Pathogenesis of acute myeloid leukaemia and inv(16)(p13;q22): a paradigm for understanding leukaemogenesis?

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Summary

Acute myeloid leukaemia (AML) has been proposed to arise from the collaboration between two classes of mutation, a class I, or proliferative, mutation and a class II, or blocking, mutation. A limitation of this so-called 'two-hit' hypothesis has been the lack of identifiable proliferative and blocking mutations in most AML cases. However, it is now known that the CBFβ–MYH11 fusion gene in AML and inv(16), by disrupting the normal transcription factor activity of core binding factor (CBF), functions as a class II mutation. In addition, nearly 70% of patients with AML and inv(16) are known to possess mutually exclusive mutations of the receptor tyrosine kinases (RTKs), c-KIT and FLT3, as well as RAS genes, that provide a class I, or proliferative, signal. AML and inv(16), therefore, is one of the best understood of the acute leukaemias at the genetic level and so provides a paradigm for the 'two-hit' hypothesis of leukaemogenesis. This paper reviews the recent advances in the molecular pathology of AML and inv(16) and discusses possible therapeutic implications of the current pathogenetic model.

Keywords: acute myeloid leukaemia, c-KIT, core binding factor, core binding factorβ–MYH11, pathogenesis.

Acute myeloid leukaemia (AML) is a heterogeneous clonal disorder with individual cases exhibiting variability in clinical presentation, cellular morphology, therapeutic response and overall prognosis. However, although this heterogeneity also extends to the underlying mutations, the end effect is similar, in that each patient's genotype confers deregulated proliferation, impaired differentiation and a survival advantage for the leukaemic cells. The number of known mutations associated with AML continues to grow at an unprecedented pace, with over 300 different chromosomal translocations and other mutational events having been described. It is obvious, therefore, that there are many more leukaemic genotypes than phenotypes. In an attempt to provide a unified molecular theme to explain how different mutations can generate essentially similar phenotypes, Gilliland (2001) has proposed a 'two-hit' model for leukaemogenesis. The basis of the hypothesis is that AML is the consequence of a collaboration between at least two broad classes of mutation; class I mutations that confer a proliferative and/or survival advantage to cells (e.g. *BCR–ABL* and oncogenic RAS) and class II mutations that primarily impair haematopoietic differentiation and subsequent cellular apoptosis (e.g. *CBFβ–MYH11* and *PML–RARα* fusion genes) (Table I). At its simplest, the model predicts that AML results from the combined effects of only two mutations, one from each class. However, a limitation of the model has been the lack of identifiable class I and class II mutations in the majority of AML cases.

Recently, however, it has become apparent that mutations of receptor tyrosine kinases (RTKs) class III and *RAS* frequently provide the 'missing' proliferative signal in AML. The purpose of this review is to discuss these findings in the context of the pathogenesis of AML and inv(16)(p13;q22) and to highlight that this leukaemic subtype provides a paradigm for the 'two-hit' model of leukaemogenesis.

AML M4Eo and inv(16)

The association of abnormal eosinophils and structural alterations of chromosome 16 was first reported by Arthur and Bloomfield (1983). The karyotypic abnormalities were described as del(16)(q22), and all five patients were noted to have AML (three with AML-M2 and two with AML-M4), and a bone marrow eosinophilia that ranged from 8% to 54%. In the same year, Le Beau *et al* (1983) reported a related cytogenetic–clinicopathological association, that of chromosome 16 inversion (inv16) and AML M4; a finding that was soon confirmed by others (de la Chapelle & Lahtinen, 1983; Testa *et al*, 1984; Schmitz *et al*, 1984; Tantravahi *et al*, 1984). This association between abnormal eosinophils, AML and structural rearrangements of chromosome 16 (AML FAB subtype M4Eo) was confirmed at the Fourth International Workshop on chromosomes in leukaemia: a prospective study of acute non-lymphocytic leukaemia (1984). However, it is now recognized that breakpoints at both 16p13 and 16q22 are required for manifestations of the complete M4Eo syndrome, as patients with the originally described del(16)(q22) have

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Table I. A list of class I and II mutations. The 'two-hit' hypothesis implies that AML is the consequence of at least two mutations, a class I and class II mutation.

Class I mutations	Class II mutations
BCR–ABL	CBFβ–MYH11
N-RAS	AML1–ETO
K-RAS	TEL–AML1
c-KIT (exon 8)	PML–RARα
c-KIT (Asp 816)	NUP98–HOXA9
FLT3 (ITD)	PU.1
FLT3 (Asp 835)	C/CEPα
PTPN11	AML1
NF1	AML1–AMP19
TEL–PDGFRβ	
? AML1–COPINE VIII	

Class I mutations confer a proliferative and/or survival advantage, while class II mutations impair haematopoietic differentiation. AML-Copine VIII may be a unique mutation since the resultant fusion protein has been proposed to possess both class I and II activities (see text).

different morphological and clinical features (Larson *et al*, 1986). Subsequently, Wessels *et al* (1991) reported that both inv(16)(p13;q22) and t(16;16)(p13;q22) have the same short-arm breakpoint and suggested that they be considered variations of the same mechanism. The bone marrow of patients with M4Eo exhibit a variable number of eosinophils at all stages of maturation without obvious maturation arrest. Characteristically, the eosinophilic granules are purple-violet in colour, often larger than those normally present in immature eosinophils, and may be so dense as to obscure the cell morphology. As might be predicted, the eosinophils derive from the leukaemic clone since they possess the inv(16)(p13;q22) rearrangement (Haferlach *et al*, 1996).

