



Contents lists available at ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint



Cytogenetic and genetic pathways in therapy-related acute myeloid leukemia[☆]

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ARTICLE INFO

Article history:
Available online xxx

Keywords:
Therapy-related myeloid leukemia
Cytogenetic analysis
Leukemogenesis
Haploinsufficiency
Tumor suppressor genes

ABSTRACT

Therapy-related myelodysplastic syndrome and acute myeloid leukemia (t-MDS/t-AML) are late complications of cytotoxic therapy used in the treatment of malignant diseases. The most common subtype of t-AML (~75% of cases) develops after exposure to alkylating agents, and is characterized by loss or deletion of chromosome 5 and/or 7 [–5/del(5q), –7/del(7q)], and a poor outcome (median survival 8 months). In the University of Chicago's series of 386 patients with t-MDS/t-AML, 79 (20%) patients had abnormalities of chromosome 5, 95 (25%) patients had abnormalities of chromosome 7, and 85 (22%) patients had abnormalities of both chromosomes 5 and 7. t-MDS/t-AML with a –5/del(5q) is associated with a complex karyotype, characterized by trisomy 8, as well as loss of 12p, 13q, 16q22, 17p (*TP53* locus), chromosome 18, and 20q. In addition, this subtype of t-AML is characterized by a unique expression profile (higher expression of genes involved in cell cycle control (*CCNA2*, *CCNE2*, *CDC2*), checkpoints (*BUB1*), or growth (*MYC*), loss of expression of *IRF8*, and overexpression of *FHL2*. Haploinsufficiency of the *RPS14*, *EGR1*, *APC*, *NPM1*, and *CTNNA1* genes on 5q has been implicated in the pathogenesis of MDS/AML. In previous studies, we determined that *Egr1* acts by haploinsufficiency and cooperates with mutations induced by alkylating agents to induce myeloid leukemias in the mouse. To identify mutations that cooperate with *Egr1* haploinsufficiency, we used retroviral insertional mutagenesis. To date, we have identified two common integration sites involving genes encoding transcription factors that play a critical role in hematopoiesis (*Evi1* and *Gfi1b* loci). Of note is that the *EVI1* transcription factor gene is deregulated in human AMLs, particularly those with –7, and abnormalities of 3q. Identifying the genetic pathways leading to t-AML will provide new insights into the underlying biology of this disease, and may facilitate the identification of new therapeutic targets.

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1. Introduction

t-MDS and t-AML are late complications of cytotoxic therapy (radiation and/or chemotherapy) used in the treatment of both malignant and non-malignant diseases [1–4]. Several distinct cytogenetic and clinical subtypes of t-MDS/t-AML are recognized that are closely associated with the nature of the preceding treatment. Rowley et al. first noted the association of loss or deletion of chromosomes 5 and/or 7 with t-MDS/t-AML [5]. Subsequently, it was recognized that abnormalities of chromosomes 5 and/or 7 are the hallmark of t-MDS/t-AML following alkylating agent therapy

[4,6,7]. Patients who develop t-MDS/t-AML in this setting typically show a latency of 3–7 years from alkylating agent exposure (median 5 years), insidious disease onset with an antecedent MDS with peripheral cytopenias, and a poor prognosis (median survival <8 months) [1]. Typically, all three hematopoietic cell lineages (erythroid, myeloid, and megakaryocytic) are involved in the dysplastic process (trilineage dysplasia), suggesting that the disease arises in a multipotent hematopoietic stem (HSC) or progenitor cell (HPC).

In contrast, patients who develop t-AML following treatment with drugs targeting topoisomerase II are younger, have a shorter latency period (2–3 years), and rarely present with MDS. Overall, these patients have a more favorable response to intensive remission induction therapy than do t-AML patients with abnormalities of chromosomes 5 and/or 7 [4,8]. Balanced translocations involving *MLL* at 11q23, *RUNX1* at 21q22, *CBFB* at 16q22, or *PML* (15q22) and *RARA* (17q12) are common in this subgroup, suggesting that these cytogenetic subsets of t-AML arise in a lineage committed progenitor cell. t-MDS/t-AML arising after alkylating agent therapy represents the largest subgroup of patients (80–85%), whereas t-AML with balanced translocations represents ~15% of

Abbreviations: CIS, common insertion sites; ENU, N-ethyl-nitrosourea; HPC, hematopoietic progenitor cell; HSC, hematopoietic stem cell; ITD, internal tandem duplication; MPD, myeloproliferative disorder; t-AML, therapy-related acute myeloid leukemia; t-MDS, therapy-related myelodysplastic syndrome; TSG, tumor suppressor gene.

[☆] Supported by grants CA40046 and CA14599 from the National Cancer Institute.

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cases [4]. Survival times of t-AML patients are often short, because this disorder is less responsive to current forms of therapy than is AML *de novo* [1,3,4].

t-AML represents an important model for cancer. The incidence of t-AML is rising, as a result of the increasing number of cancer survivors at risk of developing this disorder and the changes in therapeutic trends. t-AML also provides a unique opportunity to examine the effects of mutagens on carcinogenesis in humans, as well as the role of genetic susceptibility to cancer [3]. In this regard, Knight et al. used a low-resolution genome-wide association study, and identified novel loci associated with susceptibility to t-AML, providing proof-of-principle for this methodological approach [9]. Finally, the mechanisms of leukemogenesis that are uncovered in t-AML will likely apply to those subtypes of AML *de novo* which share the same cytogenetic abnormalities, e.g., AML *de novo* with abnormalities of chromosome 5 or 7. In this study, we review the genetic characteristics of t-MDS/t-AML with an emphasis on defining the genetic pathways leading to t-AML with a del(5q).

