

# symposium article

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## New insights into molecular mechanisms of haematological malignancies

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### introduction

Chromosomal translocations are a hallmark of haematological malignancies and present as clonal abnormalities in both leukaemias and lymphomas. Whilst these chromosomal aberrations were initially described with cytogenetic techniques, the genes involved have in most instances been cloned and their functions well characterized.

In more recent times, molecular abnormalities have been discovered in leukaemia and lymphoma which largely escape detection through classical and molecular cytogenetics. A good example to illustrate this field are acute myeloid leukaemias (AMLs) with a 'normal' karyotype [1], which will be a focus of this review. Information has largely been collected from recent authoritative reviews available in this field, and from a few very selected and recent original articles; readers interested in a broader collection of original papers are asked to consult the references in the summary articles.

### pathology of transcription factor genes

#### CEBPA

The main role of the transcription factor CCAAT/enhancer binding protein  $\alpha$  (CEBPA) in haematopoiesis is the development of mature granulocytes. Disruption of CEBPA accordingly results in a blockade of early granulocyte maturation. Molecular studies as well as mouse models suggest that whilst disrupting the cell-cycle regulatory function of CEBPA may be sufficient to initiate AML-like transformation, other CEBPA functions must be preserved in leukaemic stem cells.

Clinically, AML patients with CEBPA mutations enjoy a longer relapse-free or overall survival than patients with other molecular abnormalities, although the reason for this remains unclear. The majority of AML patients have more than one CEBPA mutation [2, 3]. Among patients with a single CEBPA mutation, the predominant types are frameshift N-terminal mutations (40%) or in-frame C-terminal insertion/deletion mutations (30%). Patients with multiple CEBPA mutations mainly show the combination of one N-terminal frameshift mutation and one C-terminal in-frame mutation, with the two mutations typically being located on different alleles. This may explain why additional genetic abnormalities are hardly ever identified in AML patients with CEBPA

mutations, since the most frequent 'second' genomic abnormality in such patients is an additional mutation in the very same gene. These observations indicate that one CEBPA mutation might predispose to the occurrence of an additional CEBPA mutation as a secondary hit, ultimately inducing AML. This would also be consistent with the observation that both CEBPA mutation types do not develop at the time of relapse, indicating that CEBPA mutations are a primary event in the pathogenesis of AML rather than being involved in disease progression.

#### AML1-ETO

Dysregulated expression rather than gene mutation is another mechanism to disrupt function of transcription factors crucial for normal haematopoiesis [3–5]. CEBPA mRNA expression is suppressed in the presence of the AML1-ETO fusion protein both *in vitro* and *in vivo*. The leukaemic fusion protein AML1-ETO, i.e. the product of the chromosomal translocation t(8;21)(q22;q22), occurs frequently in human AML with granulocytic differentiation. AML with t(8;21) tends to occur in early adulthood and has a better prognosis than most other types of AML. Morphologically, it shows enhanced marrow granulopoiesis with inhibition of erythropoiesis. Blasts display with solitary Auer rods and abnormal myelocytes with increased cytoplasmic granulation.

The AML1-ETO fusion protein comprises a moiety from the haematopoietic transcription factor RUNX1 (also known as AML1), on chromosome 21, which is joined to the majority of the ETO repressor protein, encoded on chromosome 8. RUNX1 normally functions as a transcriptional activator, promoting granulocytic differentiation through the coordinated upregulation of lineage-specific target genes such as myeloperoxidase. ETO is a scaffolding factor for the assembly of protein complexes. Through the fusion with ETO, RUNX1 function changes from activator to repressor, and thus AML1-ETO predictably acts as a transcriptional repressor for RUNX1 target genes at several nodal points during haematopoietic differentiation. As a consequence, AML patients with t(8;21) have much less CEBPA mRNA than AML with a normal karyotype, and AML1-ETO is thought to inhibit CEBPA mRNA expression through inhibition of autoregulation. Through the repression of several key haematopoietic transcription factors, AML1-ETO may thereby expand stem cell pools. In addition, repression of genes involved in DNA base excision repair conveyed by AML1-ETO may promote

genetic instability within this expanded stem cell pool. In particular, lineage commitment defects induce expansion of a self-renewing progenitor compartment, which may then result in leukemic transformation through additional mutations.