In the early 1990s, positional cloning revealed the breakpoints of inv(16) and t(16;16) to lie within the introns of *CBFβ* and *MYH11* (Liu *et al*, 1993, 1995). *CBFβ* at 16q22 encodes the β-subunit of core binding factor (CBF), whereas *MYH11* at 16q13 encodes the smooth muscle myosin heavy chain (SMMHC). The resultant chimaeric protein, CBFβ–SMMHC, is of variable size (Fig 1), resulting from the incorporation of the first five, or rarely, the first four exons of *CBFβ* fused, in frame, to variable lengths of the C-terminal region of *MYH11* (Liu *et al*, 1995). Rarely, the *CBFβ–MYH11* fusion can arise from either variant translocations (Martinez-Climent *et al*, 1999) or insertion mutations (Aventin *et al*, 2000; O'Reilly *et al*, 2000). The reciprocal fusion product (*MYH11–CBFβ*) is not thought to be important since it has not been detected in leukaemic cells and it is deleted in some cases with an unbalanced inversion (Liu *et al*, 1993, 1995; Marlton *et al*, 1995). The inv(16) is difficult to detect by standard cytogenetic analysis but the molecular characterization of the breakpoints has resulted in the development of additional diagnostic strategies, including reverse transcription-polymerase chain

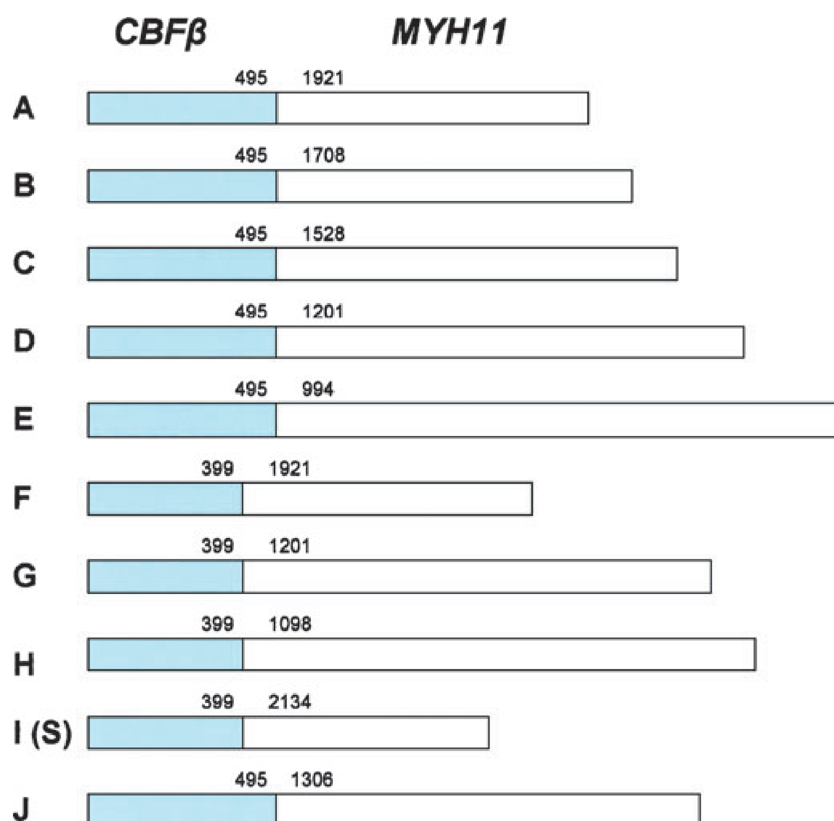

Fig 1. A schematic illustration of the 10 reported CBFβ–MYH11 fusion transcripts (A–J), with nucleotide numbering as in Liu *et al* (1995).

reaction (RT-PCR) (Claxton *et al*, 1994; Hébert *et al*, 1994; Poirel *et al*, 1995), Southern blot analysis (Van der Reijden *et al*, 1995a) and two-colour interphase fluorescence *in situ* hydridization (Dauwerse *et al*, 1999). To date, 10 differently sized *CBFβ–MYH11* transcripts (Fig 1), have been identified, types A-H (Liu *et al*, 1995; Shurtleff *et al*, 1995; Van der Reijden *et al*, 1995b; Costello *et al*, 1997a), type I or S (Dissing *et al*, 1998; Grardel *et al*, 2002; Van der Reijden *et al*, 2001) and type J (Springall *et al*, 1998; Trnkova *et al*, 2003). The majority (85%) of cases of inv(16), or t(16;16), are associated with the type A fusion transcript, corresponding to an in-frame CBFβ nt 495-MYH11 nt 1921 junction (Liu *et al*, 1995), while transcript types B-J are rare. Whether the different fusion genes have any clinical or biological significance is unknown, although there is preliminary evidence that the rare CBFβ–MYH11 transcripts may be associated with therapy-related inv(16) AML (Dissing *et al*, 1998; Grardel *et al*, 2002). The mechanisms that favour the formation of the *CBFβ–MYH11* fusion gene are unclear. Van der Reijden *et al* (1999), following the subcloning and sequencing of the major breakpoint regions, reported that sites of genetic instability and V(D)J recombination may be involved. Recently, it has been suggested that variant *CBFβ–MYH11* fusions may result from the same genotoxic stresses that give rise to *FLT3* and *MLL* intragenic abnormalities (Libura *et al*, 2003).

Structure and function of CBFβ

Core binding factor is a family of heterodimeric transcription factors containing a common β subunit (CBFβ) and one of three CBFα subunits, CBFα1, AML1 (also known as RUNX1 or CBFα2) or CBFα3, all of which encode the so-called runt domain (Ogawa *et al*, 1993a,b) that is required for both DNA binding and interaction with CBFβ (Meyers *et al*, 1993). CBFβ does not bind DNA directly (Wang *et al*, 1996a), but increases the affinities of the CBFα subunits for the consensus DNA sequence TGT/cGGT, which is present in a number of promoters and enhancers of viral and cellular genes. The CBFβ core domain interacts with the AML1 Runt domain at a site distant from the AML1–DNA interface and enhances DNA binding by stabilization of the protein's conformation. CBFβ markedly augments the metabolic stability of AML1, which by itself is highly susceptible to proteasome-mediated degradation (Huang *et al*, 2001). CBF, although originally identified as a transcription regulator of the Moloney murine leukaemia virus (Wang & Speck, 1992) and polyomavirus (Kamachi *et al*, 1990) in mice, is now known to be an important transcriptional activator of genes involved in mammalian haematopoiesis and bone development (Takahashi *et al*, 1995; Komori *et al*, 1997). Indeed, CBF regulates the transcription of a large number of haematopoietic-specific genes, including cell surface receptors, such as the subunits of the T-cell antigen receptor (Redondo *et al*, 1992) and macrophage colony stimulating factor (M-CSF) receptor (Rhoades *et al*, 1996), myeloid associated enzymes, such as myeloperoxidase (Nuchprayoon *et al*, 1994), and granzyme B (Wargnier *et al*, 1995) and cytokines including interleukin-3 (IL-3) (Cameron *et al*, 1994) and granulocyte macrophage colony-stimulating factor (GM-CSF) (Takahashi *et al*, 1995). The crucial involvement of CBF in haematopoieisis is supported by mouse 'knock-out' models, in which homozygous disruption of either *AML1* (Wang *et al*, 1996a,b) or *CBFβ* (Niki *et al*, 1997) resulted in an identical phenotype with failure of definitive haematopoiesis in the liver and death from extensive haemorrhage at around day 12·5.

