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Modeling Interactions Between Leukemia-Specific Chromosomal Changes, Somatic Mutations, and Gene Expression Patterns During Progression of Core-Binding Factor Leukemias

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Abstract

In cancer genomes, changes observed during tumor progression can be difficult to separate from non-specific accumulation of cytogenetic changes due to cancer-associated genetic instability. We studied genetic changes occurring over time in cancers presenting with a relatively simple karyotype, namely two related core-binding factor (CBF) acute myeloid leukemias (AMLs), to assess how specific chromosomal changes are selected based on tumor subtype and acquired somatic mutations. Expression profiles for DNA replication/repair genes and the mutation status of *KRAS*, *NRAS*, *FLT3*, and *KIT* were compared with the karyotypic changes at diagnosis and relapse(s) in 94 cases of inv(16)(p13.1q22)-AML and 82 cases of t(8;21)(q22;q22)-AML. The majority of both AML types demonstrated a simple aneuploid pattern of cytogenetic progression, with highly distinctive patterns of chromosome copy number changes, such as +22 and +13 exclusively in inv(16)-AML and –Y and –X in t(8;21)-AML. Selection of certain cytogenetic changes correlated with particular somatic mutations, such as +8 with RAS mutation, and absence of kinase pathway mutations in t(8;21)-AML with localized deletions at chromosome band 9q22. Alterations in transcript levels of mitotic spindle kinases such as *CHEK1*, *AURKA*, and *AURKB* were associated with the aneuploid progression pattern, particularly in t(8;21) cases. Despite the similarity in the initiating genetics of the two CBF AML types, highly tumor-specific patterns of limited aneuploidy are noted that persist and continue to accumulate at relapse. Thus, activation of genetic instability, possibly through mitotic spindle dysregulation, leads rapidly to selection of advantageous single chromosome aneuploidy.

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INTRODUCTION

Genetic progression in cancer can be modeled as initial acquisition of a somatic mutation or gene fusion that activates a growth-promoting oncogene followed by dysregulation of one or more pathways of genetic instability often by deletion of tumor suppressor genes involved in control of DNA replication and repair. These later stages of tumor development produce changes in chromosome copy number (i.e. aneuploidy) or structural chromosomal changes due to defects in DNA break repair and homologous and non-homologous recombination. However, these events are difficult to model for most tumor types since karyotypes are usually highly complex by the time of diagnosis.

Acute myeloid leukemia (AML) carrying either the t(8;21)(q22;q22) [abbreviated t(8;21)] or inv(16)(p13q22) or other chromosome band 16q22 alteration [abbreviated inv(16)] comprise the core-binding factor (CBF) leukemias and have among the best outcomes of all AML types. In t(8;21) leukemias, the *RUNX1* gene (previously *AML1*), which encodes the alpha subunit of the CBF transcription factor, is fused with the *RUNX1T1* transcription factor (also known as *ETO* or *MTG8*) and functions in part by disrupting the normal CBF transcription complex (Lorsbach and Downing, 2001). The CBF complex is also disrupted by the *CBFB-MYH11* fusion in inv(16) leukemias, thus producing abnormal differentiation and maturation arrest.

These AML subtypes usually manifest a simple karyotype at diagnosis and thus represent an opportunity to model the interaction between initiating oncogenes with the cytogenetic changes which occur over the disease course. Using a large set of CBF AML, we examine the relationship between leukemia type, somatic mutation in growth factor genes, expression levels of growth and cell cycle regulators, and the type of genetic progression observed. We note that both AML subtypes show a predominantly aneuploid pattern of progression that was accompanied by shared changes in the transcript levels of mitotic spindle kinases. However, each leukemia type showed highly stereotypic patterns of chromosome gains/losses indicative of strikingly different biological selective factors following genomic progression.