2. Cytogenetic analyses

Table 1 summarizes the cytogenetic pattern in the recently updated University of Chicago series of 386 consecutive patients with t-MDS/t-AML. Of these, 349 (90.4%) had a clonal chromosomal abnormality, including 259 (67%) with a clonal abnormality leading to loss or deletion of chromosomes 5 and/or 7 ([4], Le Beau and Larson, unpublished data). Overall, 164 patients (42%) had abnormalities of chromosome 5, and 180 (47%) had abnormalities of chromosome 7. Eighty-five patients had abnormalities of both chromosomes 5 and 7. A del(5q) was the most common structural abnormality. The pattern of numerical and structural abnormalities is shown in Fig. 1, and illustrates that t-MDS/t-AML is associated

Table 1
Cytogenetic abnormalities in 386 patients with t-MDS/t-AML.

Karyotype	No. of patients (%)
Normal karyotype	37 (9.6)
Clonal abnormalities	349 (90.4)
Clonal abnormalities of chromosomes	259 (67)
5, 7, or both (+/– other abnormalities)	
Abnormal chromosome 5 only	79 (20)
Abnormal chromosome 7 only	95 (25)
Abnormal chromosomes 5 and 7	85 (22)
Recurring balanced rearrangements ^a	41 (10.6)
t(11q23)	16 (3)
t(3;21) or t(8;21) or t(21q22)	10 (3)
t(15;17)	8 (2)
inv(16)	7 (2)
Recurring unbalanced abnormalities ^a	21 (5)
+8	10 (3)
–13/del(13q)	3 (1)
–Y, +11, del(11q), del(20q), +21	8 (2)
Other clonal abnormalities	29 (7.5)

^a One patient with an abnormality of chromosome 5 and t(3;21), and is listed twice in the table.

with a complex karyotype, with a predominance of the loss of genetic material.

In other studies, we evaluated the cytogenetic pattern of 3444 patients with primary MDS, AML *de novo*, or t-MDS/t-AML evaluated over the past 35 years by our Cancer Cytogenetics Laboratory. Of these, 553 (16%) patients had –5/del(5q), and 597 (17.3%) had –7/del(7q) (Tennant and Le Beau, unpublished data). Complex karyotypes were associated with abnormalities of chromosome 5,