The fundamental role of the CBF complex in haematopoiesis is underscored by the reports that *CBFβ* and *AML1* are targeted by chromosomal rearrangements in nearly 30% of patients with AML (Look, 1997). Indeed, *AML1* located at 21q22, is involved in a number of different chromosomal translocations including t(8;21)(q22;q22), t(X;21)(p22;q22) and t(19;21)(q13;q22) in AML (Miyoshi *et al*, 1991; Zhang *et al*, 2004; Ramsey *et al*, 2004), t(3;21)(q26;q22) in myelodysplasia and blast crisis of chronic myeloid leukaemia (Mitani *et al*, 1994), and t(12;21)(p13;q22) in pre-B cell acute lymphoblastic leukaemia (Golub *et al*, 1995). Although the CBF leukaemias represent a wide range of clinical features, morphological and immunological phenotypes, the recurrent involvement of the AML1/CBFβ complex suggests a similar underlying pathogenesis. Indeed, all of the translocations highlighted generate chimaeric proteins that retain the Runt domain of AML1 and are predicted to interfere with normal CBF activity. Furthermore, acquired point mutations of *AML1*, mostly incurring loss of function, have been reported in cases of AML (Preudhomme *et al*, 2000), while inherited mutations of *AML1* have been associated with a familial platelet disorder and a propensity to develop AML (Michaud *et al*, 2002). A crucial question, therefore, is how does CBFβ–MYH11 and other CBF fusion genes perturb the normal function of AML1 and contribute to leukaemogenesis?

Leukaemogenetic consequences of CBFβ/MYH11

An early clue to the pathogenetic consequences of CBFβ–SMMHC expression was the finding, in a mouse 'knock-in' model, that the fusion protein exerted a dominant negative effect (Castilla *et al*, 1996). In these experiments, *CBFβ–MYH11* was introduced into the mouse genome to replace a single copy of the *CBFβ* gene, with the chimaeric gene expression being controlled by the endogenous CBFβ promoter, so as to recapitulate the condition in leukaemic cells. It was noted that CBFβ–SMMHC expression dominantly suppressed the function of the AML1–CBFβ heterodimer, with the mice failing to develop definitive haematopoiesis and exhibiting mid-gestational lethality, as reported for $AML1^{-/-}$ and $CBF\beta^{-/-}$ 'knock-out' mice (Okuda *et al*, 1996; Wang *et al*, 1996a,b). Kundu *et al* (2002) have documented a deficiency of haematopoietic stem cells and progenitors in *CBFβ–MYH11* 'knock-in' mice and concluded that the fusion protein blocks embryonic haematopoiesis at the stem-progenitor cell level.

In addition, expression of CBFβ–SMMHC in myeloid lineage cells impairs neutrophilic differentiation (Kogan *et al*, 1998), while increasing the number of blast-like cells in culture (Miller *et al*, 2001). A similar interference of AML1-dependent transcriptional activation has also been reported for the AML1–ETO and AML1–EVI1 fusion proteins (Meyers *et al*, 1995; Zent *et al*, 1996).

Two co-existing mechanisms, referred to as the 'sequestration' and 'co-repressor-recruiting' models, have been postulated to explain the dominant negative effect of chimaeric CBFβ–SMMHC (Fig 2). The 'sequestration' model is based on the observations that CBFβ–SMMHC can retain AML1 in the cytoplasm as deposits on cytoskeletal filaments or aggregates (Adya *et al*, 1998), or in the nucleus as multimerized complexes that can form large rod-like inclusion bodies (Wijmenga *et al*, 1996). The end result is that AML1 is physically confined to the complexes and has only limited access to cognate binding sites on chromosomes, thereby disrupting its regulation of gene expression. These observations are compatible with the finding that CBFβ–SMMHC slows cell cycle progression from G_1 to S phase (Cao *et al*, 1998). The 'co-repressor-recruiting' model, first proposed by Lutterbach *et al* (1999), followed the discovery that the inv(16) fusion protein associates with AML1 in a ternary complex with the mSin3A corepressor (Fig 2). The functional relevance of this observation was confirmed by the demonstration that the C-terminal 163 amino acid region of the myosin tail acts as a transcriptional repressor when fused to the Ga14 protein and assayed using a Ga14-TK reporter system. Subsequently, Durst *et al* (2003) showed that CBFβ–SMMHC specifically associates not only with mSin3A but also histone deacetylase 8 (HDAC 8) via a repression domain in the C-terminal SMMHC region. These events convert AML1 into a constitutive transcriptional repressor, since HDACs induce deacetylation of nucleosomal core histone tails, leading to a tight chromatin conformation and resultant gene silencing (Heinzel *et al*, 1997; Alland *et al*, 1997). Both models, however, are dependent on the inactivation of normal AML1 function, despite the presence of residual wild-type CBFβ. The explanation appears to be that CBFβ–SMMHC can heterodimerize with the Runt domain of AML1 with a much greater affinity compared with that of CBFβ (Lukasik *et al*, 2002; Huang *et al*, 2004). Indeed, it had already been appreciated that CBFβ-MMHC can stabilize AML1 against proteasome-mediated degradation to a greater degree than CBFβ (Huang *et al*, 2001).

The characteristic maturation block of CBF leukaemias may also result from the down-modulation of CCAAT/enhancer binding protein (C/EBPα), a myeloid transcription factor required for granulocytic differentiation. Interestingly, C/EBPα point mutations were already known to provide a 'type II mutation' in approximately 7–11% of AML cases (Pabst *et al*, 2001a; Preudhomme *et al*, 2002) although not in CBF leukaemias. However, recent studies have shown that decreased levels of C/EBPα expression can frequently occur in AML with inv(16)(Cilloni *et al*, 2003), as well as AML t(8;21) (Pabst *et al*, 2001b), a feature that is likely to contribute to the maturation block.