MATERIALS AND METHODS

Patients and Samples Included

All well-characterized newly diagnosed cases of inv(16)- and t(8;21)-AML presenting at the University of Texas M.D. Anderson Cancer Center between 4/1/99 (when fusion transcript PCR detection began) and 7/31/2007 were included. Patients referred after induction therapy was started or those referred early in first remission for consideration of stem cell transplant were also included. The revised World Health Organization (WHO) classification was used for diagnosis including the criterion that detection of inv(16) or t(8;21) was sufficient for AML regardless of bone marrow (BM) or peripheral blood blast count (Swerdlow et al., 2008). Some of these cases have previously reported in small series or cases reports (Sarriera et al., 2001; Sun et al., 2003; Yin et al., 2004; Appelbaum et al., 2006; Huang et al., 2006; Valbuena et al., 2006; Sun et al., 2007; Gustafson et al., 2009).

Routine workup included bone marrow biopsy and aspirate, cytochemistry on aspirate slides for myeloperoxidase (MPO), and non-specific/butyrate esterase (NSE), flow cytometry with a panel of myeloid (CD13, CD33, and CD117), monocytic (CD14, CD68, and CD56) and lymphoid antigens (including CD19 and CD2), G-banded karyotype on culture marrow aspirate, as well as reverse transcription polymerase chain reaction (RT-PCR) studies for *CBFB-MYH11* and/or *RUNX1-RUNX1T1* fusion transcripts, and dual-fusion fluorescence in situ hybridization (FISH), in a subset of cases including those with variant translocations. RT-PCR for both fusion transcripts was performed on RNA extracted from total peripheral blood or BM leukocytes following red blood cell lysis using a qualitative RT-PCR assay until 6/7/05 and thereafter by commercial quantitative TaqMan-based assay (Ipsogen, Marseille, France).

Interpretation of Karyotypes

All cases had standard Giemsa (G)-banded karyotype prepared from short-term cultures of leukocytes from BM aspirate material. All but two cases had the defining translocated chromosomes detected by karyotype, with the *RUNX1-RUNX1T1* or *CBFB-MYH11* fusion, respectively, in these two cases, detected by fusion FISH probes. Extra copies or altered translocated chromosome [e.g., der(8)t(8;21)(q22;q22), der(21)t(8;21)del(8)(q22;q22)] were seen in 5 and 3, cases of t(8;21)- and inv(16)-AML, respectively, and were not regarded as genetic progression for the purposes of this study. Only 3 cases had marker chromosomes whose identity could not be ascertained and were classified in Table 1 as having a mixed pattern of cytogenetic progression. The degree of subclonal complexity was also categorized as the percentage of the 20 analyzed metaphases with any particular secondary chromosomal change.

FLT3 Mutation Detection

FLT3 internal tandem duplication (ITD) and mutations in codon 835 or 836 in the *FLT3* kinase domain were tested in 73 t(8;21)- and 84 inv(16)-AML at diagnosis, and 23 t(8;21)- and 33 inv(16)-AML at relapse. Products were assessed by PCR on genomic DNA followed by capillary electrophoresis (CE) with products labeled with 6-carboxyfluorescein (FAM) for detection. For codon 835/836 analysis, PCR products were digested by *EcoRV* (whose recognition site spans these two codons) prior to sizing, as described (Lin et al., 2006). Mutation level was determined by a ratio of the area of the mutated CE peak(s) divided by the sum of mutated and unmutated peaks, with an assay sensitivity of 1% mutation-bearing cells, as established by a dilution series.

KIT and *RAS* Sequencing

NRAS and *KRAS* mutation status was assessed in 66 t(8;21)- and 84 inv(16)-AML at diagnosis, and 13 t(8;21) and 26 inv(16)-AML at relapse. PCR-based direct DNA sequencing was done for exons 1 and 2 of *NRAS* and *KRAS* using direct sequencing (until 2006) or pyrosequencing (2006–2009). Mutation percentage in the pyrosequencing assay was determined by a ratio of the mutant and wild-type peak heights, with a sensitivity of mutation detection of 2% mutation-bearing cells. *KIT* mutation status in exon 8 and 17 were assessed in 60 t(8;21)- and 57 inv(16)-AML at diagnosis. PCR-based DNA sequencing of

KIT was done by the Sanger (dideoxy chain-termination) methods, which has a sensitivity of 20%. In most cases with no *KIT* mutation identified by Sanger method, a pyrosequencing assay was also performed covering codons 814 to 822, which had a sensitivity of 5% (Zhao et al., 2007).

