Gene expression profiling identifies FLT3 mutation-like cases in wild-type FLT3 acute myeloid leukemia

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Abstract

Background

FLT3 mutation is present in 25–30% of all acute myeloid leukemias (AML), and it is associated with adverse outcome. FLT3 inhibitors have shown improved survival results in AML both as upfront treatment and in relapsed/refractory disease. Curiously, a variable proportion of wild-type FLT3 patients also responded to these drugs.

Methods

We analyzed 6 different transcriptomic datasets of AML cases. Differential expression between mutated and wild-type FLT3 AMLs was performed with the Wilcoxon-rank sum test. Hierarchical clustering was used to identify FLT3-mutation like AMLs. Finally, enrichment in recurrent mutations was performed with the Fisher’s test.

Results

A FLT3 mutation-like gene expression pattern was identified among wild-type FLT3 AMLs. This pattern was highly enriched in NPM1 and DNMT3A mutants, and particularly in combined NPM1/DNMT3A mutants.

Conclusions

We identified a FLT3 mutation-like gene expression pattern in AML which was highly enriched in NPM1 and DNMT3A mutations. Future analysis about the predictive role of this biomarker among wild-type FLT3 patients treated with FLT3 inhibitors is envisaged.
1. Introduction

FMS-like tyrosine kinase-3 (FLT3) is a receptor tyrosine kinase commonly mutated in acute myeloid leukemia (AML) [1]. FLT3 internal tandem duplication (ITD) is the most common mutation, affecting 25% of de novo AML cases [2], and additional tyrosine kinase mutations (FLT3-TKD) in codons D385 and I386 have been observed in 5–10% of cases [3]. Both types of mutations induce constitutive activation of FLT3 kinase activity, inducing pro-survival and proliferative signals [4]. FLT3-ITD mutation confers a poor outcome in de novo and relapsed AML, and it is currently suggested to include these patients in clinical trials [5, 6].

A number of promising FLT3 inhibitors are under development for AML treatment. The addition of the multi-kinase inhibitor midostaurin [7] to standard chemotherapy in newly diagnosed FLT3-mutated (FLT3mut) AML significantly improved event-free survival and overall survival in all FLT3 mutation subtypes [8]. Several novel FLT3 inhibitors with increased specificity for FLT3 are under study, such as gilteritinib, crenolanib and quizartinib [3], with encouraging results from phase 2 and 3 trials in the relapsed & refractory AML setting [9–11]. Curiously, overall responses among wild-type FLT3 AML patients were reported for both gilteritinib (12% of composite overall responses) and quizartinib (30–36% of complete remissions) [9, 11]. It has been hypothesized that this effect could be due to nonspecific inhibition of other kinases, cryptic FLT3 activation by other mutations or hyperactivation of FLT3 by its ligand [9]. Not surprisingly, 2 clinical trials are currently underway in order to study the activity of FLT3 inhibitors in wild-type FLT3 patients [12, 13], but there is still no biomarker to predict which patients will respond to these drugs. Therefore, an interesting point would be to identify gene expression changes associated with FLT3 mutation in order to search for wild-type FLT3 cases that resemble FLT3mut AMLs at the transcriptomic level.

In this study we analyzed the transcriptomic pattern of 6 different AML cohorts, which enabled the identification of a FLT3 mutation-like gene expression pattern highly enriched in NPM1 and DNMT3A mutants. Our results indicate common deregulated pathways among these leukemias, opening the way to study the role of this biomarker in order to predict responses to FLT3 inhibitors in wild-type FLT3 AML.

2. Materials and methods

We analyzed five AML gene expression datasets available in the Gene Expression Omnibus (GEO): GSE14468 (461 cases of de novo cytogenetically normal AML), GSE10358 (188 cases of de novo AML), GSE61804 (279 de novo AML cases), GSE17855 (237 pediatric AML cases) and GSE15434 (251 cases of normal karyotype AML). In the case of GSE14468 and GSE10358, both FLT3-ITD and D385 mutation status were reported, whereas in the remaining datasets only the FLT3-ITD status was informed. All datasets used the same type of gene expression arrays: Affymetrix Human Genome U133 Plus 2.0 Array. Signal intensities were rank-normalized, and differential expression between FLT3mut (either ITD or TKD) and wild-type samples was performed in the largest cohort (GSE14468) using the two-sided Wilcoxon-rank sum test. P-values were adjusted for multiple testing using the Bonferroni method. Hierarchical clustering and heatmap plots were created using the heatmaps3 package with default parameters [14], and enrichment analysis was performed with the two-sided exact Fisher’s test. Gene ontology analysis was performed on WebGestalt with default parameters (FDR significance threshold, 0.05) [15].

RNaseq and mutation data from 246 AML patients treated with intensive chemotherapy was retrieved from Bamopoulos et al. (GEO identification GSE146173) [16]. Raw count data was transformed to normalized transcripts per million using the fpkm function implemented in the DESEQ2 package [17]. Afterwards, rank-transformation was applied. 595 genes
matching genes with the FLT3-like pattern were selected, followed by standard hierarchical clusterization. Differential mutation between the FLT3-like and no-FLT3-like group was performed with Fisher’s test.