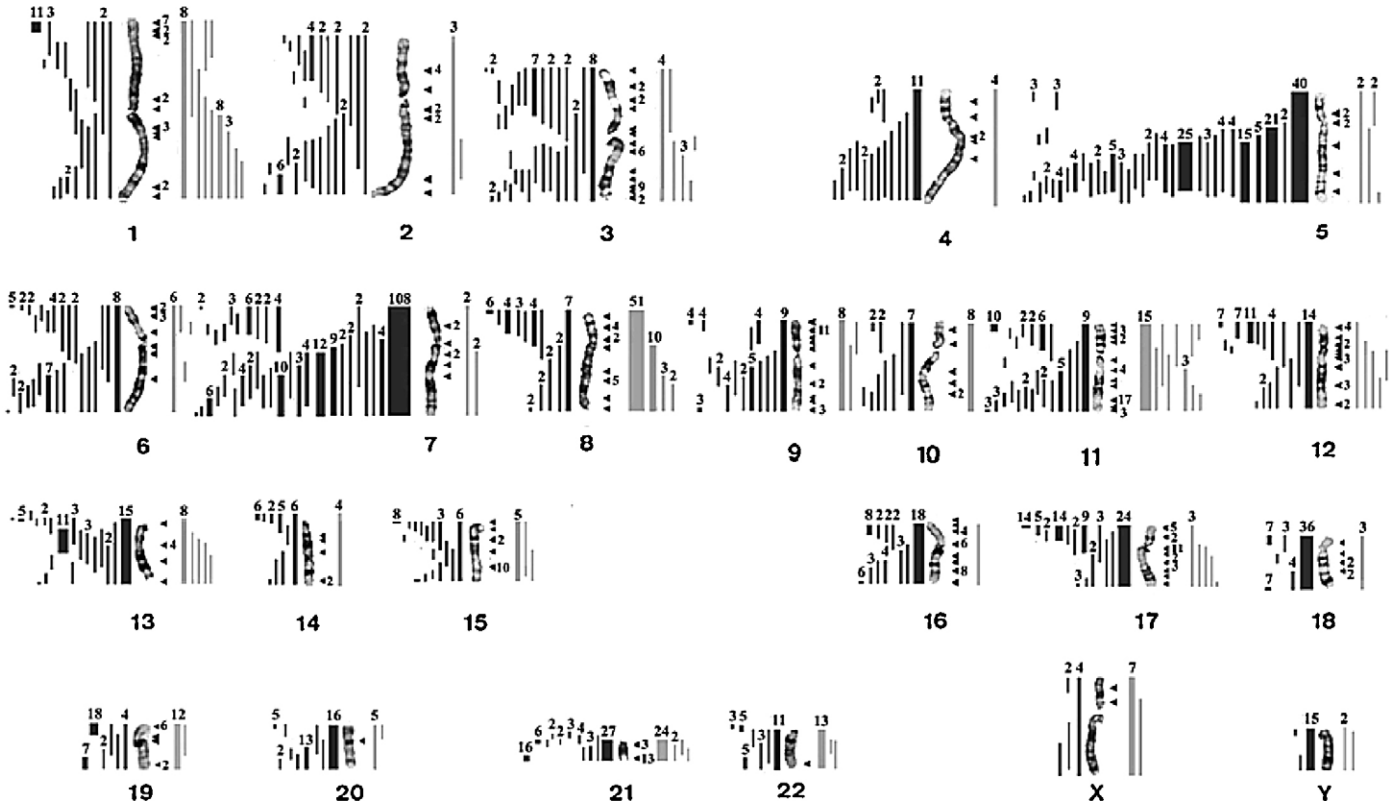


Fig. 1. Cytogenetic abnormalities in patients with t-MDS/t-AML (N = 386) in the University of Chicago series. Gain of chromosomal material is depicted by green bars to the right of each chromosome, and loss of chromosomal material is depicted by red bars to the left of each chromosome. Numbers above the bars indicate the number of patients with this abnormality. Arrowheads identify the location of the breakpoints of structural rearrangements, and the numbers next to the arrowheads indicate the number of rearrangements with the corresponding breakpoint. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2Frequency of molecular mutations in AML *de novo* and t-MDS/t-AML.

Mutated gene	AML <i>de novo</i>	t-MDS/t-AML
<i>FLT3</i> (ITD)	35%	0%
<i>FLT3</i> (TKD)	9%	<1%
<i>NRAS</i>	10–15%	10%
<i>KIT</i> ^{D816}	~5%	NA
<i>MLL</i> (ITD)	3%	2–3%
<i>RUNX1</i>	10–15%	15–30%
<i>TP53</i>	10%	25–30%
<i>PTPN11</i>	~2%	3%
<i>NPM1</i>	35–50%	4–5%
<i>CEBPA</i>	6–15%	Rare
<i>JAK2</i> ^{V617F}	2–5%	2–5%

rather than 7. Recurring abnormalities observed at a high frequency (>20%) in patients with $-5/\text{del}(5q)$ included +8, and loss of 13q, 16q, 17p (40% of cases), chromosome 18, and 20q, which frequently occurred in the same clone (Fig. 1, and data not shown).

3. Alterations in gene function

A growing body of evidence suggests that mutations of multiple genes are involved in the pathogenesis and progression of t-MDS/t-AML. The involved genes fall into two main classes, namely, genes encoding hematopoietic transcription factors, or proteins that regulate cytokine signaling pathways (Table 2). The RAS signaling cascade is downstream of a number of activated cytokine receptors, including the FLT3, IL3, and GM-CSF receptors; thus, this signaling pathway plays a pivotal role in hematopoiesis. Constitutively activating point mutations of *NRAS*, typically involving codons 12, 13, or 61, have been detected at high frequency in hematological malignancies (10–15% in t-MDS/t-AML) [10].

Mutations of the FMS-like tyrosine kinase 3 (*FLT3*) gene, including both point mutations within the tyrosine kinase domain and internal tandem duplications (ITDs), are among the most common genetic changes seen in AML *de novo* (15–35% of cases), but are not seen in t-MDS/t-AML [11]. *FLT3*-ITD mutations are common in patients with a normal karyotype, and are associated with a poor prognosis, particularly in those cases with loss of the remaining wild type *FLT3* allele.

The Runt-related transcription factor 1 (*RUNX1*), also known as AML1, encodes the DNA-binding subunit of the heterodimeric core-binding factor (CBF) complex, which is essential for definitive hematopoiesis. Point mutations in the *RUNX1* Runt (DNA-binding) domain have been reported in AML and MDS (10–15%), particularly in MDS secondary to atomic bomb radiation exposure or treatment. Similarly, the incidence is higher in t-MDS/t-AML (15–30%) [12]. Moreover, *RUNX1* mutations are associated with activating mutations of the RAS pathway, $-7/\text{del}(7q)$, and a shorter overall survival.

Mutations of *NPM1* also occur frequently in AML (35% of adult cases), but are less frequent in patients with recurring cytogenetic abnormalities, and in t-AML (5%). *NPM1* mutations, most commonly involve exon 12, resulting in alterations at the C-terminus, i.e., replacement of tryptophan(s) at position 288 or 290 and the creation of a nuclear export signal motif, which mediates aberrant localization of the protein to the cytoplasm. In the absence of *FLT3* mutations, *NPM1* mutations are associated with a favorable prognosis [13]. Of note, the *NPM1* gene located at 5q35 is not mutated in MDS with a $\text{del}(5q)$ [14].

The *TP53* tumor suppressor gene encodes an essential checkpoint protein that monitors the integrity of the genome, and arrests cell cycle progression in response to DNA damage. Mutations of *TP53* are observed in primary MDS and AML *de novo* (5–10%) and, more commonly, in t-MDS/t-AML (25–30%) [10,15]. The spectrum

of mutations includes missense mutations in exons 4–8, as well as loss of the wild type allele, typically as a result of a cytogenetic abnormality of 17p. In t-MDS/t-AML, *TP53* mutations are associated with $-5/\text{del}(5q)$ and a complex karyotype.

The role of epigenetic changes in the pathogenesis and treatment of MDS and AML is becoming increasingly important. Transcriptional silencing via DNA methylation of the *CDKN2B* (*p15^{INK4B}*) gene is observed in a high percentage of patients with t-MDS/t-AML, and is associated with $-7/\text{del}(7q)$, and a poor prognosis [16,17]. Other genes that may be affected by DNA methylation include the *CTNNA1* gene on 5q.

