Prognostic significance of high \( \text{GFI1} \) expression in AML of normal karyotype and its association with a FLT3-ITD signature


Growth Factor Independence 1 (GFI1) is a transcriptional repressor that plays a critical role during both myeloid and lymphoid haematopoietic lineage commitment. Several studies have demonstrated the involvement of GFI1 in haematological malignancies and have suggested that low expression of \( \text{GFI1} \) is a negative indicator of disease progression for both myelodysplastic syndromes (MDS) and acute myeloid leukaemia (AML). In this study, we have stratified AML patients into those defined as having a normal karyotype (CN-AML). Unlike the overall pattern in AML, those patients with CN-AML have a poorer survival rate when \( \text{GFI1} \) expression is high. In this group, high \( \text{GFI1} \) expression is paralleled by higher FLT3 expression, and, even when the FLT3 gene is not mutated, exhibit a FLT3-ITD signature of gene expression. Knock-down of \( \text{GFI1} \) expression in the human AML Fujioka cell line led to a decrease in the level of FLT3 RNA and protein and to the down regulation of FLT3-ITD signature genes, thus linking two major prognostic indicators for AML.

The concept of disease stratification promises to provide great improvements in the diagnosis, prognosis, and treatment of cancer, but requires robust and readily measurable biomarkers in order to be feasible. In the case of haematological malignancies, much has been possible through traditional phenotypic categorisation and the identification of associated genetic mutations. More recently, genome wide approaches, both at the level of gene mutations and transcriptome analysis, have enabled refinement of the classification of haematological malignancies, but there is still a great need to identify reliable molecular differences not only as prognostic indicators but also as potential therapeutic targets.

Acute myeloid leukaemia (AML) is a malignant myeloproliferative disease of the bone marrow accounting for ~10% of all haematological disorders\(^1,2\). Currently, risk classification of AML with normal karyotype, representing 50% of the cases, is based on molecular biomarkers including mutations in the genes encoding FLT3, NPM1, NRAS, KRAS, KIT and CEBPA\(^3\). FLT3 is a tyrosine kinase receptor that is expressed in early haematopoietic cells and is essential for their proliferation and differentiation\(^4,5\). FLT3 is known to be expressed at elevated levels on the AML blast cells in over 70% of AML patients and it is among the most commonly mutated genes in AML\(^6\). The mutations are typically internal tandem duplications within the juxta-membrane domain of the receptor (FLT3-ITD), occurring in 20–30% of AML cases\(^7,8\). FLT3-ITD mutations lead to constitutive signalling and factor-independent cell survival and proliferation\(^9\), and are associated with adverse clinical outcome. The leukaemogenic effects of the FLT3-ITD mutations are reflected in a distinct gene regulation signature, which is characterised by up and down regulation of DNase I hypersensitive sites and genes encoding key haematopoietic regulators and functional proteins\(^10\).

