CLONALITY STUDIES IN MYELODYSPLASIA AND ACUTE MYELOID LEUKEMIA

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Klonaliteitsstudies in myelodysplasie en
acute myeloïde leukemie

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List of frequently used abbreviations

AML	acute myeloid leukemia
AML1	acute myeloid leukemia-1 gene
BM	bone marrow
CD	cluster of differentiation/ cluster of designation
CLSM	confocal laser scanning microscope
CMML	chronic myelomonocytic leukemia
CR	complete remission
DNA	deoxyribonucleic acid
ETO	eight-twenty-one gene
FAB	French-American-British classification of MDS and AML
FISH	fluorescence in situ hybridization
FITC	fluorescein isothiocyanate
ISH	in situ hybridization
JMML	juvenile myelomonocytic leukemia
LT-MDS	leukemia transformed MDS
MDS	myelodysplastic syndrome
MGG	May-Grünwald Giemsa
MoAb	monoclonal antibody
PB	peripheral blood
PCR	polymerase chain reaction
RA	refractory anemia
RAEB	RA with excess of blasts
RAEB-t	RAEB in transformation
RARS	RA with ringed sideroblasts
RT-PCR	reverse transcriptase PCR
SSC	standard saline citrate: 150 mM NaCl + 15mM sodium citrate

CHAPTER 1

Introduction

1.1 Hematopoiesis and malignant transformation

In adult humans, the production of blood cells or hematopoiesis is mainly restricted to the bone marrow. A small number of pluripotent stem cells, which are capable of self-renewal, can generate committed progenitor cells. The latter are irreversibly committed to the granulocytic, monocytic, erythroid, megakaryocytic or the lymphoid cell lineage. After proliferation and differentiation, the mature blood cells enter the circulation (Figure 1) (1-6,8). The granulocytic and monocytic cells may be designated as myeloid cells; the erythroid cells are sometimes also included in this group. The blood cell formation is regulated by hematopoietic growth factors and cellular interactions *e.g.* with bone marrow stromal cells (3,6-11).
Malignant transformation of hematopoietic cells results in ineffective hematopoiesis. Progenitor and/or precursor cells may accumulate due to a maturation arrest. Depending on the cell lineage involved, these disorders are referred to as myelodysplastic syndrome, acute myeloid or lymphoblastic leukemia, myeloproliferative or lymphoproliferative disease. The myelodysplastic syndrome and acute myeloid leukemia are the subjects of investigation of this thesis.

1.2 Myelodysplastic syndrome

The myelodysplastic syndrome (MDS) comprises a group of clonal stem cell disorders characterized by ineffective hematopoiesis. In most patients this results in anemia, leukopenia and/or thrombocytopenia, often in association with an increased bone marrow cellularity. Morphological abnormalities such as hypogranulation of granulocytes, nuclear deformation of erythroblasts and micro-megakaryocytes, are frequently seen (12-17). The classification of the different types of MDS according to the French-American-British (FAB) cooperative group has been based on the presence of refractory anemia, or, less frequently, a refractory neutropenia or thrombocytopenia. According to the FAB-system, the myelodysplastic syndrome can be classified into refractory anemia (RA), refractory anemia with ringed sideroblasts (RARS), refractory anemia with excess of blasts (RAEB), refractory anemia with excess of blasts in transformation (RAEB-t) and chronic myelomonocytic leukemia (CMML) depending on the percentages of blasts in blood and bone marrow, percentages of ringed sideroblasts in the bone marrow, numbers of monocytes in the blood and the presence of Auer rods (Table 1) (12).