Expression Analysis

Gene expression levels were analyzed from 33 cases of inv(16)-AML and 26 cases of t(8;21)-AML. Total RNA was extracted from leukocytes in BM aspirate samples containing at least 20% blasts, reverse transcribed using random hexamers and Superscript RT (Invitrogen, Carlsbad, CA) and subjected to TaqMan-based quantitative RT-PCR using inventoried gene expression primer/probe assays (Applied Biosystems, Foster City, CA) on the BioMark platform using 96.96 Dynamic Arrays (Fluidigm, South San Francisco, CA). Four measurements for each gene in each sample were obtained and values for the threshold cycle averaged with all expressed gene measurements showing a standard error of less than 2.2. Genes analyzed included DNA repair genes (*ERCC* and *RAD51*), chromosomal maintenance/cell cycle regulators (*PCNA*, *CHEK1*, *AURA*, *AURB*, *ARF*, *P19*, *CDK4*, *CDKN2B*, and *INK4D*), transcription factors regulating myeloid differentiation (*WT1*, *HOXA10*, *HOXB2*, *HOXB5*, *BCL11A*, *ERG*, *IRF4*, *MNI*, *JUNB*, *GATA2*, *CEBPA*, and *EVII*), myeloid-associated kinases (*BAALC*, *FLT1*, *FLT3*, and *NME4*) and myeloblast markers (*CD34*, *ABCG2*, *ABCC1*, *WT1*, *HOXB5*, and *HOXA10*). Cts were normalized against the median value for all expressed genes for group comparisons.

Statistical Analysis

The Chi-square tests were used to examine the associations of categorical variables. The Mann-Whitney U test (for 2 samples) and the Kruskal-Wallis tests (for more than 2 samples) were used to compare location parameters for continuous variables. Kaplan-Meier curves in combination with the log-rank test were applied to the analysis of overall survivals. We carried out the above computations using the STATISTICA software (StatSoft, Tulsa, OK). Only two-sided P-values were reported and the significance level was set as $P < 0.05$.

The hierarchical clustering analysis, using Ward's minimum variance method and Euclidean distance, was performed on 33 inv(16)- and 26 t(8;21)-AML based on expression levels of a panel of 45 genes. We also performed principal components analysis (PCA) on the 59 cases by applying singular value decomposition to compute the principal components. A nonparametric bootstrap test sampled 500 times was conducted to determine whether the clusters found by hierarchical clustering were robust. Analyses were performed using a set of R packages developed in the Department of Bioinformatics and Computational Biology at MD Anderson Cancer Center (<http://bioinformatics.mdanderson.org/Software/OOMPA/>).

RESULTS

Outcome in the Two Subtypes of CBF AML Are Similar

We included 82 cases of t(8;21)-AML and 94 cases of inv(16)-AML whose demographic and diagnostic features are summarized in Supplementary Table 1. There was a trend towards superior outcome in inv(16)-AML that was borderline statistically significant ($P = .$

067, Fig. 1). All patients received anthracycline/cytarabine-containing polychemotherapy with 30 undergoing stem cell transplant (17 in 1st remission). Median follow-up was 28.1 mos (range 1.1–178 mos). For t(8;21)-AML, 3 patients had disease unresponsive to initial chemotherapy and 4 died of complications in induction phase, 32 patients had 1 relapse, 6 patients relapsed multiple times. There were 31 deaths at a median of 22 mos (range 2–107 mos) with 27 due to progressive disease or complications and 4 due to transplant. For inv(16)-AML, 1 patient had disease unresponsive to chemotherapy, 4 died in induction phase of complications, 37 patients had 1 relapse, and 9 patients relapsed multiple times. There were 30 deaths (28 due to disease or complications, and 2 due to transplant) at a median of 25 mos (range 1.1 to 107 months). Considering all CBF AML, cases arising as MDS had worse OS than other cases (median survival 43 vs. 71 months, $P = .02$). Cases arising secondary following treatment for another malignancies compared to de novo cases had significantly inferior in OS for t(8;21)-AML ($P = .04$) not for inv(16)-AML ($P = 0.5$).