Growth Factor Independence 1 (GFI1), a zinc-finger transcriptional repressor that plays several critical roles in haematopoietic lineage commitment and development\(^11-13\) is being increasingly associated with haematological malignancies\(^14-18\). Mutations in \( \text{GFI1} \) that lead to a dominant-negative loss of function have been reported in a number of patients with congenital neutropenia\(^16\), whilst investigation of single nucleotide polymorphisms (SNP)
and observed this to be higher in 
GFI1 high 
expression surveyed by Hönes and EVI1 
els are associated with differences in their expression in CN-AML patients. Comparing 
RNA. Strikingly, this analysis revealed that in 
GFI1 high 
to the presence or absence of FLT3-ITD. Surprisingly, 
of 
for the whole cohort with those for patients with CN-AML, distinguishing patients with low or high expression 
(From the CN-AML group we selected the bottom 30% of the whole GFI1 expression range as low expressers 
classified in the M3 FAB subgroup, as those patients are treated differently. The distribution of 
cohort reported by Verhaak 
marker of inferior outcome in AML/MDS patients. The authors of the latter study considered all patients in the 
expression of genes making up the FLT3-ITD signature was largely elevated compared to AML with low 
expression corresponds with a FLT3-ITD gene expression signature in FLT3-WT 
levels and FLT3-ITD signature genes in another independent CN-AML cohort from a study performed on 251 
seen in patients carrying FLT3-ITD mutations (Fig. 1F). Similar to the sub-cohort of CN-AML patients carrying 
expression signature incorporates 134 genes, which were further validated against the dataset from Verhaak et al. 
expression signature has a worse clinical outcome. The analysis of AML 
profile array data presented by Hönes et al. focused on the importance of low GFI1 expression as a 
marker of inferior outcome in AML/MDS patients. The authors of the latter study considered all patients in the 
whole cohort with those for patients with CN-AML, distinguishing patients with low or high expression of GFI1. Our CN-AML category (n = 178) excluded patients displaying abnormal or unknown karyotype or 
classified in the M3 FAB subgroup, as those patients are treated differently. The distribution of 
expression amongst all AML patients is not significantly different to that seen in the CN-AML subgroup (Fig. 1A). 
From the CN-AML group we selected the bottom 30% of the whole GFI1 expression range as low expressers (GFI1low, n = 27) and the top 30% as high expressers (GFI1high, n = 29), which closely encompasses the range of 
expression surveyed by Hönes et al. (Fig. 1B). In this cohort, GFI1low patients demonstrated a significantly 
inferior outcome compared to the GFI1high patients (p = 0.027) (Fig. 1C). 
Samples, these being found in 59% (p = 0.0001) and 52% (p = 0.079) of patients, when compared to the 
GFI1high subgroup in which they were found in 11.2% and 29% of the patients, respectively (Table 1). No statistically 
significant difference was observed for IDH1, IDH2, NRAS, KRAS, CEBPA and EVI1 expression. Since the FLT3-ITD mutation 
generally associated with higher FLT3 expression, we next analysed the abundance of FLT3 transcript and 
observed this to be higher in GFI1high samples (p = 0.0002, Fig. 1D). No differences between GFI1low and 
GFI1high groups were seen with respect to FAB classification, age, or sex (Table 1). Considering that FLT3-ITD is the poorest diagnostic marker in CN-AML, we speculated that this could account for the inferior survival 
observed in the GFI1high patient subgroup. We therefore sub-classified the GFI1high CN-AML patients according to the 
presence or absence of FLT3-ITD. Surprisingly, GFI1high FLT3-WT patients (n = 10) still displayed lower overall 
survival (p = 0.0046) (Fig. 1E) and higher FLT3 expression (p = 0.000033) that is comparable to the levels 
seen in patients carrying FLT3-ITD mutations (Fig. 1F). Similar to the sub-cohort of CN-AML patients carrying 
FLT3-ITD mutations, no differences in the FAB classification, age or sex were observed.

High GFI1 expression corresponds with a FLT3-ITD gene expression signature in FLT3-WT 
CN-AML. It was recently reported that FLT3-ITD is associated with a distinct gene expression profile, the 
specific signature being defined as those genes expressed at least one log2-fold higher in FLT3-ITD AML compared to 
CD34+ peripheral blood stem cells (PBSC), excluding those genes linked to mature myeloid differentiation that are expressed in CD14+ bone marrow cells at a level twice as high in PBSC. This FLT3-ITD AML gene expression 
signature incorporates 134 genes, which were further validated against the dataset from Verhaak et al. 
We therefore investigated whether high GFI1 expression in the CN-AML FLT3-WT leukaemias corresponds to 
higher abundance of the FLT3-ITD molecular signature genes. Using the AML gene expression dataset of Verhaak et al., 
we determined the expression of the FLT3-ITD signature genes in FLT3-WT CN-AML patients and stratified these according to the level of GFI1 RNA. Strikingly, this analysis revealed that in GFI1high AML 
the expression of genes making up the FLT3-ITD signature was largely elevated compared to AML with low 
GFI1 expression (Fig. 2A). Further to validate these findings, we confirmed the association between high GFI1 
levels and FLT3-ITD signature genes in another independent CN-AML cohort from a study performed on 251 
CN-AML samples, reported by Kohlmann et al. (Fig. 1). 