4. Gene expression profiling of CD34+ progenitor cells in t-AML patients

To expand our understanding of the molecular basis of t-AML, we performed expression profiling of CD34+ progenitor cells from bone marrow samples from 14 t-AML patients using the Affymetrix platform [18]. Although many of the leukemias contained multiple cytogenetic abnormalities, we identified two major groups (A and B) with unique expression profiles. Group A included all patients with -7 , but no abnormality of chromosome 5. In contrast, patients with an abnormality of chromosome 5 (with or without abnormalities of chromosome 7) clustered into a second group (Group B). t-AML patients with abnormalities of chromosomes 5 or 7 have similar clinical and morphological features; thus, the possibility that these leukemias would segregate into distinct expression subgroups was not anticipated. The remainder of the patients with other abnormalities or with a normal karyotype clustered into Group A. In Group A t-AML cases, we observed loss of expression of the genes encoding the TAL1, GATA1, and EKLf transcription factors, which are key regulators of early hematopoiesis (TAL1), and the development of the erythroid (EKLf, GATA1) and megakaryocyte (GATA1) lineages. In contrast, *FLT3*, *PI3K/PIK3C2B*, and *BCL2* were highly expressed in Group A leukemias. The *FLT3*-ITD mutations noted in AML *de novo* do not occur in t-MDS/t-AML, suggesting that *FLT3* is deregulated by an alternative mechanism in t-AML. Downstream targets of the *FLT3* kinase include the p85 subunit of PI3K, which activates the AKT serine/threonine kinase, resulting in expression of antiapoptotic proteins, such as BCL-X_L and BCL2. The coordinated overexpression of *FLT3*, *PI3K*, and *BCL2* suggests that FLT3 may play a role as a survival factor by regulating the expression of BCL2 through the PI3K pathway.

Group B t-AML cases with $-5/\text{del}(5q)$ have high expression of proliferation signature genes, such as the genes encoding cyclin A2, cyclin E2, CDC2, CKS2, BUB1, and MYC. In contrast, the expression of the *IRF8* (*ICSBP*) gene (interferon consensus sequence-binding protein) was significantly down-regulated in Group B leukemias when compared with CD34+ cells from healthy individuals. *IRF8* functions as a negative regulator of IFN-induced genes, and plays a role in regulating proliferation, differentiation, and apoptosis of hematopoietic cells. Taken together, these results suggest that t-AML is characterized by deregulation of genes that are critical to the development of HSCs, as well as proliferation, survival and differentiation of hematopoietic cells.

5. Molecular models for abnormalities of chromosomes 5 and 7 in myeloid disorders

The genetic consequences of a deletion may be a reduction in the level of one or more critical gene products (haploinsufficiency), or complete loss of function. The latter model, known as the “two-hit model”, predicts that loss of function of both alleles of the target gene would occur, in one instance through a detectable chromosomal loss or deletion and, in the other, as a result of a subtle

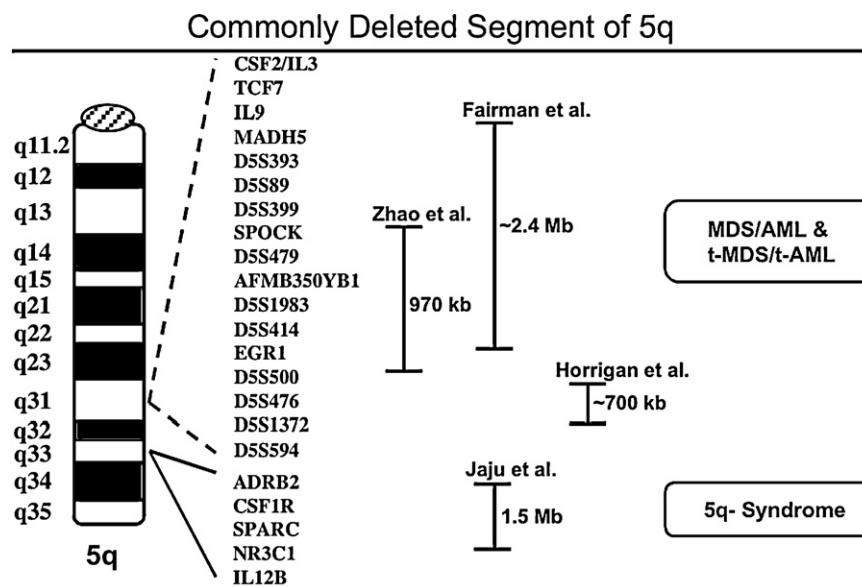


Fig. 2. Idiogram of the long arm of chromosome 5 showing chromosome markers and candidate genes within CDSs as reported by various investigators. The proximal CDS in 5q31.2 was identified in MDS, AML and t-MDS/t-AML, whereas the distal CDS in 5q33.1 was identified in MDS with an isolated del(5q) (the 5q– syndrome).

inactivating mutation, or other mechanisms, such as transcriptional silencing. The relatively long latency period between the time of exposure and t-MDS/t-AML with abnormalities of chromosomes 5 or 7 (~5 years) is compatible with a two-step mechanism in which two mutations of a target gene must occur in a stem/progenitor cell. Efforts to identify putative myeloid leukemia genes on 5q and 7q have been complicated for a number of reasons, including (1) clinical heterogeneity and the possibility that different genetic lesions of 5q or 7q may be associated with specific disease phenotypes; (2) the absence of families with an inherited leukemia predisposition gene mapping to chromosomes 5 or 7 (note, a predisposition gene has been mapped to 16q) [19]; (3) the lack of homozygous deletions of 5q or 7q in myeloid leukemias, which have facilitated tumor suppressor gene (TSG) identification in many solid tumors [20,21]. Thus, unlike solid tumors, TSG inactivation in MDS/AML may rarely be associated with homozygous deletions.