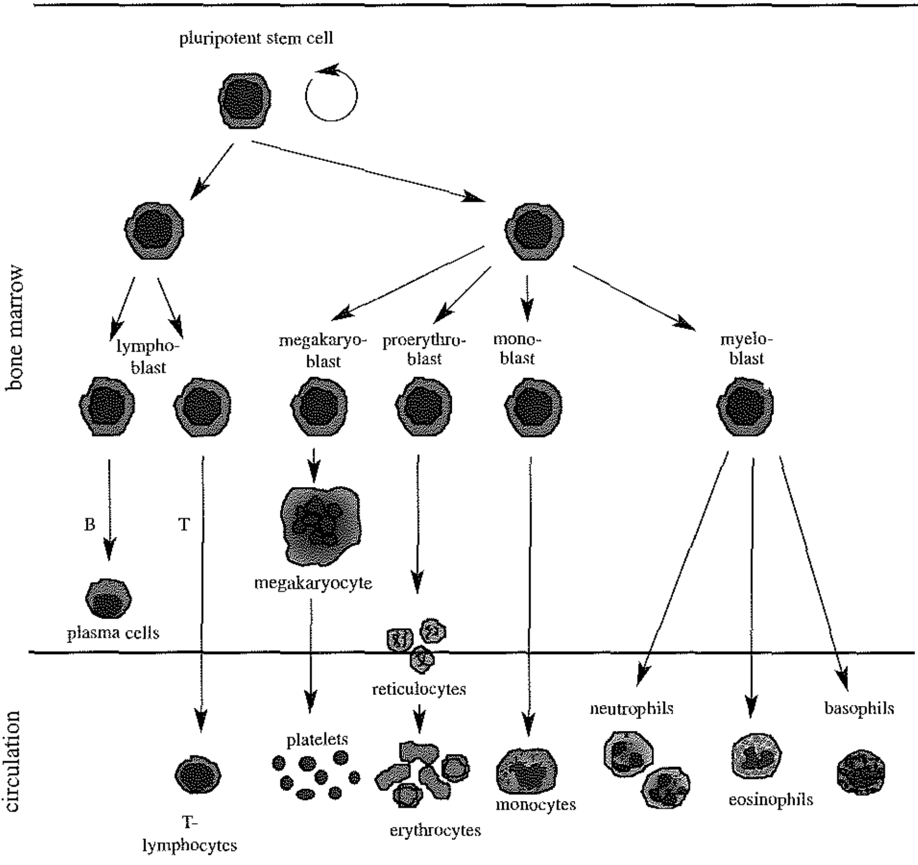

Figure 1.
Schematic representation of the blood cell production.

In CMML, leucocytosis, splenomegaly and tissue infiltration by monocytes may be present. Consequently, this type of MDS is sometimes considered as a myeloproliferative disease (MPD)(15,16). The FAB classification is relatively easy in use and widely applied for diagnostic distinction of MDS in adults and childhood. In children, CMML is usually referred to as juvenile myelomonocytic leukemia (JMML) (14,17-19). In approximately 30% of patients with MDS, disease progression will occur leading to an increase of the percentages of bone marrow blasts, and development into full AML (or leukemia transformed (LT)-MDS) (17,18,20-23).

Table 1. FAB classification of the myelodysplastic syndromes

FAB type	Criteria: Refractory anemia, neutropenia or thrombocytopenia
Refractory anemia (RA)	**PB**: Blasts ≤ 1% Monocytes ≤ 1x 10^9/l and **BM**: Blasts < 5% Ringed sideroblasts ≤ 15% of erythroblasts
Refractory anemia with ringed sideroblasts (RARS)	**PB**: Blasts ≤ 1% Monocytes ≤ 1 x 10^9/l and **BM**:Blasts < 5% Ringed sideroblasts > 15% of erythroblasts
RA with excess of blasts (RAEB)	**PB**: Blasts > 1% or **BM**: Blasts ≥ 5% but **PB**: Blasts < 5% and **BM**: Blasts ≤ 20% **PB** Monocytes ≤ 1 x 10^9/l
RAEB in transformation (RAEB-t)	**PB**: Blasts ≥ 5% or **BM**: Blasts > 20% but < 30% or Auer rods
Chronic myelomonocytic leukemia (CMML)	**PB**: Blasts < 5% Monocytes > 1 x 10^9/l **BM**: Blasts ≤ 20%