Known regulators of FLT3 are over expressed in GFI1high CN-AML. Recent reports have demonstrated a mechanism for the Gfi1-dependent acceleration of K-Ras driven myeloproliferative disorders in mice 
through the over expression of HoxA9 and other AML-related genes, eventually leading to the development of 
AML. This study also revealed elevated expression of the HoxA9 partner proteins Meis1 and Pbx1, and of their 
known target genes Myb and Spi1/PU.1. Considering that these transcription factors, together with CEBPα, are among the main regulators of FLT3 activity in AML, we next sought to determine whether high GFI1 
levels are associated with differences in their expression in CN-AML patients. Comparing GFI1high versus GFI1low 
FLT3-WT CN-AMLs, we found higher expression in the former of MYB (p = 0.0029), CEBPA (p = 0.0043), 
HoxA9 (p = 0.0025), Hoxa7 (p = 0.0033), Meis1 (p = 0.000043) and Pbx3 (p = 0.0001), while lower abundance 
was observed for Pbx1 (p = 0.011) and Pbx2 (p = 0.00004). No differences were observed in the levels of 
Meis2 and PU.1 (Fig. 2B). The latter is unsurprising as we have previously shown that PU.1 is directly involved in 
regulating FLT3 in haematopoietic progenitor cells but not in leukaemic cells. While the lower levels of
PBX1 and PBX2 observed in high GFI1 patients are in agreement with the findings presented by Horman et al.\textsuperscript{15}, higher expression of HOXA9 and its partner MEIS1 are in contrast with their observations. This difference could be explained by the fact that our analysis was performed in CN-AML patients only, therefore excluding patients harbouring chromosomal translocations, such as those involving MLL, in which HOXA9 expression is often deregulated\textsuperscript{26, 27}. To address this, we performed the same analysis using patient samples from the whole AML cohort, classified into GFI1 low and high expressers. In line with previous observations, this analysis revealed lower expression levels of HOXA9, HOXA7 and MEIS1 (Fig. 2B).

Reduction of GFI1 in AML cell lines leads to decreased expression of FLT3-ITD signature genes. In order to assess the significance of our observed correlation between GFI1 expression and the level of FLT3-ITD signature genes in FLT3-WT CN-AML, we next investigated whether manipulation of GFI1 expression in a human AML cell line would lead to corresponding changes in the expression of the FLT3-ITD signature genes.
genes. First, we measured the relative expression of GFI1 RNA in human AML cell lines characterised by the expression of wild type FLT3 receptor, including KG1a, Kasumi-1, THP1, and Fujioka. Quantitative RT-PCR revealed that GFI1 is least expressed in KG1a and most highly expressed in Fujioka cells (Fig. 3A). To determine if higher expression of the FLT3-ITD signature genes also correlates with GFI1 level in these cell lines, we then tested the transcript abundance of the FLT3-ITD signature genes NFIX, FAM92A1, BIK, IGFBP2, LYRM1, IRX3, PBX3, PRTN3, AK2, CTSG, FLT3, ELANE and AZU1. Interestingly, this analysis demonstrated higher expression of most of these genes, with the exception of FAM92A1, in those cell lines with the highest expression of GFI1 (Fig. 3B).

Next, we performed siRNA-mediated knockdown of GFI1 in the Fujioka line to determine if the highlighted FLT3-ITD signature genes were dependent on the high level of GFI1 expression in these cells. Fujioka cells were electroporated with siRNA targeting either GFI1 or a scrambled negative control. Cells were harvested 24 hours post transfection to assess GFI1 expression levels by quantitative RT-PCR, revealing a highly significant down regulation of both GFI1 RNA (p < 0.001) (Fig. 4A). Cells were additionally collected at 48 hours for preparation of protein extracts and assessment of GFI1 protein levels by immunoblotting (Fig. 4B). Cell counts performed every 24 hours post transfection revealed that GFI1 down regulation had no significant effect on cell growth (Fig. 4C). Fujioka cells were maintained in culture for 96 hours post transfection at which point the expression of several surface markers associated with myeloid differentiation was tested by immunofluorescence / flow cytometry. CD34, CD38, CD11b, CD14, and CD56 showed no measurable differences (data not shown), whereas FLT3 surface expression was significantly decreased (Fig. 4D). Importantly, analysis of the expression of FLT3-ITD signature genes demonstrated a reduction in the abundance of several of these, the most notable being NFIX, BIK, IGFBP2, LYRM1, PRTN3, AK2, CTSG, FLT3, ELANE and AZU1 (Fig. 4E).

Discussion

In this study, we show that higher levels of GFI1 expression can be used to predict unfavourable outcome in AML patients with normal cytogenetics, and thereby provide a possible stratification for therapy choices. Additionally, we found that higher GFI1 levels are also associated with higher FLT3 expression and elevated levels of FLT3-ITD signature genes even in patients with non-mutated FLT3.

Compared to earlier findings by other groups, the way in which we have distinguished between CN-AML and AML with abnormal cytogenetics illustrates how stratification based on a single factor such as GFI1 expression can lead to quite different conclusions. Hence, Hönes et al. sub-fractioned AML patients purely according to GFI1 expression revealing that overall inferior survival associated with low GFI1 levels. In the present study we first classified AML based on karyotype, dividing them into abnormal karyotype only and CN-AML only and then sub-fractioning these two groups into low and high GFI1 expressers. In the first group we observed that low GFI1 expressers were indeed associated with inferior outcome, in agreement with findings reported previously. Strikingly, when the same criterion was applied to the CN-AML group, we observed that high GFI1 expressers were instead characterized by worse overall survival.