In conclusion, the functional activity of the *CBFβ–MYH11* fusion gene clearly fulfills the requirements for a class II, or 'blocking', mutation and so provides a paradigm for the molecular understanding of impaired haematopoietic differentiation that characterizes AML. It is now known that other leukaemogenic fusion proteins, whether they affect AML1-dependent transcriptional control or not, such as AML1–ETO, TEL–AML1 or PML–RARA, repress transcription through similar interactions with mSin3A, N-CoR and HDACs (Amann *et al*, 2001; Fenrick *et al*, 1999; He *et al*, 1998).

Is expression of CBFβ/MYH11 sufficient for AML?

There is strong evidence that CBF oncoproteins cannot transform haematopoietic stem cells alone, but instead must co-operate with additional mutations to induce acute leukaemia. This concept has emerged from the collective results of animal model studies, minimal residual disease monitoring and the finding that translocations associated with some paediatric leukaemias may be prenatal in origin.

Chimaeric mice, for example, created by inserting the *CBFβ–MYH11* fusion gene into the mouse CBFβ locus in embryonic stem cells, failed to develop leukaemia at high

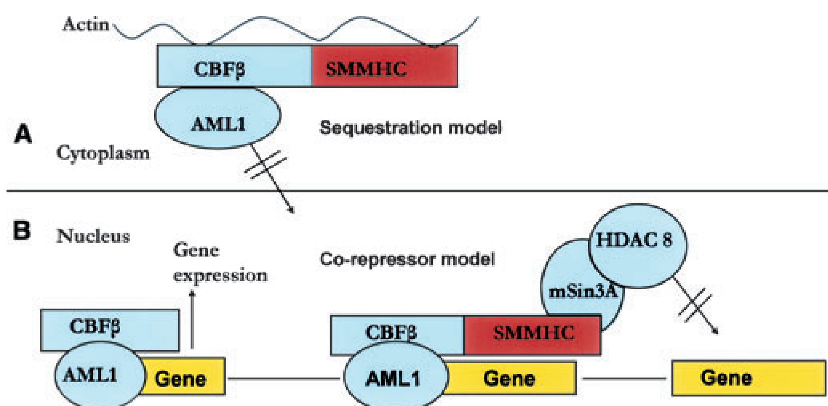

Fig 2. Pathogenetic effects of the CBFβ–SMMHC fusion protein. (A) The 'sequestration' model is based on the observation that CBFβ–SMMHC retains AML1 in the cytoplasm as deposits on cytoskeletal filaments. (b) The 'co-repressor-recruiting' model reflects the known ternary complex formation of CBFβ–SMMHC, AML1 and the mSin3A corepressor. The resultant effects of sequestration and co-repressor recruitment is gene silencing, because of impaired AML1 function, and the eventual blocking of haematopoietic differentiation.

frequency within the first year of life (Castilla *et al*, 1996). Similarly, no leukaemia developed in the mouse model in which *CBFβ–MYH11* was driven by a myeloid-specific promoter, *MRP8* (Kogan *et al*, 1998). It seems likely, therefore, that CBFβ–MYH11 by itself is insufficient to initiate leukaemia, at least in the mouse. In contrast, Castilla *et al* (1999) reported that 4–16 week-old chimaeras developed leukaemia, with some features of inv(16)-containing AML, several months after exposure to one dose of the DNA-alkylating agent, *N*-ethyl-*N*-nitrosourea (ENU), a potent mutagen. Furthermore, co-expression of CBFβ–SMMHC with the human papillomavirus E7 oncogene, or expression of the fusion protein in the absence of the tumour suppressor genes $p16^{INK4a}$ and $p19^{ARF}$ leads to acute leukaemia (Yang *et al*, 2002). The need for additional genetic events for full transformation has also been demonstrated for AML1–ETO, as mice expressing the fusion protein only develop AML, and to a lesser extent T-cell acute lymphoblastic leukaemia, after exposure to ENU (Rhoades *et al*, 2000; Higuchi *et al*, 2002). In the study by Higuchi *et al* (2002), for example, ENU treatment resulted in 40% of the AML1–ETO-expressing animals developing granulocytic sarcomas and AML that was highly reminiscent of human t(8;21) disease. AML1–ETO expression alone, however, resulted in only minimal haematopoietic abnormalities (Yuan *et al*, 2001). Collectively, these animal model experiments suggest that CBF-translocations predispose mice to leukaemia, but that secondary, or additional, mutations are necessary for the onset of AML.

There is also mounting clinical evidence that CBFβ–MYH11 on its own is insufficient for AML development. Cells expressing CBFβ–MYH11, for example, lack a significant growth advantage and are able to maintain their capacity for terminal differentiation. Several groups have reported persistence of chimaeric CBFβ–MYH11 transcripts even after allogeneic bone marrow transplantation (Tobal *et al*, 1995; Costello *et al*, 1997b), while some AML patients in long-term remission retain the *AML1–ETO* fusion gene in a small, but stable, fraction of their bone marrow cells (Nucifora *et al*, 1993; Miyamoto *et al*, 1996). In addition, AML1–ETO expressing cells can retain their capacity to undergo terminal differentiation and form mature cells of myeloid, erythroid and B-cell lineages (Miyamoto *et al*, 2000). Recently, the prenatal origin of childhood leukaemia harbouring *CBFβ–MYH11* has been reported with postnatal latencies of approximately 10 years (McHale *et al*, 2003), while Wiemels *et al* (2002) detected AML–ETO in Guthrie blood spots of children who developed a corresponding AML many years later. The long latency periods have been interpreted to reflect postnatal persistence of translocation-positive, quiescent multi-potent cells, which, upon later recruitment into the myeloid differentiation pathway, acquire additional secondary changes necessary for leukaemia. Two independent groups have extended these observations and demonstrated that CBF translocations are present in cord blood at a rate that is significantly greater than the cumulative risk of the corresponding leukaemia (Mori *et al*, 2002; Basecke *et al*, 2002). Taken together, these clinical and experimental studies strongly support the concept that the CBF fusion genes are present in potential leukaemic precursor cells but that additional mutagenic 'hits' are necessary for transformation. However, these studies have not provided any clues as to the nature of the secondary events.