Different Aneuploid Patterns of Genetic Progression Distinguish CBF AML Subtypes but Both Correlate With Superior Outcome

The chromosomal changes seen in CBF AML were examined by G-banded karyotypes from involved BM samples. At both diagnosis and relapse, t(8;21)-AML showed a higher rate of secondary genetic changes due to slightly more frequent secondary structural chromosomal changes; however, both tumor types showed a similar high rate of “simple” aneuploidy with gains or losses in one to several chromosomes (Table 1 and Supplementary Table 2). Inv(16)-AML had higher rates of aneuploid progression at relapse whereas t(8;21)-AML showed more frequent structural changes such as del(7q).

Although the overall degree of genetic complexity was similar, the chromosomal changes underlying this simple aneuploid pattern were strikingly different between the two AML subtypes. For example, 27 cases of t(8;21)-AML (33.0%) had either $-X$ or $-Y$ present at diagnosis, with 3 additional cases showing $-Y$ and 1 with $-X$ at relapse, with no case of inv(16)-AML showing either of these changes ($P < .0001$). In contrast, 22 cases of inv(16)-AML had +22/i(22q) at diagnosis, with an additional 10 cases occurring at relapse (overall incidence 34.0%); 6 cases had +13 (6.4%). No case of t(8;21)-AML had +22/i(22q) ($P < .0001$). Trisomy 8 was found in 9 (11.0%) of t(8;21)-AML but in 23 (24.5%) of inv(16)-AML ($P = .03$). Aneuploidy involving other chromosomes was unusual in either leukemia type (Fig. 2).

The rate/persistence of aneuploidy at diagnosis and relapse was also distinct for different chromosomes. In inv(16)-AML, +22, +13 and +8 were mostly subclonal at diagnosis or relapse meaning that they were not present in all analyzed metaphases, and increased, decreased, or were lost in frequency with relapse(s). In contrast, $-Y$ and $-X$ were almost always present as the dominant clone at diagnosis and persisted at relapse in all but 1 case.

Among structural chromosomal changes, localized deletions spanning chromosome 9q22 were found in 11 cases of t(8;21)-AML but not in any cases of inv(16)-AML ($P = .0001$). High-risk AML changes (i.e. -5, -7, del5q, del7q and del11q) were seen at similar rates in 10 (8.2%) t(8;21)-AML and 8 (8.5%) inv(16)-AML. The presence of aneuploidy at diagnosis was associated with better outcome than both the presence of other structural changes or no

additional cytogenetic aberration at all (Fig. 1B). This superior outcome was also noted for the most common aneuploid subgroups of $-X/-Y$ in $t(8;21)$ -AML and $+22$ in $inv(16)$ -AML (Fig. 1C and not shown).

Receptor Kinase Pathway Mutation Status Correlates With Specific Secondary Cytogenetic Changes

At least one tyrosine kinase (TK) pathway mutation was detected in the *FLT3*, *KIT*, *NRAS*, or *KRAS* genes at diagnosis in 31/72 (42.5%) of $t(8;21)$ -AML and 54/84 (64.3%) $inv(16)$ -AML, with a higher rate of *NRAS* or *KRAS* point mutation in $inv(16)$ -AML ($P = .0007$), particularly those with $+8$. The overall incidence of *FLT3*-ITD, *FLT3*-KD mutation or exon 8 and 17 *KIT* mutations was not significantly different (Table 2). In the 63 cases with mutation quantitation in the diagnostic samples, blast-normalized mutation ratios were over 50% in 58 (92% of cases) consistent with presence in the vast majority of blasts.

