (yr.1,3,4 and 6) stained with May-Grünwald Giemsa staining. Magnification: 20×. **(C)** Secondary *ASXL1* mutations: variant allele frequencies of *GATA2* germline mutation and *ASXL1* acquired mutation. Samples from 3 individuals were sequenced: one asymptomatic parent (III.7), one deceased MDS/AML cousin (IV.6) and across 3 time-points (yr.1, 4 and 6) from the symptomatic patient (IV.10) reflecting disease evolution.

(D) *GATA2* global expression measured by qRT-PCR of bone marrow samples and normalized to healthy bone marrow control: downregulation in IV.10_yr.1 compared with III.7 and downregulation in IV.10_yr.1-3 *GATA2* expression compared with IV.10_yr.4-6. The average of five independent experiments is shown. Statistical significance was determined at $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***) using a *t*-test with Bonferroni correction. Error bars represent standard error of the mean (SEM). **(E)** *GATA2* monoallelic expression of the mutant allele in symptomatic (IV.10) vs. asymptomatic carriers (III.5 and III.7) as measured by cDNA sequencing of bone marrow samples. **(F)** Correlation of monoallelic *GATA2* expression with disease symptoms across the time-points studied in IV.10 with reactivation of the WT allele **"C"** expression noted 3 years after presentation, concurrent with improvements in hematological parameters. **(G)** RNA-seq analysis: principal component analysis (PCA) plot showing a good separation between *GATA2* biallelic (green) and monoallelic (blue) groups based on all transcriptomes.

Figure 2: Elucidating the molecular mechanisms driving allele-specific changes in *GATA2* expression. **(Ai)** A non-coding SNP (rs1806462 [C/A]) located within the second *GATA2* promoter region overlapping a CpG island was detected in the symptomatic (IV.10) but not in asymptomatic members (III.7). **(Aii)** Given the location of promoter 2 SNP within the 5'UTR, a haplotype between the SNP allele "A" and the germline mutant allele "T" was established, providing a means of distinguishing between mutant and WT alleles in subsequent experiments. **(Aiii)** This promoter SNP[C/A] removes a CpG methylation site in the mutant allele "A" and generates a CpG methylation site in the WT allele "C". **(B)** The proportion of methylated CpGs between mutant and WT alleles across the 3 time-points of IV.10. WT allele is significantly more methylated than mutant allele in monoallelic samples (yr.1 and yr.3) whereas no significant allele-specific differences in methylation were observed in a biallelic expressing sample (yr.6). The average of three independent experiments is shown. (**C**) Quantification of mutant and WT allele ChIP sequence peak heights across the time-points of IV.10 based on Sanger sequencing. H3K4me3 activation mark favoring the mutant

allele was enriched in monoallelic samples (yr.1 and yr3) compared with the biallelic sample (yr.4). The average of three independent experiments is shown. Statistical significance was determined at $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***) using a *t*-test with Bonferroni correction. N.S corresponds to non-significant comparisons. Error bars represent SEM.

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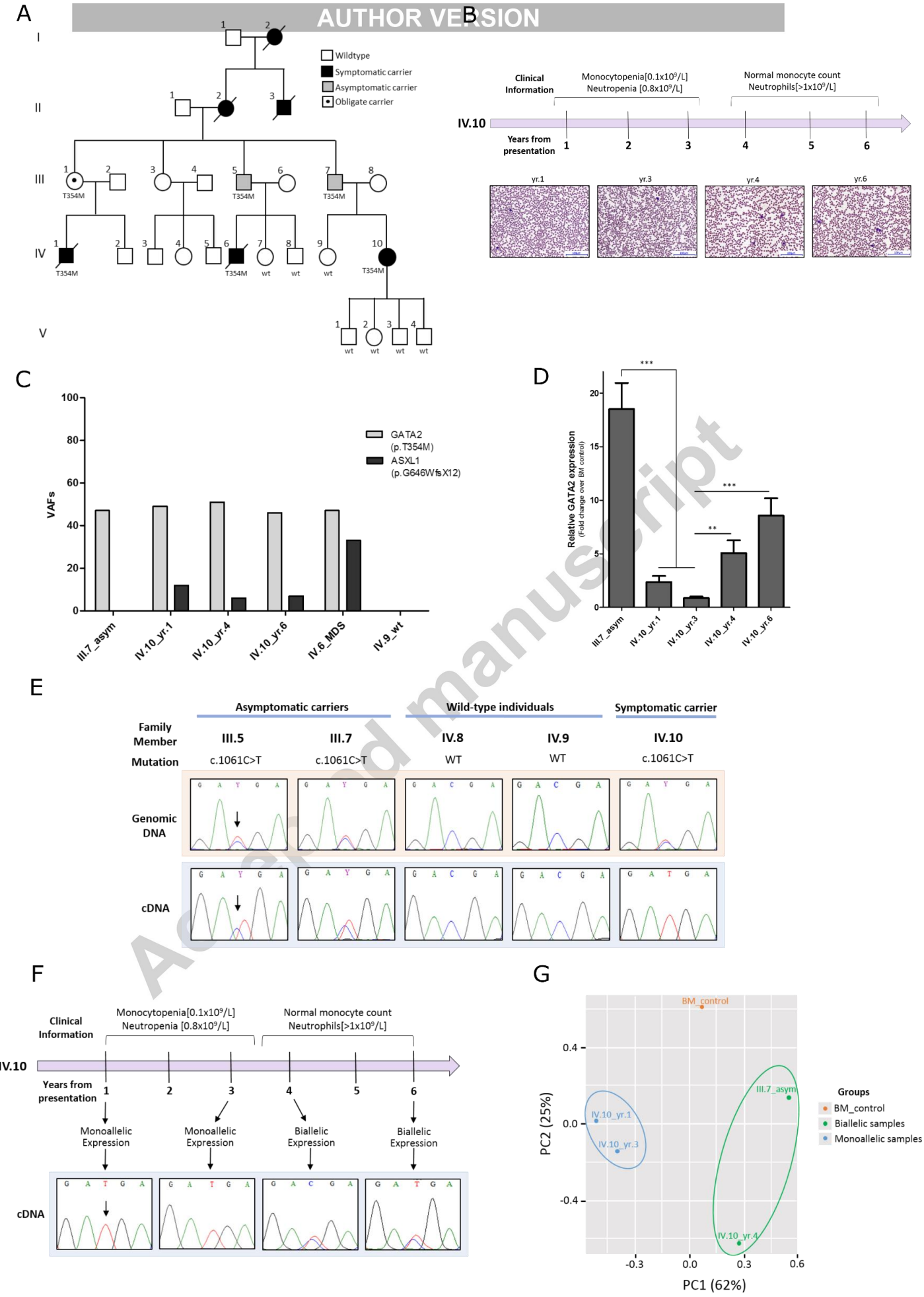
Figure 1



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