What is the nature of the 'second hit'?

Early evidence that activated tyrosine kinases might provide a proliferative signal in the pathogenesis of AML came from rare, but highly informative, cases of chronic myeloid leukaemia (CML) in transformation. For example, cases of CML that acquired an additional inv(16)(p13;q22) in blast crisis (Asou *et al*, 1992; Heim *et al*, 1992; Evers *et al*, 1992; Mohamed *et al*, 2003), or accelerated phase (Colovic *et al*, 1998; Enright *et al*, 1992) have been documented, with most of the patients having myelomonocytic characteristics associated with marrow eosinophilia, i.e an M4Eo phenotype. Because *BCR/ABL* alone is sufficient to initiate CML, it has been argued that the cooperation of the two mutations is all that is required for the development of the acute leukaemic transformation, with *CBFβ–MYH11* dictating the phenotype. Similar conclusions can be drawn from the rare interactions of *BCR–ABL* with other class II mutations, namely *PML–RARA* (Scolnik *et al*, 1998), *AML1–ETO* (Ferro *et al*, 1992; Kojima *et al*, 1999), *AML1-EVI1* (Cuenco & Ren, 2001) and *NUP98–HOXA9* (Yamamoto *et al*, 2000). However, the universality of the 'two-hit hypothesis' would require all acute leukaemias to harbour genetic changes that confer proliferative and/or survival signals. The problem has been that, until recently, the nature of the predicted 'second-hit' has not been known for most AMLs.

Role of RTK/RAS signalling pathway

Support for the 'two-hit' hypothesis came unexpectedly in 1996 when Nakao *et al* (1996) reported somatic alterations of the FLT3 gene in a small study of AML and suggested that these mutations might play an important role in leukaemogenesis (Fig 3). The changes consisted of internal tandem duplications (ITD) of the juxtamembrane domain coding sequence, primarily involving exon 14, but occasionally involving intron 14 and exon 15 (see Abu-Duhier *et al*, 2001a, for revised exon numbering). Irrespective of the type of mutation, however, all FLT3-ITDs appear to result in constitutive dimerization and activation of the receptor (Kiyoi *et al*, 1998; Hayakawa *et al*, 2000; Tse *et al*, 2000). Furthermore, Mizuki *et al* (2000) demonstrated that FLT3-ITD mutations induce factor-independent growth and leukaemogenesis of 32D cells and that this effect was mediated by the RAS and STAT5 pathways. The presence of FLT3 ITD was subsequently confirmed by many groups to be present in approximately 25% of AML cases and to be a poor prognostic factor

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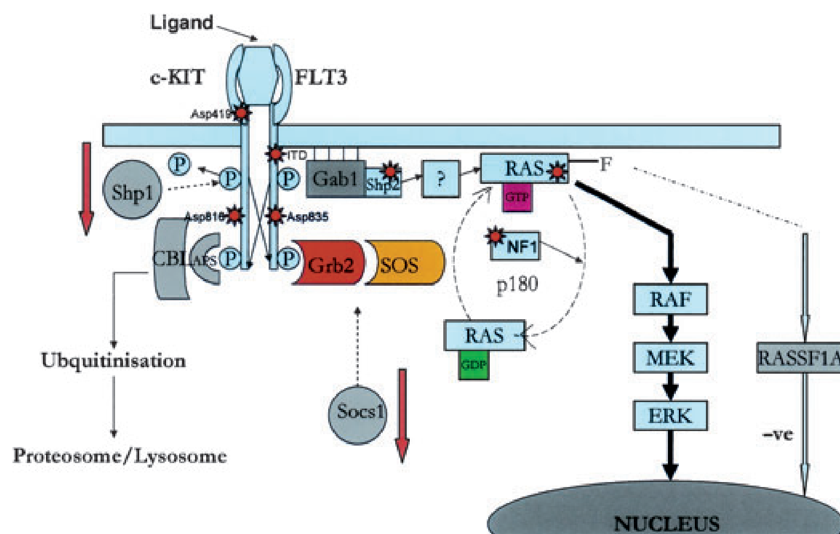

Fig 3. A schematic representation of reported class I, or proliferative, mutations affecting the RTK/RAS signalling pathway in myeloproliferative disorders. ★, location of class I mutations: arrows, reduced levels of Shp1 and Socs1 (negative regulators of RTK/RAS) secondary to gene hypermethylation.

(Abu-Duhier *et al*, 2000; Kottaridis *et al*, 2001; Stirewalt *et al*, 2001; Whitman *et al*, 2001). However, *FLT3* ITD mutations, although common in AML, are not randomly distributed within the various cytogenetic subgroups. Kottaridis *et al* (2001), for example, reported *FLT3* ITDs to be most common in AML patients exhibiting either a t(15;17) (37%) or a normal karyotype (34%) and to be uncommon in patients with CBF AML, occurring in only 9% and 7% of cases with t(8;21) and inv(16) respectively. Nevertheless, the association of FLT3 ITDs and CBF leukaemias, has now been confirmed by a number of groups (Abu-Duhier *et al*, 2000; Frohling *et al*, 2002; Schnittger *et al*, 2002), and suggests that this mutation can provide the 'missing' proliferative, or class I, signal in a minority of cases. In support of this conclusion, FLT3 ITD has been reported to cause a myeloproliferative disorder in an animal model, characterized by leucocytosis with normal myeloid maturation, but not AML (Kelly *et al*, 2002a). Two independent groups subsequently reported FLT3 activation loop mutations involving Asp835 in 7% of AML cases (Abu-Duhier *et al*, 2001b; Yamamoto *et al*, 2001). Interestingly, these mutations, which occur in approximately 5% of AML with inv(16) (Care *et al*, 2003), appear to be independent of FLT3 ITDs, suggesting that FLT3 is the target gene most commonly mutated in adult AML. In addition, it has been suggested that Asp835 mutations may be more commonly associated with other molecular abnormalities, especially inv(16) (Nomdedeu *et al*, 2001; Thiede *et al*, 2002).