In the alternative model, loss of only a *single copy* of a relevant gene (haploinsufficiency) perturbs cell fate [22,23]. There is growing evidence that a number of cancer-related genes are haploinsufficient, e.g., *TP53*, *SPI1*/*PU.1*, and *RUNX1*. We, and others, have not detected submicroscopic deletions on the “intact” chromosome 5 homologs or mutations of the remaining allele in any of the genes within the commonly deleted segment (CDS), an observation that is compatible with a haploinsufficiency model [20,21]. In this case, traditional positional cloning approaches are highly problematic, as these ultimately rely upon demonstrating mutations in the non-deleted TSG homolog. Finally, it remains theoretically possible that loss of more than one gene, i.e., a contiguous gene syndrome, is necessary to give rise to the characteristic features of MDS/AML or t-MDS/t-AML with a –5/del(5q).

6. Cytogenetic and molecular delineation of the commonly deleted segment of 5q

Several groups of investigators have defined a CDS on the long arm of chromosome 5 predicted to contain a myeloid TSG (Fig. 2) [24–26]. To determine the location of genes on 5q that may be involved in leukemogenesis, we previously examined the deletions in 177 patients, including 44 patients who had t-MDS/t-AML, 116 patients who had MDS or AML *de novo*, and 17 patients who had the 5q– syndrome [20,24]. This analysis revealed that these deletions

were interstitial, and allowed us to define the CDS of 5q as band q31. By using fluorescence in situ hybridization of genomic clones to leukemia cells with a del(5q), we defined a 970 kb CDS within 5q31.2 flanked by D5S479 and D5S500, generated a genomic contig, and determined the genomic sequence of this region [20,24]. In subsequent studies, we generated a transcript map of the CDS, and identified and cloned 20 genes [20]. The functions of the encoded proteins are diverse, and include the regulation of mitosis and the G2 checkpoint, transcriptional control, and translational regulation.

MDS with an isolated del(5q) (the 5q– syndrome) is a distinct subtype of MDS, characterized by a macrocytic anemia, female predominance, and a favorable outcome, with a low risk of transformation to AML. Boulton et al. identified a 1.5 Mb CDS within 5q33.1 between D5S413 and *GLRA1* [26]. This region is distal to the CDS in 5q31.2 found in the patients with AML with del(5q). In summary, the existing data suggest that there are two non-overlapping CDSs in 5q31.2 and 5q33.1 in *de novo* and t-MDS/t-AML, and the 5q– syndrome, respectively (Fig. 2).

7. Identification of haploinsufficient myeloid suppressor genes on 5q

Despite intense efforts, the identification of TSGs on chromosomes 5 and 7 has been challenging. Molecular analysis of the 20 candidate genes within the CDS of 5q31.2 performed in our laboratory did not reveal inactivating mutations in the remaining alleles, nor was there evidence of transcriptional silencing ([20], and Godley and Le Beau, unpublished data). Similarly, molecular analysis of all 44 genes mapping to the CDS in 5q33.1 in the 5q– syndrome did not reveal inactivating mutations [26]. These observations are compatible with a haploinsufficiency model in which loss of one allele of the relevant gene(s) on 5q perturbs cell fate [22]. A number of genes located on 5q including *RPS14* [27], *EGR1* [28], *NPM1* [29], *APC* [30], and *CTNNA1* [31] have been implicated in the development of myeloid disorders due to a gene dosage effect.

The gene encoding *RPS14*, which is required for the processing of 18S pre-rRNA, was identified as a candidate disease gene in the 5q– syndrome by Ebert et al. [27]. Down-regulation of *RPS14* in CD34+ bone marrow cells blocks the differentiation of erythroid cells, and increases apoptosis in differentiating erythroid cells *in*

vitro. However, haploinsufficiency of RPS14 does not account for several features of the 5q– syndrome, including megakaryocytic dysplasia, neutropenia, and clonal dominance. NPM1 is involved in ribosome biogenesis and centrosome duplication, and modulates the activity of the TP53 and CDKN2A tumor suppressors. Absence of *Npm1* results in embryonic lethality, and *Npm1* heterozygous mice develop erythroid dysplasia with elevated mean corpuscular volume and red cell distribution width, normal red blood cell counts and hemoglobin (Hb) levels, and dysplastic megakaryocytes [29]. However, the role of *NPM1* in the pathogenesis of MDS/AML is unclear, since *NPM1* is not deleted in many patients with a del(5q), and mutations have not been identified in the remaining allele [14]. Liu et al. showed that the α -catenin gene (*CTNNA1*) is down-regulated in stem and progenitor cells from MDS and AML patients with a del(5q) as compared to patients lacking del(5q), or normal HSCs [31]. *CTNNA1* is suppressed due to epigenetic silencing in HL-60 cells, a myeloid leukemia cell line used as a model for del(5q) leukemia. Reinduction of *CTNNA1* expression led to reduced proliferation, and an increased frequency of apoptosis, suggesting that down-regulation of α -catenin in HSCs may contribute to transformation of myeloid cells in AML patients with a del(5q) [31]. Our laboratory has investigated the *APC* and *EGR1* genes on 5q, and we discuss the role of these genes in myeloid leukemias below.