All computations except gene ontology analysis were performed in R. All data used for this analysis is readily accessible from public repositories.

3. Results

FLT3-like gene expression pattern

We chose the largest database (GSE14468) as the discovery set, and we identified 911 probes mapping to 649 different genes which were differentially expressed between FLT3\textsuperscript{mut} (ITD and/or TKD) and wild-type FLT3 samples (Bonferroni p-value < 0.05, S1 Table). None of the probes mapped to FLT3. These probes were significantly enriched in 52 gene ontology terms, among which 17 terms were associated with hemopoiesis & immunology, and 3 terms were specifically linked to myeloid differentiation, namely myeloid cell homeostasis, q-value 5.01 x 10\textsuperscript{-3}; myeloid cell differentiation, q-value 4.86 x 10\textsuperscript{-3}; and negative regulation of myeloid cell differentiation, q-value 0.03 (S2 Table). Hierarchical clusterization revealed two broad groups. A cluster of 46.20% of patients was substantially enriched in FLT3 mutants (p-value < 1 x 10\textsuperscript{-4}), since it contained 81.67% of all FLT3-ITD cases, 61.36% of all FLT3-TKD cases and 83.33 of composed mutants (FLT3-ITD plus FLT3-TKD; Fig 1A). 28.52% of wild-type FLT3 cases were also clustered within this group. A similar finding was identified in the independent GSE10358 database, where a cluster of 51.06% of patients contained 81.08% of all FLT3-ITD mutants and 70.00% of all FLT3-TKD mutants (p-value < 1 x 10\textsuperscript{-4}). Furthermore, 42.15% of wild-type FLT3 cases were also clustered within this group (Fig 1B).

The same clustering was repeated in 3 different datasets that only reported FLT3-ITD mutation status. In GSE61804 a cluster comprising 58.06% of all patients harbored 78% of all FLT3-ITD cases (p-value 1.50 x 10\textsuperscript{-3}), and 53.71% of all non FLT3-ITD cases were grouped within this cluster (Fig 1C). In GSE17855, a cluster of 47.67% of patients contained 81.25% of all FLT3-ITD patients (p-value < 1 x 10\textsuperscript{-4}), but it also included 39.15% of all non FLT3-ITD cases (Fig 1D). Finally, in GSE15434, a cluster of 64.14% of patients was enriched in FLT3-ITD cases, comprising 86.67% of the whole cohort (p-value < 1 x 10\textsuperscript{-4}); and additionally 49.11% of all non FLT3-ITD cases co-clustered within this group (Fig 1E). We replicated the FLT3-like pattern in GSE146173 using RNAseq data, identifying 28.49% of wild-type FLT3 AMLs as FLT3-like (Fig 2).

Mutation landscape of FLT3-like leukemias

Four of the microarray databases provided data about driver mutations in a few genes (S3 Table). The most significant finding was the enrichment of FLT3-like AMLs in NPM1 mutants, which was observed in the 4 cohorts. This proportion was variable, ranging from 80.72% in karyotype normal AML (GSE15434) to just 12.16% in pediatric AML (GSE17855), indicating that the existence of the FLT3-like pattern is not explained by co-occurring NPM1 mutations. Additionally, a relative enrichment in IDH1 mutants within FLT3-like leukemias was detected in the discovery set GSE14468 (p-value 2.07 x 10\textsuperscript{-4}). Furthermore, depletion of CEBPA mutants in FLT3-like AML was a common phenomenon in all 3 cohorts that reported mutations in this gene, and a reduced frequency of KIT mutations was also discovered in the pediatric AML dataset (GSE17855).

Using the most recent RNAseq data published by Bamopoulos et al. [16] we observed that FLT3-like leukemias were highly enriched in concurrent NPM1 and DNMT3A mutants, whereas 19.60% of FLT3-like AMLs were NPM1 and DNMT3A wild-type. Additionally, there
was a tendency for an enrichment in IDH1 mutations (p-value 0.09). On the contrary, FLT3-like AMLs were depleted in ASXL1, CEBPA, RUNX1, TP53, SF3B1 and U2AF1 mutations (Table 1).

4. Discussion
In this report we describe the existence of a FLT3 mutation-like gene expression pattern in wild-type FLT3 AMLs. The FLT3-mutation like pattern was enriched in NPM1 and DNMT3A mutant leukemias. This is probably related to the significant co-occurrence of FLT3 mutations with those of NPM1 and DNMT3A [18], which probably leads to partially overlapping transcriptomic fingerprints. Not surprisingly, the FLT3-like pattern contains numerous HOX genes, which have been previously vinculated with the NPM1-transcriptional pattern [19]. Nevertheless, the heterogeneous frequency of NPM1 mutations among FLT3-like leukemias,
and particularly its low frequency in pediatric AML, along with the reproducibility of the \textit{FLT3}-like pattern in all cohorts, indicate that co-occurring \textit{NPM1} mutations are insufficient to explain the existence of the \textit{FLT3}-like pattern.