<table>
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<td>Median age</td>
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Table 1. Genetic and phenotypic characteristics of the GFI1low and GFI1high leukemias in the CN-AML category. The table shows data relating to the 56 patient AMLs included in the CN-AML sub group, including the occurrence of common specific leukaemia-associated mutations, the FAB categorisation, and the sex and age of the patients.
We also considered how the status of FLT3 expression or mutation might correlate with the stratification. In their study of unfractionated AML Hönes et al. suggested that FLT3-ITD associates with high GFI1 levels and favourable outcome measures, which is a surprising finding given that FLT3-ITD is the most significant prognostic factor in AML and is associated with unfavourable outcome. However, as we have discussed, patients from this cohort were not subcategorized based on karyotype, raising the possibility that GFI1 impacts on leukaemia in different ways according to the driver mutations. When we looked at the CN-AMLs we also observed an association of FLT3-ITD with the GFI1 high group, suggesting that FLT3-ITD was responsible for the inferior outcome observed in those patients. To test this, we looked instead at only those CN-AML that had a wild type FLT3 status, again classifying into GFI1low versus GFI1high. This analysis showed that the overall survival of the GFI1 high AML was much worse, and moreover, these AML exhibited a significantly higher expression of FLT3.

Several studies, mainly in mouse models, have addressed the role of GFI1 in haematological malignancies. In line with an association between high GFI1 expression and poor outcomes it has been reported that Gfi1 cooperates with Pim-1 and Myc in the genesis of T-cell lymphoma. High levels of Gfi1 expression were also found to be important in accelerating T-cell proliferation and preventing induced cell death in Jurkat T-cells.

Figure 2. Analysis of FLT3-ITD signature genes and known FLT3 regulators in CN-AML (A) Histogram representing gene expression ratio of FLT3-ITD molecular signature in GFI1high versus GFI1low FLT3-WT CN-AML patient samples from the Verhaak dataset. (B) Analysis of expression levels of known regulators of FLT3 activity in leukaemia, comparing GFI1low versus GFI1high FLT3-WT CN-AML samples (blue bars) or WHOLE AML samples (red bars). Statistical significance: ***<0.001, **<0.01, *<0.05
Furthermore, Khandanpour et al.\textsuperscript{21} reported a requirement for Gfi1 in the establishment and progression of murine B-cell lymphoma and T-cell acute lymphoblastic leukaemia driven by various genetic lesions, the ablation of Gfi1 leading to significant tumour regression and increased host survival in a p53-dependent manner\textsuperscript{30}. In apparent contrast to these latter findings, the role of Gfi1 in the regulation of HoxA9, Meis1 and Pbx1 in murine myeloid cells appears to prevent predisposition to haematological malignancies, its loss-of-function decreasing the latency of KRas-driven MDS\textsuperscript{15}. These observations were further supported by the identification of a SNP in the \textit{GFI1} gene that generates a variant protein (GFI1 [\textit{36N}]), which experimentally accelerated KRas-driven myeloproliferative disorders by inducing epigenetic changes at the HoxA9 locus\textsuperscript{21}. Similarly, studies using a humanized \textit{GFI1} knockdown mouse model showed that lower \textit{GFI1} levels accelerate the progression of MLL-AF9- and NUP98-HoxD13-driven AML.

These contrasting observations from different model systems clearly imply that the functional consequences of GFI1 activity can be quite different and context dependent, and there is a need to investigate more broadly what mechanisms operate. For example, one could speculate that in AML involving translocations in major chromatin remodelers, such as in MLL-AF4 gene fusions, high levels of GFI1 might antagonise runaway activation while the converse would fail to compensate the latter, resulting in additionally impaired differentiation and thus contributing towards more aggressive leukaemogenesis. In CN-AML however, GFI1 might play a more physiological role with higher expression corresponding to repression of alternative lineages, thus favouring differentiation and thus more manageable AML.