For both $inv(16)$ - and $t(8;21)$ -AML, the presence of one or more *NRAS*, *KRAS*, *KIT* or *FLT3* mutation at diagnosis was highly correlated with high presenting white blood cell (WBC) count ($P < 0.0001$), with the strongest correlations seen for exon 17 *KIT* point mutations in $inv(16)$ -AML ($R = 0.58$). At least 1 RTK mutation was observed in 90% of $inv(16)$ -AML with presenting WBC above $20 \times 10^9/L$ compared with 39% with WBC count below $20 \times 10^9/L$ ($P < 0.0001$). Similarly, 91% of $t(8;21)$ -AML with presenting WBC above $10 \times 10^9/L$ had mutations as compared to 14% below that level ($P = 0.001$). The presence of any TK mutation at diagnosis was significantly correlated with monocytic blast differentiation in $inv(16)$ cases ($P = 0.005$ for CD64 expression), and with the percent of MPO+ blasts in $t(8;21)$ cases ($P = 0.029$).

The frequency of finding any TK pathway mutation was not significantly different in those cases with only $inv(16)/t(8;21)$ (35/57, 61.4%) versus aneuploid changes 21/34 (61.8%) or more complex karyotype (7/17, 41.2%) at diagnosis. However, cases of $-X/-Y$ in $t(8;21)$ -AML or $+22/+13$ in $inv(16)$ -AML had TK mutations less frequently than other cases ($P = .001$). Cases of $t(8;21)$ -AML with deletions spanning chromosome 9q12q22 also frequently lacked detectable RTK pathway mutations (6 of 7 tested, $P = 0.05$). In contrast, 12/25 (48%) of CBF AML with $+8$ had *RAS* mutations compared to 21/113 (18.6%) of other cases ($P = .0036$).

The finding of *FLT3* ITD, any *FLT3* mutation, or any ras mutation was not significantly correlated with inferior OS or PFS with the exception of inferior PFS in $inv(16)$ -AML with *FLT3* mutation ($p .016$). There was a trend towards inferior OS ($P = .3$) and PFS ($P = .33$) with *KIT* mutation in $t(8;21)$ -AML, which was borderline statistically significant if patients who received a stem cell transplant were excluded ($P = .06$).

Expression of Mitotic Spindle Kinases Genes Correlate With Cytogenetic Progression Patterns

Gene expression analysis by RQ-PCR arrays for 45 DNA replication/repair genes and myeloid differentiation genes was performed on diagnostic BM samples from 33 $inv(16)$ -AML and 24 $t(8;21)$ -AML. This panel of genes easily distinguished most cases of $t(8;21)$ -

from inv(16)-AML by unsupervised hierarchical clustering or PCA (Fig. 3A). The genes that were most significant in differentiating the two leukemias include *MNI*, *HOXA10*, *HOXB2*, and *WT1* which were increased in t(8;21) and *AURKA*, *ERG*, and *DNMT3A* which are increased in inv(16)-AML (Supplementary Table 3). Gene expression patterns also identified three clusters of inv(16)-AML by bootstrapping analysis (Fig. 3B). These inv(16)-AML clusters appeared related to maturation state and phenotype for blast markers with increased *CD34* but lower *ABCG2* and *EVII* in cluster 3 and increased *HOXA10* expression in cluster 1 (Fig. 3D and Supplementary Fig. 1). None of these genes profiled were highly correlated with OS or relapse, including the transcriptional regulators *MNI*, *ERG* and *BAALC* (not shown).

Aneuploid cytogenetic progression patterns were best correlated with differential expression of genes involved in DNA replication/chromosomal maintenance including the mitotic kinases *CHEK1*, Aurora A (*AURKA*), and Aurora B (*AURKB*) (Fig. 4A and B, and not shown). Although the genes profiled were not chosen to identify differentially expressed genes on the aneuploid chromosomes, the transcription factor *MNI* which is located on chromosome 22 did show differential higher expression only in inv(16) with +22 consistent with dosage effects (Fig. 4C).