PB: peripheral blood; BM: bone marrow

Recently, a new classification of myeloid hematological malignancies has been proposed by the World Health Organization (WHO) based on morphologic, immunophenotypic and cytogenetic abnormalities, as well as on clinical features (Table 2) (24). Here, the 5q-syndrome is acknowledged as a specific type of MDS as is therapy related MDS. CMML and J-CMML are not classified as MDS but as myelodysplastic/myeloproliferative diseases. Furthermore, the WHO classification does not recognize the FAB subtype RAEB-t; since AML is defined by the presence of more 20% blasts in the bone marrow, hence including the original FAB subtype RAEB-t.

Table 2. WHO classification of myeloid malignancies

Myelodysplastic syndromes

Refractory anemia with ringed sideroblasts
Refractory anemia without ringed sideroblasts
Refractory cytopenia with multilineage dysplasia
Refractory anemia with excess of blasts
5q- syndrome
MDS, unclassifiable

Myelodysplastic/myeloproliferative diseases

Chronic myelomonocytic leukemia
Atypical chronic myelogenous leukemia
Juvenile myelomonocytic leukemia

Acute myeloid leukemia (AML)

AML with t(8;21)(q22;q22)(*AML1/ETO*)
Acute promyelocytic leukemia with t(15;17)(q22;q11–12)(*PML/RAR*α) and variants
AML with abnormal eosinophils and inv(16)(p13q22) or t(16;16)(p13;qq11)(*CBF*β*/MYH11*)
AML with 11q23 (*MLL*) abnormalities
AML with multilineage dysplasia
-with prior myelodysplastic syndrome
-without myelodysplastic syndrome
AML and MDS, therapy related
-Alkylating agents
-Epipodophyllotoxin-related
-other types
AML not otherwise categorized

Acute biphenotypic leukemias

1.2.2 Pathogenesis and biological features

MDS is mainly a disorder of the elderly (25,26). Environmental factors, *e.g.* exposure to benzene, insecticides or certain organic solvents, may cause MDS (25,27-29). In addition, patients treated for cancer with radiation or chemotherapy *e.g.* alkylating agents, are predisposed to the development of secondary MDS (25,30-33,154,156-158). Radiation as well as chemicals may cause gene mutations or chromosome aberrations in hematopoietic progenitors which eventually may result in malignant transformation.
Several gene mutations have been implicated in the pathogenesis of MDS. A few selected examples will be mentioned here. Proto-oncogenes like *ras* can be mutated, especially in CMML (34-40). The family of *ras* genes encode for $p21^{ras}$ proteins with guanosine-

triphosphatase (GTPase) activity, which are involved in mitogenic and differentiation-related signal transduction. Mutations in the *fms* gene have also been reported for CMML (39,41). The *fms* gene at chromosome 5q33 encodes for the cell surface macrophage-colony stimulating factor (CSF-1) receptor (42). Mutations in *ras* and *fms* genes may contribute to deregulated cell growth. In addition deletions in the early growth response gene-1 (*egr-1*) and the interferon regulatory factor-1 gene (*irf-1*) at chromosome 5q31, both putative tumor suppressor genes, have been implicated in the pathogenesis of MDS (43,44). Although at low frequencies, mutations of the tumor suppressor gene *p53* at chromosome 17p13 have also been reported (39,45,46). This gene codes for an apoptosis protein which has a role in the cell cycle arrest of cells with DNA damage. As a result of mutations of p53, inappropriate expansion of abnormal cells may occur (47-50).
Abnormal cellular features in the hematopoietic progenitor cells have been demonstrated in MDS. Clonogenic assays show reduced growth of hematopoietic progenitors (51-58). The inhibited colony forming abilities cannot be overcome by adding supersaturating concentrations of growth factors, indicating that the cellular responsiveness of the progenitors to growth factor stimulation is impaired. Responsiveness to erythropoietin (EPO) is generally defective despite a normal average density of EPO receptors on the cell surface and normal ligand binding capacities of the EPO receptor. Stimulation with EPO has revealed impaired downstream receptor activation that may involve the signal transducer and activation of transcription, STAT5 (58).
The high bone marrow cellularity in the presence of cytopenia in the peripheral blood of patients with MDS can be explained by an increased rate of intramedullary cell death. Increased apoptosis has been demonstrated using the in situ end labeling (ISEL) technique and with experiments using fluorescein labeled annexin-V, which binds to phosphatidyl serine on apoptotic cells (59-64). Patients with MDS can also show aberrant expression of the FAS/APO-1 protein (CD95) in their CD34+, CD33+, glycophorin+ bone marrow cells (65,66). Fas/APO-1 is a cell surface protein which can transduce an apoptotic signal when crosslinked with an anti-Fas/APO-1 antibody and is known to play an important role in normal hematopoiesis (67-70). All these findings provide evidence for the important role of apoptosis in the hematological phenotype of MDS (71).