To gain an initial insight into the influence of high GFI1 expression in FLT3-WT CN-AML we sought to determine if there is any correspondence with the signature of gene expression that characterises FLT3-ITD AML. Interestingly, this analysis showed that, despite those samples having wild type FLT3, the molecular signature of FLT3-ITD was prominent in GFI1\textit{high} cells. More importantly, in the attempt to validate the relevance of these findings, we observed that a number of the components of the FLT3-ITD molecular signature (\textit{IGFBP2, PRTN3, AK2, CTSG, ELANE, and AZU1}) were down regulated after siRNA-mediated silencing of GFI1 in Fujioka human AML cell line. This suggests that one of the main mechanisms of GFI1 leading to inferior outcome might directly or indirectly act through these genes.

Taken together, our results call for a more elaborate stratification of AML patients to ensure proper diagnosis and effective treatment and demonstrate that high GFI1 expression is a reliable and powerful prognostic indicator for CN-AML. Our findings also suggest that it will be fruitful to investigate in detail how GFI1 is linked to driver mutations, including those involving FLT3.

**Materials and Methods**

**Patient profiling arrays.** Log\textsubscript{2} transformed, MASS normalised microarray expression data from Verhaak et al.\textsuperscript{19} and Kohlmann et al.\textsuperscript{20} were retrieved from GEO accession number GSE6891 and GSE15434, respectively. CN-AML patients reported in those cohorts were ranked according to their GFI1 expression into GFI1\textit{high} (top 30\% of expression range) and GFI1\textit{low} (bottom 30\%). Gene expression fold changes were expressed as log\textsubscript{2} ratios of GFI\textit{high}/GFI\textit{low} gene expression levels. Up- and down-regulated genes were identified as those displaying log\textsubscript{2} fold change $\geq 1$ and $p < 0.05$. For data from Cauchy et al.\textsuperscript{10}, and gene expression levels and fold changes from Verhaak et al.\textsuperscript{19} and Kohlmann et al.\textsuperscript{20} were retrieved for the previously published 134-gene FLT3-ITD signature.  

**Figure 3.** Expression analysis of a set of FLT3-ITD molecular signature genes in human AML cell lines (A) Scatter plot showing the relative abundance of GFI1 transcript in KG1A, Kasumi-1, THP1 and Fujioka human AML cell lines. (B) Histogram depicting the relative transcript abundance of FLT3-ITD signature component in the Fujioka cell line.
AML cell lines. KG1A, Kasumi-1, THP1, and Fujioka human AML cell lines were grown in RPMI 1640 medium supplemented with 10% foetal bovine serum, 50 μg/ml penicillin, 50 μg/ml streptomycin, and 2mM L-Glutamine. Cells were maintained at $0.5 \times 10^6$ cells/ml and were washed with phosphate buffered saline solution between passages.

Transfections, cell viability assays, and flow cytometry analysis. In total, $5 \times 10^6$ Fujioka cells were electroporated with 300 nM of either GFI1 siRNA (s199938, Ambion – Life Technologies) or scrambled control siRNA (4390843 Silencer Select Negative Control #1) using the BIORAD Gene Pulser XCell (BIORAD, Hercules, California, US). Cell proliferation assays were performed in triplicate with Fujioka cells transfected with GFI1 siRNA and negative control. Cells were counted every 24 hours for 4 consecutive days and growth curves were obtained by quantifying the number of viable cells. Flow cytometry analysis was performed on transfected Fujioka cells stained using antibodies against CD34, CD38, CD11b, CD14, CD56, and FLT3. All the antibodies were purchased from eBioscience.
Quantitative PCR and Western Blot. RNA extraction was performed 24 hours post siRNA transfection using RNeasy Mini kit (Qiagen) and first-strand cDNA synthesis was performed using standard protocols. Quantitative RT-PCR analysis of GFI1, FLT3, GAPDH, and the components of the FLT3-ITD molecular signature was performed using pre-designed Taqman gene expression assays (Applied Biosystems). Total proteins obtained from Fujioka mice transfected with either GFI1 siRNA or scrambled control were used for Western Blot analysis. Antibodies were as follows: anti-GFI1 mouse monoclonal (1:500, Santa Cruz Biotechnology) and anti-GAPDH mouse monoclonal (1:10000 dilution, Abcam).

Data availability. All patient data described were obtained from publically available databases.

References

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Author Contributions
G.V. conceived and designed the research, analysed the data, performed the in vitro experiments, and wrote the manuscript; D.S.W., D.E.G. and D.B. carried out the genetic manipulation studies; C.W. and P.C. performed bioinformatics analysis; D.J.L.C. and P.N.C. provided reagents/materials and useful discussions; P.G. and J.F. supervised the research and wrote the manuscript. All authors read and approved the final manuscript.

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