DISCUSSION

We note that the related CBF AMLs initiated by the inv(16) and t(8;21) gene fusions show a similar general pattern of genetic progression characterized by simple aneuploidy in most cases but had highly stereotypical chromosomal gains and losses supporting strong biological selection dependent on tumor type [-X/-Y in t(8;21) versus +22/+13 in inv(16)]. The subclonal pattern, outgrowth and persistence at relapse(s) of particular changes were also used to infer the importance of the selected changes for tumor growth. In the case of -X and -Y in t(8;21), these changes occurred early in disease course and persisted in relapsed samples, whereas for +22 and +13 in inv(16)-AML, these changes were often subclonal at diagnosis, increased in frequency with relapse and were occasionally lost during tumor progression. This differential persistence suggests that -X and -Y provide a more powerful (or less deleterious) positive growth effect for t(8;21) than +22, +13 or +8 do for inv(16)-AML.

It has been commonly stated in the cytogenetic literature that -Y and some other simple aneuploid changes are non-specific findings that are not obviously neoplastic and perhaps of limited significance. Our results, particularly the specificity and persistence of -X and -Y throughout the course in t(8;21)-AML, do not support that interpretation for CBF AML. The results from our series and comparative analyses of cytogenetic findings in other large studies on CBF AML (Delaunay et al., 2003; Schlenk et al., 2004; Marcucci et al., 2005), clearly implicate -Y/-X and +13/+22/+8 as important and specific factors in the progression of substantial proportions of t(8;21)- and inv(16)-AML, respectively. The stereotypic selection of different chromosomes might be related to the dosage effects of a particular gene (e.g. *MNI* on chromosome 22) (Mrozek et al., 2008), or the entire set of genes on these chromosomes on myeloid cells from particular stages of differentiation. A similar phenomenon may be operative in AML-M0 with *RUNX1* dominant-negative mutations that

show selective gain of chromosome 13 (Silva et al., 2007), and chronic myelogenous leukemia with *RUNX1* mutations that show gains of chromosome 21 (Roche-Lestienne et al., 2008).

Other events clearly implicated in the progression of CBF AMLs are acquisition of mutations in the TK pathway genes *FLT3*, *KIT*, and *NRAS/KRAS*. In our series as well as others (Goemans et al., 2005; Boissel et al., 2006; Cairoli et al., 2006), mutations in *KIT*, *FLT3*, and *RAS* mutations were associated with proliferative presentations (i.e. high blood WBC and blast counts) consistent with a role for these mutations in driving leukemia cell growth and adverse outcome (Nguyen et al., 2002; Care et al., 2003; Meshinchi et al., 2003; Schlenk et al., 2004; Wang et al., 2005; Paschka et al., 2006; Schnittger et al., 2006; Kim et al., 2008). We found that *FLT3* mutations were predictive of inferior PFS in inv(16)-AML but not in t(8;21)-AML. In contrast to most other studies (Boissel et al., 2006; Cairoli et al., 2006; Paschka et al., 2006; Schnittger et al., 2006), we found that *KIT* point mutations (mostly in codon 816) were only weakly correlated with inferior outcome in t(8;21)-AML and not at all for inv(16)-AML. In agreement with other studies (Boissel et al., 2006), *NRAS/KRAS* mutation status did not correlate with outcome. Although the general trend for the influence of particular RTK mutations on outcome were similar, differences in the level of significance may relate to patient populations with different age ranges and prior histories (particularly incidence of secondary AML) and in the incidence and timing of stem cell transplant, use of gemtuzumab ozogamicin, and cytarabine dose intensification (Dombret et al., 2009). For example, the adverse effect of *KIT* mutations in t(8;21)-AML was more significant in our series when only patients who did not receive stem cell transplant were considered.

We also noted that the occurrence of some TK pathway mutations correlated with the emergence of secondary aneuploid changes driving different routes of CBF AML progression. This was most prominent for the association of *RAS* mutations with +8, particularly in inv(16)-AML. There was also a lower incidence of identifiable TK mutations in +22 inv(16)- and -Y/-X t(8;21)-AML suggesting that these chromosomal changes may partially compensate for the lack of an activating growth factor mutation (Wang et al., 2005). Based on the high blast-normalized percentage of TK pathway mutations at diagnosis, mutation appears to precede aneuploid progression in most cases especially in inv(16)-AML.