7.1. APC

APC is a multifunctional tumor suppressor that is involved in the initiation and progression of colorectal cancer via regulation of the WNT signaling cascade. Qian et al. employed the *Cre-loxP* system to inactivate *Apc* in hematopoietic cells *in vivo* [30]. Conditional inactivation of *Apc* *in vivo* dramatically increased apoptosis and enhanced cell cycle entry of HSCs/HPCs, leading to their rapid disappearance and bone marrow failure. The defect in HSCs/HPCs caused by *Apc* ablation is cell autonomous. In addition, *Apc* loss led to exhaustion of the myeloid progenitor pool (common myeloid progenitors, granulocyte–monocyte progenitors, and megakaryocyte–erythroid progenitors), as well as the lymphoid-primed multipotent progenitor pool. Down-regulation of the genes encoding *Cdkn1a*, *Cdkn1b*, and *Mcl1* occurs after acute *Apc* excision in HSCs. Our preliminary studies have shown that mice with loss of a single allele of *Apc* in bone marrow cells developed a fatal disease at 3–8 months of age, characterized by a macrocytic anemia and monocytosis, characteristic features of MDS and t-MDS/t-AML (Qian et al., unpublished data). In summary, these results suggest that *Apc* is essential for the function of HSCs/HPCs, and raise the possibility that haploinsufficiency of *APC* contributes to the pathogenesis of hematological disorders.

7.2. EGR1

The early growth response 1 gene (*EGR1*) encodes a member of the WT-1 family of transcription factors and contains 3 Cys₂His₂ Zn fingers that bind the GC-rich consensus sequences, GCG(G/T)GGGCG [32]. In the mouse, *Egr1* has been shown to be an early response gene, and mediates the cellular response to growth factors, mitogens, and stress stimuli [32]. *Egr1*^{+/–} or *Egr1*^{–/–} mouse embryonic fibroblasts bypass senescence and have immortalized growth characteristics, suggesting a role for *Egr1* as a “gatekeeper” of p53-dependent growth regulation. *EGR1* has also been shown to act as a TSG in several human tumors, including breast carcinomas and non-small cell lung cancer [33]. Recently, *Egr1* has been shown to be a direct transcriptional regulator of many known TSGs, e.g., *Tp53*, *Cdkn1a/p21*, *Tgfb* and *Pten* (reviewed in [33]).

We characterized the hematopoietic potential of WT, *Egr1*^{+/–}, and *Egr1*^{–/–} mice (15 mice per cohort were monitored for 18 months), and found that heterozygous or homozygous loss of *Egr1* alone under normal physiological conditions does not affect the hematopoietic potential of murine marrow [28]. However, Wagers and colleagues have shown that *Egr1*-deficient mice show spontaneous mobilization of HSCs into the periphery, identifying *Egr1* as a transcriptional regulator of stem cell migration [34]. To investigate whether loss of *Egr1* cooperates with secondary mutations to induce leukemia in the mouse, WT, *Egr1*^{+/–}, and *Egr1*^{–/–} mice were treated with a single dose of 100 mg/kg N-ethyl-nitrosourea (ENU) at 4 weeks or 20 weeks of age. ENU was chosen because it is an alkylating agent, and may recapitulate the effects of alkylating agent chemotherapy in patients who develop t-AML. ENU treated *Egr1*^{+/–} and *Egr1*^{–/–} mice developed a high incidence of immature CD4⁺, CD8⁺ T-cell lymphomas, as compared to WT mice. In addition to the increased rate of lymphomagenesis, *Egr1*^{+/–} and *Egr1*^{–/–} mice developed a myeloproliferative disorder (MPD). With ENU treatment at 4 weeks of age, 33% of *Egr1*^{+/–} mice and 40% of *Egr1*^{–/–} mice develop a MPD, compared to only 13% of wild-type mice. With ENU treatment at 20 weeks of age, 25% of *Egr1*^{+/–} mice and 33% of *Egr1*^{–/–} mice develop a MPD (Fig. 3). Wild-type mice did not develop MPD in this cohort. *Egr1*^{+/–} and *Egr1*^{–/–} mice develop MPD at the same rate and latency, suggesting that loss of a single allele is sufficient for disease predisposition [28]. The development of a MPD following ENU treatment is notable in that MPD has not been observed previously in 20 inbred strains treated with ENU [35].

The MPD was characterized by elevated white blood cell counts (WBC > 19 × 10⁶/ml), anemia (Hb < 7.5 g/dl), and thrombocytopenia (PLTs < 800K/ml), with ineffective erythropoiesis in the bone marrow and spleen. Dysplastic neutrophils were observed in the

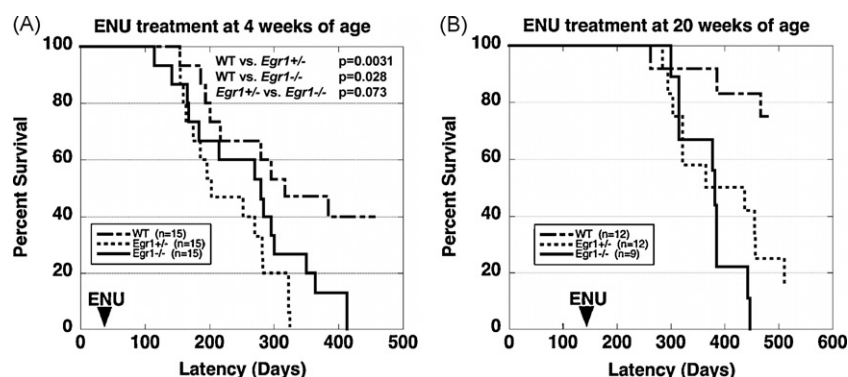


Fig. 3. Survival curves of WT and *Egr1*-deficient mice after ENU treatment at (A) 4 weeks or (B) 20 weeks of age. Note that only one WT mouse treated at 20 weeks developed a lymphoma; two were sacrificed due to severe dermatitis.