## the nuclear factor- $\kappa$ B pathway in lymphoma

Mediastinal large B-cell lymphoma (MLBCL) is a subtype of diffuse large B-cell lymphoma (DLBCL) that characteristically presents as a localized tumour in the mediastinum of young females. Although MLBCL has distinctive pathologic features, it clinically resembles the nodular sclerosis subtype of classical Hodgkin's lymphoma (cHL). Primary MLBCLs express low levels of B-cell receptor signalling pathway components and exhibit a distinctive cytokine signature, strikingly similar to those of cHL. In cHL, activation of the nuclear factor- $\kappa$ B (NF- $\kappa$ B) pathway enhances the survival of surface immunoglobulin-negative Reed–Sternberg cells. Gains in chromosome 2p found in ~20% of MLBCL and up to 50% of cHL, are associated with amplification of the *REL* locus, which encodes an NF- $\kappa$ B subunit, and thus may represent one potential mechanism for increased NF- $\kappa$ B activity and tumour cell resistance to apoptosis [6–10].

The constitutive activation of the transcription factor NF- $\kappa$ B is a hallmark of many highly malignant tumours. NF- $\kappa$ B regulates many genes for immune response, cell adhesion, differentiation, proliferation, angiogenesis as well as apoptosis, and it delivers strong anti-apoptotic stimuli to cells. NF- $\kappa$ B transcription factors are kept in an inactive state in the cytoplasm, but signalling through various cell surface receptors leads to nuclear accumulation of NF- $\kappa$ B which in turn elicits upregulation of several NF- $\kappa$ B target genes. Whereas in most germinal centre (GC) B cells, there is low activity of the NF- $\kappa$ B pathway, GC-derived lymphomas use several strategies to overcome the physiological propensity of normal GC cells to undergo apoptosis. These may include NF- $\kappa$ B gene point mutations which release NF- $\kappa$ B for transfer into the nucleus (cHL), or latent membrane protein 1 in Epstein–Bar virus (EBV)-associated lymphomas which activates NF- $\kappa$ B. Constitutive activation of the NF- $\kappa$ B pathway in lymphoma leads to nuclear localization of the protein, a prerequisite to set its downstream pathway into motion.

The known role of NF- $\kappa$ B activation in cHL and the increased expression of certain NF- $\kappa$ B target genes in primary MLBCLs prompted further investigation of this survival pathway in MLBCL. In almost all cases, *c-REL* was localized to the nucleus, indicating that the NF- $\kappa$ B survival pathway was constitutively active in these tumours. Functional assays confirmed that MLBCL cell lines exhibited increased constitutive NF- $\kappa$ B activity and relied upon the NF- $\kappa$ B pathway for survival. Therefore, the MLBCL transcriptional profile already seems to be translating into a functionally relevant immunohistochemical signature in this disease. As specific NF- $\kappa$ B inhibitors become available for clinical trials, MLBCL should be rapidly targeted for further evaluation.

## nucleophosmin protein aberrations

Nucleophosmin protein 1 (NPM1) is an abundant and highly conserved phosphoprotein that normally resides in nucleoli and shuttles between nucleus and cytoplasm. NPM1 has a role in various cellular processes, including ribosome biogenesis, response to stress such as UV irradiation and hypoxia, maintenance of genomic stability, regulation of activity and stability of tumour suppressor genes such as *p53* and *ARF*, and transcriptional regulation.

NPM1 mutations can be identified in about half of patients with cytogenetically normal AML and thus are the most frequent genetic change in this patient subset [2, 11]. Mutations in NPM, particularly those placed in exon 12, result in abnormal cytoplasmic localization of NPM1 protein. Cytoplasmic accumulation of NPM1 mutants is caused by two major alterations acting in concert: (i) loss of tryptophan residues normally required for NPM1 binding to the nucleoli, and (ii) generation of an additional nuclear export signal motif at the C-terminus by the exon 12 mutation. Moreover, NPM1 leukaemic mutants may recruit wild-type NPM1 from nucleoli to nucleoplasm and cytoplasm through dimerization.