With the exception of del(9)(q22) in t(8;21)-AML, structural chromosomal changes (such as sub-chromosomal deletions, addition and inversions) were found less commonly in CBF AML suggesting that DNA break repair defects are far less common in tumor progression than chromosome segregation defects. The reasons for the low incidence of common AML changes such as -5, -7, del(5q), del(7q), del(11q), -17 and del(20q) with progression in CBF AML are unclear but their absence likely imparts the relatively good prognosis for this AML subtype. It is possible that specific effects of the *RUNX1-RUNX1T1* and *CBFB-MYH11* fusion proteins drive the predominant simple aneuploidy pattern of progression (Peterson et al., 2007). One possible model for a direct role of altered CBF proteins in generation of aneuploidy is the recently demonstrated effect of a mouse homolog of *RUNX1-RUNX1T1* on attenuating the spindle checkpoint during mitosis (Boyapati et al., 2007).

Previous whole-genome gene expression studies have revealed that there are hundreds of genes which are differentially expressed in t(8;21)-AML and inv(16)-AML as well as multiple expression subsets within each leukemia type (Bullinger et al., 2007; Andreeff et al., 2008; Grubach et al., 2008; Verhaak et al., 2009). Many of these differentially expressed genes are related to mitosis (e.g. spindle pole, M phase genes, and those mediating polymerase and helicase activity) (Bullinger et al., 2007). It thus seems likely that the differential gene expression pattern driven by the altered CBF transcription factors directly mediates the aneuploid pattern of progression.

We thus focused the gene expression analyses in this set on myeloid differentiation factors and those involved in mitotic regulation and chromosome maintenance. Expression of these genes provided segregation of CBF AMLs along several axes. Expression of a limited set of differentiation-associated genes easily allowed separation of t(8;21)- from inv(16)-AML, except in four cases. Many of these genes were in the HOX cluster (e.g. *HOXB2* and *HOXA10*), as seen by others (Andreeff et al., 2008; Grubach et al., 2008). Furthermore, inv(16)-AML could be divided into several 3 clusters based on expression of blast genes, including *ABCG2* and *CD34*, and proliferation markers. This apparent segregation of CBF leukemias according to immaturity and proliferative signature was also noted in a recent expression array study (Bullinger et al., 2007) and likely is reflected in the differential epigenetic profiles of subgroups of CBF AMLs (Jongen-Lavrencic et al., 2008; Li et al., 2008). However, most of these differentially expression genes showed no correlation with outcome. In that regard, expression of genes reported to predict outcome in intermediate-risk AML, such as *ERG*, *BAALC* and *MNI* did not show correlation with OS or PFS in our series.

We did, however, note that differential transcript expression of kinases involved in the mitotic spindle and chromosomal segregation was associated with those cases that showed aneuploid progression particularly the -X/-Y subset of t(8;21)-AML. These gene expression findings suggest that integrated genomic-expression models for cancer progression can be defined which have important correlates with disease presentation and progression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Outcomes in CBF AML are related to chromosome changes besides the initiating translocation. (A) Overall survival (OS) by translocation type. (B) OS by presence of other chromosomal aberration, besides by $t(8;21)$ and $inv(16)$, including chromosomal copy number alterations only (simple aneuploidy) versus structural aberrations or mixed patterns, or no additional changes. (C) OS in $t(8;21)$ -AML comparing those with $-X$ or $-Y$ aberrations compared to all other cases.

Figure 2. Distribution of chromosome copy number alterations at diagnosis and relapse in CBF AML. Exclusive acquisition of chromosomes 13 and 22 was seen in inv(16)-AML and near exclusive loss of -X and -Y in t(8;21)-AML with rare loss or gain of other chromosomes in either AML type.

Figure 3.