Constitutively activating mutations of *c-KIT* have been strongly linked to the pathogenesis of mast cell disease, as well as gastrointestinal stromal tumours (GISTs) and sinonasal natural killer cell lymphomas (reviewed, Reilly, 2002). Recently, however, an intriguing association between *c-KIT* mutations and CBF AMLs has been documented. Beghini *et al* (1998), for example, reported an Asp816Tyr activating kinase (TK2) domain mutation in an AML-M2 with t(8;21) and later strengthened this link by documenting similar mutations in four of nine cases with t(8;21) and two of six cases with inv(16) (Beghini *et al*, 2000a). A case of trisomy 4 was also shown to possess duplication of the *c-KIT* mutation (Beghini *et al*, 2000b), while the t(8;21) Kasumi-1 cell line contains an activating *c-KIT* Asn(822)Lys mutation (Beghini *et al*, 2002). Subsequently, Gari *et al* (1999) reported novel *c-KIT* exon 8 deletion-plus-insertion mutations in a third of patients with AML and inv(16), a finding that has been confirmed by several groups (Böll *et al*, 2002; Goemans *et al*, 2003). Despite the marked heterogeneity of *c-KIT* exon 8 mutations, over 90% of cases have a deletion of the highly conserved Asp419 located near the fifth immunoglobulin-like domain (Care *et al*, 2003). Indeed, Böll *et al* (2002) documented a single case in which deletion of Asp419 was the sole abnormality. Structure-function analysis has indicted that the three N-terminal immunoglobulin-like repeats of c-KIT are involved in ligand binding, the fourth in receptor dimerization, while the fifth is associated with proteolytic cleavage of the receptor and modification of normal ligand binding (Broudy *et al*, 2001). It is possible therefore, that mutations around Asp419 could alter the conformation of the fourth and fifth immunoglobulin-like repeats and lead to receptor activation or altered ligand affinity. The finding that *c-KIT* exon 8 mutations increase the relapse rate of AML patients provides indirect evidence for a 'gain-of-function' (Care *et al*, 2003). Novel mutations have also been reported in c-FMS, a further member of the class III RTK, in small number of AML patients, including a single case with inv(16) (Abu-Duhier *et al*, 2003). Overall, RTK mutations may provide a class I, or proliferative, signal in approximately 40% of patients with AML and inv(16) (Care *et al*, 2003). In addition, RTK mutations appear to be mutually exclusive, suggesting that they provide an equivalent or, at least, redundant oncogenic stimulus in leukaemogenesis.

Oncogenic *RAS* mutations, which occur with a frequency of approximately 30–40% in AML (Beaupre & Kurzrock, 1999; Reuter *et al*, 2000), are known to provide the signals for

enhanced cell proliferation and survival. *N-RAS* mutations are the most common, occurring in 20–25% of AML patients, with *K-RAS* mutations being found in 10–15% of cases. These activating mutations usually involve single amino acid substitutions at codons 12, 13 and 61, which abrogate intrinsic RAS GTPase activity and lead to constitutive RAS activation. In a study by Valk *et al* (2004), *RAS* mutations were found in 33% of patients with AML and inv(16). Crucially, the incorporation of both *RAS* and RTK mutational analysis in the same cohort of patients increased the class I mutational rate to nearly 70%, the highest frequency reported for any AML subclass. The finding of mutual exclusivity was again a feature, with only four cases possessing both a RAS and a RTK mutation (Valk *et al*, 2004). The view that RAS mutations can provide a class I signal that is insufficient on its own to induce AML is supported by animal studies. For example, in a transplant model using bone marrow cells retrovirally transduced with oncogenic N-RAS, recipient animals developed a myeloproliferative disorder with a long latency, suggesting that secondary mutations were necessary for the phenotype (MacKenzie *et al*, 1999). Recently, Chan *et al* (2004) developed a mouse model in which expression of oncogenic K-RAS resulted in a myeloproliferative disease rather than AML. These two studies provide further support for the 'two-hit' hypothesis and indicate that mutations in addition to oncogenic RAS are required for the development of acute leukaemia.

Additional evidence for the 'two-hit' hypothesis of AML

A variety of mutations that function as either a class I or class II signal have now been identified (see Table I). If the two-hit hypothesis is valid, then one might predict that any two mutation combination, one from each class, could give rise to an acute leukaemia. Indeed, many such combinations have now been reported for AML, including mutations in AML1 and FLT3 in AML FAB type M0 (Matsuno *et al*, 2003), FLT3 and PML–RARα in APML (Kottaridis *et al*, 2001) and TEL/PDGFRβ and AML1–ETO in AML (Golub *et al*, 1994). Indirect evidence for the 'two-hit' hypothesis is provided by the finding of a high degree of mutual exclusivity for mutations from within each class. For example, there are only rare reports of AMLs possessing more than one class I mutation (Kiyoi *et al*, 1999; Stirewalt *et al*, 2001; Valk *et al*, 2004), while mutual exclusivity characterizes the proliferative mutations that involve *RAS*, *NF1* and *SHP1 (PTPN11)* in juvenile chronic myelomonocytic leukaemia (Tartaglia *et al*, 2003; Johan *et al*, 2004a). Mutual exclusivity is also a feature of class II mutations since mutations of C/EBF and PU.1 are rare in the CBF leukaemias (Pabst *et al*, 2001a; Mueller *et al*, 2002). Several animal models have confirmed the cooperation of class I and II mutations in the pathogenesis of acute leukaemia. For example, Kelly *et al* (2002b) have shown that PML–RARα and FLT3-ITD cooperate in the development of acute promyelocytic leukaemia, while Grisolano *et al* (2003) have shown that the class I mutation TEL/PDGFRβ cooperates with AML1–ETO to induce AML. Finally, is it possible for a single mutation to provide both a class I and II signal? This scenario has recently been suggested for a mutation reported in a case of secondary AML and t(12;21) (Ramsey *et al*, 2003). The fusion gene, *AML1–Copine VIII*, has been suggested to act as both a class I mutation, since disruption of Copine VIII would affect its role as negative regulator of cell proliferation, as well as a class II mutation, since AML1 truncation would block differentiation.