1.3 Acute myeloid leukemia

Acute myeloid leukemia (AML) is a clonal proliferation of myeloid progenitor cells in the bone marrow and often also in the peripheral blood. These cells are still capable of

Table 3. FAB classification of the acute myeloid leukemias

FAB type	Description	Criteria
M0	Acute myeloid leukemia with minimal evidence of myeloid differentiation	Blasts ≥ 30% of nucleated BM cells Blasts ≥ 30% of nonerythroid BM cells* < 3% of blasts positive for Sudan black B or myeloperoxidase staining Blasts positive for myeloid lineage by *e.g.* immunological markers
M1	Acute myeloid leukemia without maturation	Blasts ≥ 30% of nucleated BM cells Blasts ≥ 90% of nonerythroid BM cells* ≥3% of blasts positive for Sudan Black B or myeloperoxidase staining Monocytic component ≤ 10% of nonerythroid BM cells* Maturing granulocytic component ≤ 10% of nonerythroid BM cells*
M2	Acute myeloid leukemia with maturation	Blasts ≥30% of nucleated BM cells Blasts 30-89% of noneryhtroid BM cells* Maturing granulocytic cells >10% of nonerythroid BM cells*. Monocytic component < 20% of nonerythroid BM cells*
M2eo		As in M2, eosinophils are increased
M3 M3V	Promyelocytic leukemia	Presence of hypergranular promyelocytes M3-variant: hypogranular promylocytes
M4	Acute myelomonocytic leukemia	Blasts ≥ 30% of nucleated BM cells Blasts ≥ 30% of nonerythroid BM cells* Granulocytic component ≥ 20% of nonerythroid BM cells* **+** BM monocytic component ≥ 20% of non erythroid BM cells*. PB monocytic cells ≥ 5 x 10^9/l **or** BM monocytic component ≥ 20% of nonerythroid BM cells* confirmed by cytochemistry, or increased serum or urine lysozyme concentration **or** BM resembling M2 but PB monocytic cells ≥ 5 x 10^9/l confirmed by cytochemistry, or increased serum or urine lysozyme concentration
M4eo		as in M4, eosinophils are increased
M4 baso		as in M4, basophils are increased

M5	Acute monoblastic/ monocytic leukemia	Blasts ≥ 30% of nucleated BM cells Blasts ≥ 30% of nonerythroid cells * Monocytic component ≥ 80% of nonerythroid BM cells* M5a: Monoblasts ≥ 80% of BM monocytic component M5b: Monoblasts < 80% of BM monocytic component
M6	Acute erythroid leukemia	Erythroblasts ≥ 50% of nucleated BM cells Blasts ≥ 30% of nonerythroid BM cells *
M7	Acute megakaryoblastic leukemia	Blasts ≥ 30% of nucleated BM cells Blasts demonstrated to be megakaryoblasts by *e.g.* immunological markers