Gene expression profiles segregate CBF AMLs by type and subgroup. (A) Unsupervised clustering using the 45-gene set distinguishes t(8;21)-AML (blue) from inv(16)-AML (yellow) except in 4 cases. (B) Principal component analysis similarly highlights the differences between t(8;21)-AML (blue) and inv(16)-AML (yellow). (C) The boot-strapping method validated three distinct subgroups within inv(16)-AML identified in unsupervised clustering.. (D) These groups within inv(16)-AML are primarily distinguished by differential expression of genes highly expressed in myeloblasts/stem cells such as *ABCG2* and *CD34*. The boxes show the interquartile range (IQR) with black bar in box indicating median values. Values with whiskers indicate a range of 1.5 times of IQR, with circles representing outliers.

Figure 4.

Differential expression of mitotic spindle kinases related to aneuploidy progression patterns.

t(8;21) AML with -X or -Y show lower expression levels of the *CHEK1* kinase (A) and

higher expression of *AURKA* (B). (C) Higher expression of the MN1 gene located on

chromosome 22 is noted in inv(16)-AML with +22. Graphs as in Fig. 3D.

TABLE 1

Patterns of Cytogenetic Progression in CBF AML

		Cytogenetic progression pattern				
	#	No additional aberration (%)	Only chr aneuploid changes (%)	Additional chr inversions/ translocations (%)	Other chr structural changes (%)	Mixed patterns (%)
t(8;21)						
At diagnosis	82	32 (39.0)	30 (36.6)	3 (3.7)	8 (9.8)	9 (11.0)
At relapse	32	6 (18.8)	9 (28.1)	2 (6.3)	7 (21.9)	8 (25)
inv(16)						
At diagnosis	94	58 (61.7)	25 (26.6)	2 (2.1)	2 (2.1)	7 (7.4)
At relapse	37	9 (24.3)	22 (59.5)	1 (2.7)	3 (8.1)	2 (5.5)

TABLE 2

Mutational Profiling of CBF AML

<i>Diagnostic samples</i>	Genes analyzed		
	<i>NRAS/KRAS</i> ¹	<i>FLT3</i> ²	<i>KIT</i> ³
<i>t</i> (8;21)	6/66 (9.1%)	14/73 (19.2%)	12/60 (24.0%)
<i>inv</i> (16)	29/84 (34.5%)	17/84 (20.2%)	7/57 (12.3%)
<i>Correlation with OS (P value)</i>	<i>t</i> (8;21): ns <i>inv</i> (16): ns	<i>t</i> (8;21): ns <i>inv</i> (16): ns	<i>t</i> (8;21): ns, <i>p</i> = .3 <i>inv</i> (16): ns
<i>Correlation with PFS</i>	<i>t</i> (8;21): ns <i>inv</i> (16): ns	<i>t</i> (8;21): ns <i>inv</i> (16): <i>p</i> = 0.16	<i>t</i> (8;21): ns, <i>p</i> = .3 <i>inv</i> (16): ns
<i>Correlations with other features:</i>	Ras mutation with <i>inv</i> (16)-AML (<i>p</i> = .0002), ras mutation with +8 (<i>p</i> = .0007) Any RTK mutation with high presenting WBC count		

¹ 27 were *NRAS* (5 *t*(8;21), 22 *inv*(16)), 6 were *KRAS* (all *inv*(16)), 2 cases had double mutations. 1 gained *KRAS* mutation at relapse (*inv*(16)).

² 19 were ITD type (9 *inv*(16), 11 *t*(8;21)), 10 were D835/836 point mutations (8 *inv*(16), 2 *t*(8;21)), and 2 had both (both *inv*(16)). 3 gained *FLT3* mutation (2 *t*(8;21), 1 *inv*(16)) at relapse, and 1 lost *FLT3* ITD (*inv*(16)).

³ 10 were D816V (4 *inv*(16)), 4 D816Y, 3 D816H (8;21), and 2 in exon 8. No change in *KIT* status at relapse in the 6 cases studied.

ns; not significant (i.e. *P* value > .05).