Is the two-hit hypothesis for CBF AML an oversimplification?

While the 'two-hit' hypothesis has the advantage of providing a unified molecular theme for the pathogenesis of AML, there are data to suggest, at least for some CBF AML patients, that this model might be an oversimplification. Firstly, it has been well documented that certain secondary non-random chromosomal abnormalities are associated with the common AML translocations. Trisomy 22, for example, is a frequent finding in patients with inv(16) (Langabeer *et al*, 1998; Berger & Coniat, 2000), while trisomy 8 and del(9q) are associated with t(15;17) and t(8;21) respectively (Johansson *et al*, 1994). Interestingly, the link between chromosome 22 and inv(16) has been further strengthened by reports of trisomy 22 in patients with cytogenetically cryptic inv(16) (Wong & Kwong, 1999), while the coexistence of isochromosome 22q and inv(16) suggests that duplication of 22q may contain critical gene(s) involved in the pathogenesis of AML M4Eo (Gad *et al*, 1993). However, class I mutations and non-random chromosomal changes are not mutually exclusive in CBF AML, with the result that some patients may have at least three genetic abnormalities (unpublished observations). Secondly, the *CBF*β*–MYH11* fusion gene can be associated with genetic loss from chromosome 16. For example, it is well established that deletions 3′ from *MYH11*, involving a region 160–350 kb centromeric to the 16p13 short-arm inversion breakpoint, are common (Marlton *et al*, 1995; Martinet *et al*, 1997), while 10% of cases have deletions of approximately 170 kb telomeric to CBFβ (Kolomietz *et al*, 2001). These deletions are associated with the loss of genes, including *MRP*, the gene for multidrug resistance associated protein, and *ARA* (anthracycline resistance associated), an ATP-binding cassette gene (Kuss *et al*, 1996; Van der Kolk *et al*, 2000; Kuss *et al*, 1998). *MRP*, located at 16p13·13, lies proximal to the primary breakpoint and its loss of function may play a role in determining prognosis, as deletions correlate with longer periods of complete remission and survival (Kuss *et al*, 1996). In contrast, Kolomietz *et al* (2001) reported in a small study that patients with associated 3′ CBFβ deletions exhibited refractory disease. Similarly, large deletions 5′ to the ETO breakpoint are recurrent events in patients with t(8;21) AML (Godon *et al*, 2002). The reason why genetic loss is a characteristic feature of chimaeric gene formation, especially *CBF*β*–MYH11* and *BCR–ABL* (Huntly

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et al, 2003), is poorly understood, but it appears that considerable genetic heterogeneity may result from seemingly identical translocations. In the future, it will important to determine if this genetic loss is crucial for leukaemogenesis or whether it merely contributes to the observed variation in clinical behaviour. Aberrant promoter methylation of a number of genes in CBF AML has been reported, including *p15* in AML with inv(16) and MEIS I in t(8;21) (Wong *et al*, 2000; Lasa *et al*, 2004). Whether these locus-specific epigenetic inactivations are a direct consequence of the translocation, or represent additional levels of complexity unrelated to the class II mutation, remains to be determined.

Finally, the two classes of genetic abnormality may not be as functionally exclusive as previously believed. The recent finding by Zheng *et al* (2003) suggests that FLT3-ITD may exert its effect by acting both as a differentiation blocker, in that it suppresses the transcription factors C/EBPα and PU.1, as well as augmenting proliferation and survival. In addition, AML1–ETO, while resulting in a maturation block, also augments granulocyte colony-stimulating factor-dependent proliferation and expansion of human haematopoietic stem cells (Mulloy *et al*, 2002). Nevertheless, the possibility that some patients with AML and inv(16) possess additional pathogenetic events, resulting from genetic or epigenetic changes, does not detract from the basic 'two-hit' model in which the AML phenotype requires the cooperation of at least one proliferative and one blocking event.

Therapeutic implications of the 'two-hit' hypothesis of CBF AML

Clinically, the CBF leukaemias have been associated with a high rate of complete remission (CR) and favourable outcome when compared with other AML subsets (Grimwade *et al*, 1998; Byrd *et al*, 2002), with prolonged CR often being achieved with intensive postremission chemotherapy (Bloomfield *et al*, 1998; Razzouk *et al*, 2001; Byrd *et al*, 2002). Such concordant observations have raised the question of the value of allogeneic stem cell transplantation in first CR in patients with a histocompatible donor (Burnett *et al*, 2002; Delaunay *et al*, 2003). Nevertheless, a recent study of 110 cases of inv(16)/t(16;16) AML reported an estimated overall survival, disease-free survival and cumulative incidence of relapse at 3 years of 58%, 48% and 42%, respectively, clearly highlighting the need for improved treatment regimens (Delaunay *et al*, 2003). It is unlikely, however, that dose escalation of current chemotherapeutic agents will significantly improve clinical outcome and novel therapeutic approaches need to be explored. In this regard, the elucidation of the pathogenetic roles of *CBFβ–MYH11* and the various cooperating class I mutations should provide a rational basis for the development of targeted therapy. For example, it may be possible to reverse the block in differentiation mediated by CBFβ–SMMHC. There is 'proof of principle' for this approach, based on the efficacy of all-*trans* retinoic acid to overcome the dominant negative block in differentiation caused by the PML–RARA fusion protein (Melnick & Licht, 2000). In addition, it may be possible to develop small molecules that inhibit HDAC activity, as HDAC-dependent transcriptional repression appears to be a common pathway in leukaemogenesis, and so allow maturation and apoptosis of the leukaemic cells. Indeed, histone deacetylase as well as DNA methyltransferase inhibitors have been incorporated into treatment of AML1–ETO positive leukaemias, with their ability to reverse the inhibition of myeloid-specific genes helping to re-establish a normal differentiation programme (Wang *et al*, 1999; Klisovic *et al*, 2003).