NPM1 mutations are associated with several pretreatment characteristics of patients with cytogenetically normal AML, including predominantly female sex, higher bone marrow blast content, higher white blood cell and platelet counts, as well as high CD33-antigen expression but low or absent CD34-antigen expression. Clinical data on the prognostic impact of NPM1 mutations have been somewhat controversial. Whereas in some studies there was a significant effect on complete remission rate, relapse-free survival and event-free survival, other studies revealed no significant differences in these parameters. NPM1 mutation appears to be a favourable prognostic marker, but only in the absence of FLT3-ITD (see below), whilst the presence of NPM1 mutations has no influence on the adverse prognosis in patients with FLT3-ITD. Approximately 40% of patients with NPM1 mutations also carry FLT-ITD.

## tandem duplications of tyrosine kinase genes

The receptor tyrosine kinase FMS-like tyrosine kinase-3 (FLT3) and its ligand are important for the proliferation and differentiation of early haematopoietic progenitor cells. In patients with AML, somatic mutations that result in the constitutive activation of FLT3 have been identified in two functional domains of the receptor, the juxtamembrane domain and the split tyrosine kinase domain [2, 12]. Specifically, one mutation type is characterized by internal tandem-duplications (ITDs) of particular excerpts of the gene. Activating mutations of the FLT3 receptor occur in ~30% of AML. FLT3 mutations trigger downstream signalling pathways including RAS-MAP/AKT kinases and signal transducer and activator of transcription-5 (STAT5). FLT3-ITD mutations start a cycle of genomic instability whereby increased reactive oxygen species production leads to increased DNA double strand breaks and repair errors that may explain aggressive AML in FLT3-ITD patients.

From a clinical perspective, FLT3 mutations are relevant because of their prognostic impact, and because constitutively active FLT3 may be an attractive target for molecular therapy. Patients with cytogenetically normal AML harbouring FLT3-ITD have a significantly inferior outcome compared with patients without FLT3-ITD, although the prognostic relevance of FLT3 mutations remains somewhat controversial. Currently, there are a number of FLT3 inhibitors at various stages of clinical development. When used as single agents, these compounds unfortunately have limited activity in AML with mutant FLT3. Data look more promising when these inhibitors are combined with conventional chemotherapy. Results from clinical trials will tell us whether inhibition of mutant FLT3 will be a successful targeted approach for these patients.

## aberrations of microRNA in tumour cells

In contrast to most haematological malignancies, the neoplastic lymphoid cells in B-cell chronic lymphocytic leukaemia (CLL) mostly show chromosomal deletions or loss of heterozygosity (LOH) rather than translocations. It was therefore suspected for a long time, that in analogy to solid cancers, eventually tumour suppressor genes inactivated through the classical 'Knudson' mechanism would be found in CLL. However, they proved pretty elusive. For example, detailed genetic analyses failed to demonstrate the consistent involvement of any of genes located in or close to a region at 13q14.3, which is frequently deleted in B-cell CLL. Instead, another molecular principle has now been discovered that seems to operate in CLL. MicroRNAs (miRNAs) have been linked to the initiation and progression of CLL. A cluster of two miRNAs, *MIRN15A* and *MIRN16-1*, is located exactly inside the minimal region of loss at 13q14, and both genes are deleted or downregulated in the majority of CLL samples [13, 14].

MiRNAs are a group of small non-coding RNA molecules distinct from but related to small interfering RNAs (siRNAs) that have been identified in a variety of organisms. These small 19- to 24-nucleotide (nt) RNAs are transcribed as parts of longer molecules named pri-miRNA, several kilobases (kb) in length, that are processed in the nucleus into hairpin RNAs of 70–100 nt named pre-miRNA by the double-stranded RNA-specific ribonuclease. The hairpin RNAs are transported to the cytoplasm, where they are digested by a second double-stranded-specific ribonuclease. In animals, single-stranded miRNA binds specific messenger RNA (mRNA) through sequences that are significantly, though not completely, complementary to the target mRNA, mainly to the 3' untranslated region. By a mechanism that is not fully characterized, the bound mRNA remains untranslated, resulting in reduced levels of the corresponding protein. Alternatively, in some cases bound mRNA can be degraded, resulting in reduced levels of both the corresponding transcript and protein. The number of miRNAs is growing rapidly. Today the number of miRNAs, including those electronically cloned, is >1000 and still growing.