bone marrow and peripheral blood with hypersegmented and atypical nuclei and a hypogranular cytoplasm. The bone marrow was hypercellular with full maturation of all lineages, but no increase in blasts. The mice presented with severe splenomegaly, and histological analysis revealed a dramatic increase in early erythropoiesis. By flow cytometric analysis, there was an expansion of erythroid (Ter119+, CD71+) and myeloid (Gr1+, Mac1+) populations in the spleen; however, immature cells (Kit+, CD34+) were not increased. Following the Bethesda Criteria, the disease is classified as a MPD with ineffective erythropoiesis. This MPD recapitulates some, but not all, of the clinical features seen in patients with a $-5/\text{del}(5q)$.

To date, we have not identified mutations of *Egr1* in malignant cells from WT mice, or of the remaining allele or *Egr1*+/- mice, suggesting that *Egr1* acts by haploinsufficiency. We also performed mutation analysis on candidate genes that are known to be involved in primary MDS, AML *de novo*, or t-MDS/t-AML, including *Tp53* (exons 4–9), *Nras* and *Kras* (exons 1–3 encoding amino acids 12, 13, 59, and 61), and *Jak2* (exon 13 encompassing the codon for conserved amino acid 617). No mutations of these genes were detected in MPDs arising in *Egr1*+/- and *Egr1*-/- mice treated with ENU [28]. To identify genes that cooperate with loss of *Egr1* in murine leukemogenesis, we are using a forward genetic screen by retroviral insertional mutagenesis [35]. We have injected cohorts of wild type, *Egr1*+/-, and *Egr1*-/- neonate mice with the MOL4070LTR retrovirus [36]. WT and *Egr1*+/- mice developed disease at 10.5–15 months of age. *Egr1*+/- mice injected with MOL4070LTR develop MDS, MPD, or AML with a shorter latency and at a higher overall frequency than littermate controls. Of note is that the incidence of myeloid disease is higher in *Egr1*+/- mice (44%) than in WT mice (10%), indicating that loss of one allele of *Egr1* shifts the disease spectrum to myeloid neoplasms.

To identify cooperating cancer genes, we have cloned 81 retroviral integrations from 5 AMLs developing in *Egr1*+/- mice. Common insertion sites (CISs) were compared to those in the Retroviral Tagged Cancer Gene Database (RTCGD) maintained by Copeland et al. (<http://rtcgd.ncicfcrf.gov>). To date, we have identified two CISs, both of which have been identified by retroviral insertional mutagenesis in other mouse models of AML. The first CIS is 15 kb upstream of the *Evi1* locus in 3/5 mice. The second insertion site is 4 kb downstream of the *Gfi1b* locus and 23 kb upstream of the *Gtf3c5* locus in 2/5 mice. Of note is that the *EVI1* transcription factor gene is deregulated in human AMLs, particularly those with loss of chromosome 7, and abnormalities of chromosome 3. The *Gfi1* and *Gfi1b* transcription factors play a critical role in HSC survival, as well as erythroid and megakaryocytic development, and their corresponding genes have also been identified as CISs by retroviral mutagenesis studies.

A core set of genes has been identified as “master regulators” of myeloid differentiation. At the level of the granulocyte–monocyte progenitor, overall fate determination involves a balance of two opposing forces: PU.1 promotes monocytic differentiation, whereas CEBPA promotes granulocytic differentiation. Monocytic differentiation is promoted by the PU.1-induced transcription factors EGR2 and NAB2, whereas granulocytic differentiation is promoted by GFI1. EGR1/2 and NAB2 have been found to suppress the expression of *Gfi1* and its downstream targets; conversely, GFI1 suppresses *EGR1*, *EGR2* and *NAB2* (Fig. 4) [37]. In the context of the $\text{del}(5q)$, EGR1 haploinsufficiency would be expected to deregulate myeloid cell differentiation, favoring granulocytic over monocytic differentiation. The *HNRNPA0* gene is also located within the CDS of 5q31.2, and is expressed at reduced levels in CD34+ cells from patients with MDS characterized by a $\text{del}(5q)$ (Young and Le Beau, unpublished data). The *HNRNPA0* protein is a member of the hnRNPA/B family of RNA-binding proteins, and has been shown to regulate transcript stability via binding to the AU-rich element of mRNAs. Using shRNAs in mouse hematopoietic cells, we demonstrated that

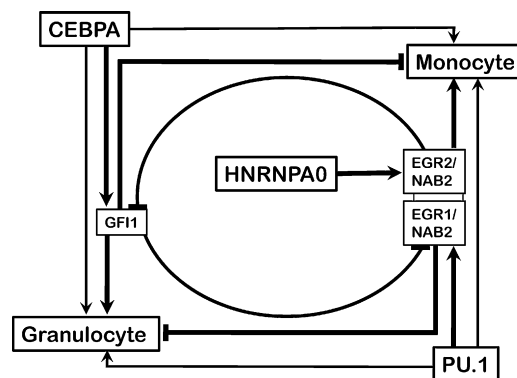


Fig. 4. The network of genes regulating myeloid cell fate at the level of the granulocyte–monocyte progenitor, illustrating some, but not all, of the factors that regulate cell fate. Lines ending in arrows indicate positive regulation, and lines ending in bars indicate inhibitory effects. The thickness of the lines indicates the relative strength of the effect. See text for a description of this network. Adapted from reference [37].

knockdown of *Hnrnpa0* leads to a decrease in the stability of *Egr2* transcripts (Young and Le Beau, unpublished data). Thus, loss of a single allele of *EGR1* and *HNRNPA0* as a result of a $\text{del}(5q)$ may lead to a synergistic disruption of EGR1/2 activity during leukemogenesis.