The identification that activating *c-KIT*, *FLT3* and *RAS* mutations act as class I mutations and provide a proliferative signal in AML has stimulated a number of groups to search for inhibitors as possible therapeutic interventions. This drive has been stimulated by the clinical success of imatinib mesylate, a relatively potent inhibitor of *BCR–ABL*, in CML (Druker & Lydon, 2000). Interestingly, imatinib also inhibits wild type c-KIT, some forms of mutated c-KIT, PDGFRα and β, although not c-FMS or FLT3 (Buchdunger *et al*, 2000; Ueda *et al*, 2002). The frequent mutation of *c-KIT* in CBF-AML suggests a role for imatinib in these disorders, although specific inhibitors will need to be developed to block the constitutive activating mutations at codon 816 (Ueda *et al*, 2002). A number of tyrosine kinase inhibitors with activity against FLT3 have been reported, including CEP-701, PKC412, SU5416, SU5614 and MLN518, some of which are in phase I testing (reviewed in Brown & Small, 2004). Several farnesyl transferase inhibitors (FTIs) are known to inhibit the activation of RAS, as well as a large number of uncharacterized farnesylated proteins, and may have a role in AML therapy (Lancet & Karp, 2003). It is encouraging, therefore, that inhibitors to the majority of the reported class I mutations in AML and inv(16) have been developed, although it will require large well-designed clinical trials to determine their clinical efficacy.

Future challenges

Recent advances have greatly increased our understanding of the pathogenesis of AML and inv(16), with class I mutations having been identified for the majority of cases. As a result, CBFβ–MYH11 expressing M4-Eo is one of the best understood of all AMLs at the molecular level and so provides a paradigm for understanding leukaemogenesis. However, despite this increased knowledge, many key questions remain unanswered. What, for example, are the additional class I mutations that cooperate with CBFβ–MYH11? What are the precise secondary genetic consequences of chimaeric gene expression that leads to impaired haematopoietic differentiation and subsequent cellular apoptosis and how does the expression of CBFβ–SMMHC dictate phenotype?

A number of strategies are being employed to answer the first two questions, including retroviral insertional mutagenesis (RIM), candidate gene analysis and gene expression profiling (GEF). RIM provides an efficient approach to alter

gene expression and identify candidate cancer genes (Jonkers & Berns, 1996). Using this technique, Castilla *et al* (2004) reported that the neonatal injection of *CBF*β*–MYH11* knock-in mice with retrovirus 4070A lead to the development of AML within 2–5 months. Interestingly, each resultant leukaemia contained only one, or at most, a few integrated proviruses, suggesting that alteration of a single gene is sufficient to synergise with *CBF*β*–MYH11*; a conclusion that supports the two-hit hypothesis. Analysis of common insertional sites (CISs) identified the transcription factors *PLAG1* and *PLAGL2* as potential cooperating genes. Furthermore, preliminary data from the same group revealed that the introduction of either *PLAG1* or *PLAGL2* cDNA into bone marrow cells expressing CBFβ–MYH11 resulted in the development of AML 2–5 months after transplantation into irradiated mice (Landrette *et al*, 2002). *MYB* and *RUNX2* were similarly identified as CISs in the CBFβ–MYH11-induced AML mouse model (Castilla *et al*, 2004), while analysis of CISs has identified *HOXA7* and *HOXA10* as potentially co-operating genes in *AML1–ETO* mouse models (Stocking *et al*, 2002). A caveat to this approach, however, is that, while RIM is undoubtably a powerful technique for identifying cooperating genes in animal models, the clinical relevance of the such findings will need to be confirmed.

A second approach has been to screen for activating mutations in other candidate genes, including haematopoietic tyrosine kinases and their downstream effectors. Hiwatari *et al* (2003), for example, identified novel activation loop mutations in the RTK class III PDGFRα in two cases of childhood CBF AML, one AML-M1 and t(8;21) and an AML and inv(16). However, we have not been able to detect PDGFRα or PDGFRβ mutations in a series of 30 CBF leukaemias, suggesting that such findings are uncommon (Johan *et al*, 2004b). In the future, high-throughput analysis of genome-wide RTKs should facilitate this approach and determine whether additional RTKs can function as class I mutations in AML. Currently, little is known about the mechanisms that regulate the termination of cellular signalling and whether defects in these systems could provide a proliferative signal and contribute to oncogenetic transformation. It is possible that defects of tyrosine phosphatases could lead to excessive signalling by RTKs. A potential candidate is SHP-1, a protein-tyrosine phosphatase expressed primarily in haematopoietic cells (Yi *et al*, 1992) and which has been shown to be aberrantly methylated in a high percentage of AML cases (Chim *et al*, 2004). The suppressor of cytokine signalling-1 (SOCS1) is known to down-regulate the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathways and, as a result, to have tumour-suppressor activity. It is of interest, therefore, that SOCS methylation has recently been reported in 60% of AML cases, including 11% of patients with t(8;21) (Chen *et al*, 2003).

Gene expression profiling offers exciting opportunities for the identification of cooperating mutations and the determination of the secondary effects of class II mutations. In preliminary studies, high levels of expression of NT5E (5′ nucleotidase) (Bullinger *et al*, 2004) as well as high levels CDC37 homolog with under-expression of Cyclin C, and HOXA9 appear to be characteristic of specimens with inv(16) (Lacayo *et al*, 2003). It is to be hoped that future GEP studies, utilizing whole-genome gene arrays, will help identify dysregulated pathways that contribute to transformation in this patient group.

Finally, the mechanisms that link genotype to phenotype remain unknown. The timing of fusion protein expression during myeloid development may be important. For example, expression of PML–RARA in late myeloid cells, in contrast to early myeloid precursors, has little effect on myeloid development and suggests that the cellular milieu in which the transcript is expressed may be relevant for leukaemogenesis. Recently, Lane and Ley (2004) have provided supportive evidence for this concept by showing that the leukaemic potential of PML–RARA requires proteolytic cleavage by neutrophil elastase, a neutral serine protease that is maximally produced in promyelocytes.

Conclusions

Significant advances have been made during the last decade in the elucidation of the pathogenesis of AML and inv(16). Cooperating class I mutations, involving *RTKs* and *RAS* genes, have been identified in approximately 70% of cases, making this subtype one of the best understood acute leukaemias at the molecular level. As a result, AML and inv(16) acts as a paradigm for the interacting processes of differentiation block and proliferation that characterize leukaemogenesis. Hopefully, these advances will lead to improved therapies since, although CBF AMLs are regarded as good prognostic diseases, only half of the patients are alive at 5 years with standard chemotherapeutic regimes.

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