Our results suggest that a group of AMLs with \textit{FLT3} plus \textit{NPM1} and/or \textit{DNMT3A} mutations share a similar transcriptomic background. Noteworthy, responses to the \textit{FLT3} inhibitor gilteritinib among relapsed \textit{FLT3} mut AMLs are superior in those patients with mutations in \textit{NPM1} or \textit{DNMT3A}, and particularly in those with both genes mutated [20], and similar findings were

Table 1. Differential distribution of recurrently mutated genes in wild-type \textit{FLT3} AML patients according to the presence or absence of the \textit{FLT3}-like pattern (GSE146173).

| Gene ID | \( p \)-value | \textit{FLT3}-like (%) | No \textit{FLT3}-like (%) |
|--------|---------------|------------------------|-------------------------|
| \textit{ASXL1} | 5.29E-04 | 1.96 | 21.87 |
| \textit{CEBPA} | 2.66E-15 | 0 | 5.47 |
| \textit{DNMT3A} | 1.61E-03 | 41.17 | 17.19 |
| \textit{EZH2} | 1 | 1.96 | 1.56 |
| \textit{IDH1} | 0.09 | 15.69 | 7.03 |
| \textit{IDH2} | 0.81 | 13.73 | 12.5 |
| \textit{NPM1} | 3.40E-24 | 74.51 | 2.34 |
| \textit{RUNX1} | 2.30E-05 | 1.96 | 28.12 |
| \textit{TET2} | 0.29 | 23.53 | 16.41 |
| \textit{TP53} | 0.02 | 1.96 | 14.06 |
| \textit{SRSF2} | 0.62 | 9.8 | 13.28 |
| \textit{U2AF1} | 7.95E-14 | 0 | 7.03 |
| \textit{SF3B1} | 8.10E-18 | 0 | 3.12 |
| \textit{Concurrent NPM1 & DNMT3A} | 7.11E-05 | 19.61 | 1.56 |

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reported with crenolanib [21]. Furthermore, FLT3-ITD leukemias with mutations in NPM1 or DNMT3A exhibit a different drug response mechanism to the FLT3 inhibitor quizartinib, where the cell differentiation effect predominates over the cytotoxic mechanism [22].

The use of FLT3 inhibitors in wild-type FLT3 AML has been tested in some trials. For example, the FLT3 inhibitor midostaurin evidenced blast reduction responses in 53% of relapsed & refractory AML cases [23]. Similarly, responses to novel FLT3 inhibitors in a variable proportion of wild-type FLT3 cases have been observed in phase I & II trials [24–26]. This has motivated the development of new studies to specifically test the possible benefit of adding these drugs in the upfront treatment for AML [27, 28]. As it is expected that only a subgroup of patients might benefit from FLT3 inhibitors, it is imperative to develop and test new predictive biomarkers of response. Therefore, it becomes necessary to test the predictive value of the FLT3-like pattern in these clinical trials. Additionally, the enrichment of FLT3-like leukemias in IDH1 mutations (which are correlated with NPM1 mutation [29]) could set the basis for the development of new clinical trials testing the combination of different check-point inhibitors in AML [30].

This study has some limitations. Firstly, a variable proportion of FLT3 mutation-like samples were clustered near FLT3 mutants across the different cohorts, which probably reflects substantial heterogeneity between them. One of the possible explanations for this could be a differential distribution of NPM1 and DNMT3A mutants, since these are drivers of cytogenetically-normal AMLs (such as in the case of GSE15434) [31]. Finally, some of the datasets had only FLT3-ITD annotation, and a minority of the FLT3-like cases might indeed have FLT3-TKD mutations.

5. Conclusions
Our results are concordant with the existence of a FLT3 mutation-like transcriptomic pattern with a different mutational background which clusters a proportion of wild-type FLT3 AMLs with FLT3mut samples. These leukemias were highly enriched in NPM1, and particularly in composed NPM1/DNMT3A mutants, but NPM1 status alone was insufficient to explain the existence of the FLT3-like pattern. The analysis of wild-type FLT3 AML patients treated with FLT3 inhibitors in clinical trials is envisaged in order to study its possible role as a drug response biomarker.

Supporting information
S1 Table. Annotation of all probes significantly associated with FLT3 mutation status in AML.
(XLSX)
S2 Table. Significantly enriched gene ontology (biological process) terms from the list of genes associated with FLT3 mutation status.
(XLSX)
S3 Table. Differential distribution of recurrently mutated genes in wild-type FLT3 AML wild-type according to the presence or absence of the FLT3-like pattern. Data reported for mutations analyzed in GSE14468, GSE10358, GSE15434 and GSE17855.
(XLSX)

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