In summary, *Egr1*-deficient mice may represent the first animal model for AML characterized by $-5/\text{del}(5q)$. Our data suggests that loss of a single allele of *Egr1* cooperates with mutations induced by an alkylating agent in the development of malignant lymphoid and myeloid diseases in mice. Nevertheless, *Egr1* haploinsufficiency alone *in vivo* does not result in expansion of the HSCs or abnormalities in adult hematopoiesis [28]. Collectively, these data support a haploinsufficiency model, in which loss of a single allele of more than one gene on 5q contributes to the pathogenesis of t-MDS/t-AML with $-5/\text{del}(5q)$.

8. Models for the pathogenesis of t-AML

Extensive experimental evidence indicates that more than one mutation is required for the pathogenesis of hematological malignant diseases. Moreover, these mutations cooperate to confer a proliferative and/or antiapoptotic activity, as well as impair normal differentiation pathways. Haploinsufficiency for a gene(s) on 7q and 5q is likely to be an initiating mutation. Pedersen-Bjergaard et al. have proposed eight different pathways that are involved in progression to t-AML [38]. Pathway I consists of patients who have abnormalities of chromosome 7, without chromosome 5 abnormalities. These patients often present with mutations of the RAS pathway (*KRAS*, *NRAS*, *NF1*, *PTPN11*), and methylation silencing of *p15* (*CDKN2B*), and they have a poor prognosis. Loss of *TAL1*, *GATA1*, and *EKLf* expression in t-AML with a $-7/\text{del}(7q)$ may result in impaired differentiation, whereas overexpression of *FLT3*, *PIK3C2B*, and *BCL2* result in a proliferative/survival advantage. Pathway II comprises patients with a $-5/\text{del}(5q)$ with or without abnormalities of chromosome 7, and a poor prognosis (Fig. 5). Haploinsufficiency of multiple genes on 5q is likely to be the initiating event. Genomic instability and complex karyotypes with gain of chromosome 8, and loss of 12p, 13q, 16q, 17p (*TP53* locus), chromosome 18, and 20q, as well as mutations of *TP53* are often observed in this subgroup. In t-AML patients with $-5/\text{del}(5q)$, loss of expression of *IRF8* may lead to impaired differentiation and/or a survival advantage, whereas increased expression of cell cycle regulatory proteins (*CCNA2*, *CCNE2*, *CDC2*) would result in a proliferative advantage.

Pathway III consists of patients with translocations of 11q23. Alterations of pathway IV convey the best prognosis for patients

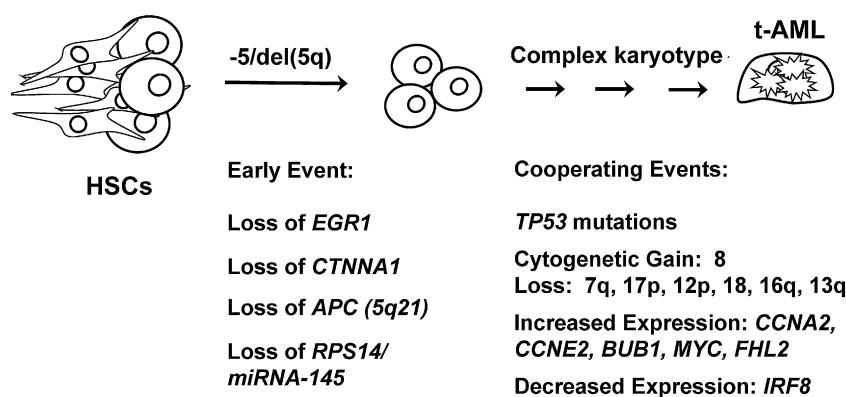


Fig. 5. Cooperating genetic mutations leading to t-MDS/t-AML with a del(5q).

with t-AML, and include the t(8;21) or inv(16). Pathway V comprises patients who present with therapy-related acute promyelocytic leukemia with the t(15;17) resulting in the *PML-RARA* fusion and a good prognosis. Pathway VI involves balanced translocations of *NUP98* at 11p15. Pathway VII includes t-MDS/t-AML with a normal karyotype. Recently, internal tandem duplications of *FLT3* and *MLL* have been described in a few of these patients. Pathway VIII includes patients with other chromosomal abnormalities. New technologies, such as high-throughput genomics technologies, will facilitate further delineation of the genetic pathways leading to t-AML.

9. Concluding remarks

t-MDS/t-AML remains one of the most adverse complications of successful therapy for a variety of malignant and non-malignant conditions. The factors that place individual patients at risk are beginning to be elucidated, and are critical for risk-assessment, and to allow individualized therapy directed at minimizing the development of this disease. Moreover, characterizing the genetic pathways that give rise to t-MDS/t-AML will lead to a greater understanding of the molecular features of the disease and, ultimately, may lead to more targeted therapies for its treatment.

Conflict of interest statement

None

Acknowledgements

We thank the patients, and the many members of the Leukemia Program at the University of Chicago who participated in these studies, especially Drs. Sonali M. Smith, Theodore Karrison, and Lucy A. Godley.

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