The function of most miRNAs is not known. In spite of this, for a few members the participation in essential biological

processes for the eukaryotic cell is proven. The list of proposed functions includes haematopoietic B-cell lineage fate (*miR-181*), B-cell survival (*miR-15a* and *miR-16-1*) and cell proliferation control (*miR-125b* and *let-7*). The direct evidence of the involvement of miRNAs in a human disease came from the link between *miR-155* and B-cell CLL. *MIRN15A* and *MIRN16-1* form a small cluster at chromosome 13q14.3. Both genes are ubiquitously expressed as non-coding RNAs (*miR-15a* and *miR-16-1*), with highest levels in normal CD5+ lymphocytes, highlighting their relevant role in normal CD5+ B-cell homeostasis. The majority of CLL patients present a downregulation of *MIRN15A* and *MIRN16-1* expression correlating with homozygous LOH at 13q14.3. The two-hit inactivation model of tumour-suppressor genes applies unequivocally to the *MIRN15A/MIRN16-1* locus *per se*, as one allele can be deleted by heterozygous LOH and the second may be eliminated by a germline mutation in a region which compromises its procession. E-miμ-miR-155 transgenic mice initially exhibit a pre-leukaemic pre-B-cell proliferation evident in spleen and marrow, followed by frank B-cell malignancy. These findings indicate that the role of *miR-155* is to induce polyclonal lymphoid expansion, enhancing the chance capture of secondary genetic changes for full transformation. Currently collective evidence strongly indicates a role for miRNAs in haematological cancers.

## haploinsufficiency of genes encoding ribosomal subunits in myelodysplastic syndrome

Patients with a 5q- myelodysplastic syndrome (MDS) or 5q- syndrome have severe macrocytic anaemia, normal or elevated platelet counts, normal or reduced neutrophil counts, erythroid hypoplasia in the bone marrow and hypolobated micromegakaryocytes. They also have a propensity to progress to AML, although more slowly than other forms of MDS. The 5q- MDS shows a remarkable response to treatment with the thalidomide analogue lenalidomide.

No patients with 5q- MDS were reported to have biallelic deletions within a common deleted region (CDR) on 5q, and no point mutations were found in the remaining allele of any of the genes in this region. Thus, the 5q- syndrome was suspected to be caused by protein haploinsufficiency which is a loss of only one allele at a given locus, implicating reduced protein expression from the remaining single allele, i.e. a reduction in gene dosage. Data from both mouse models and human tumours point out that allelic heterozygosity, leading to protein haploinsufficiency, may in fact convey tumour predisposition and progression. An elegant report very recently pointed out that the principal hallmarks of 5q- MDS (erythroid maturation block with preservation of megakaryocyte differentiation) can be recapitulated experimentally with short hairpin RNAs (shRNAs) targeting each of the genes within the CDR [15, 16]. Partial loss of function, or haploinsufficiency of a gene called *RPS14* in fact recapitulates the phenotype of the 5q- syndrome. *RPS14* is a component of the 40S ribosomal subunit. Decreased expression of *RPS14* results in an accumulation of the 30S pre-rRNA species with a concomitant decrease in 18S/18SE rRNA

levels which is consistent with reports that *RPS14* is specifically required for the processing of 18S pre-rRNA.

The same genetic principle also operates in a familial autosomal dominant platelet disorder with predisposition to AML [17]. These families show germline heterozygous nonsense mutations or intragenic deletion of one allele of the haematopoietic transcription factor CBFA2 (formerly AML1). Bone marrow or peripheral blood cells from affected individuals shows a decrement in megakaryocyte colony formation, demonstrating that CBFA2 dosage affects megakaryopoiesis. These findings support a model for this rare disorder where haploinsufficiency of CBFA2 causes an autosomal dominant congenital platelet defect and predisposes to the acquisition of additional mutations that cause leukaemia.

## conclusion

In summary, the molecular pathology of leukaemias and lymphomas has today been extremely well worked out, including as it were a great potential for the development of new 'targeted' treatments (to use a fashionable phrase). The extraordinary success story of imatinib mesylate treatment in chronic myelogenous leukaemia will still have to be matched in other malignancies, but a number of approaches are on the way that may eventually prove to be equally rewarding.

## disclosures

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