* Excludes also lymphocytes, plasmacells, macrophages and mast cells
BM: bone marrow; PB: peripheral blood

selfrenewal but have generally lost the ability to undergo terminal differentiation. In such cases, normal hematopoiesis in the bone marrow is diminished due to the excessive proliferation of the leukemic cells. This may result in anemia, granulocytopenia and thrombocytopenia. According to the classification of the FAB cooperative group, AML can be subdivided into various subtypes depending on the percentages and cytological features of the bone marrow blasts, erythroblasts and the presence of leukemic promyelocytes (Table 3) (72-75). In AML type M1 and M2, myeloblasts dominate, in AML type M3 leukemic promyelocytes are present. In AML M4 and M5 the leading cytological phenotype is that of monoblasts, in AML type M6 not only myeloblasts but also erythroblasts are increased in numbers. Additional immunological analysis is required for the classification of AML type M0, undifferentiated AML, and megakaryoblastic leukemia, AML type M7 (74,75). The FAB classification is relatively easy in use and therefore highly reproducible.

In the recent WHO classification of AML, in addition to the morphologic and immunophenotypic results, the cytogenetic and clinical features are considered (24). Thus, four subtypes of AML are recognized: AML with recurrent cytogenetic translocations, AML with multilineage dysplasia, therapy related AML, and the remaining, not otherwise categorized, types of AML (Table 2). The WHO classification will be more difficult to apply then the FAB classification. Cytogenetic data have to be available. If metaphase analysis is not possible, molecular analysis or FISH on routinely made blood or bone marrow smears could be a useful alternative. But then, molecular techniques should be standardized. Moreover, international guidelines how to establish "dysplasia" and how to define therapy-related, still have to be made. However, it seems an improvement that now a distinction is made between *de novo* AML, with or without

specific chromosome aberrations, therapy related AML and LT-MDS. The WHO classification will prove even more useful when therapies become available for the different types of diseases.

1.3.2 Pathogenesis and biological features

Several risk factors for developing AML have been reported. Patients with genetic syndromes such as Down's syndrome and Fanconi's anemia are predisposed to AML (76-80). Exposure to radiation or chemicals increases the risk of developing AML (30,31,33,148,152-158). As in MDS, the malignant transformation of hematopoietic progenitor cells is probably a multistep process and may involve alterations in a variety of genes involved in cellular growth, differentiation and apoptosis, *e.g.* genes encoding signaling molecules and transcription factors (see paragraph 1.2.2) (41,43,44,46,60,81-87).

1.4 *De novo* and secondary MDS and AML

When patients present with MDS or AML and neither have a history of prior treatment with chemo- or radiotherapy, nor an antecedent myelodysplastic phase, the hematological conditions are operationally termed as *de novo*. In contrast to *de novo* AML, the bone marrow of therapy related MDS or AML (or secondary MDS/AML), often shows dysplastic features of the erythroid, myeloid as well as the megakaryocytic cells. In addition to the trilineage dysplasia, an increased reticulin fibrosis can be seen in bone marrow tissue sections (14,30,32). Therapy with alkylating agents can induce secondary MDS or AML (29-33,154,156-158). These types of therapy related MDS and AML are frequently associated with abnormalities of chromosomes 5 and or 7 (29,33,154,156-158). Secondary AML following exposure to topoisomerase-II-inhibitors, usually develops without an apparent preceding MDS phase and with a short latent period. Rearrangements of chromosome 11q23 are often found (148,152-154,158).

1.5 Chromosome analysis: cytogenetic and molecular methods

Cytogenetic and molecular techniques, applied to detect genetic abnormalities in hematopoietic cells, contribute in a major way to the diagnosis MDS or AML. These techniques, which can reveal chromosome aberrations, are briefly summarized below.

Conventional chromosome analysis

Conventional chromosome analysis requires metaphase preparations which are stained with various banding techniques. Chromosomal aberrations found on karyotypic analysis may include numerical abnormalities (*e.g.*, loss or gain of a chromosome) and structural aberrations (*e.g.*, translocations, amplifications or deletions). The findings are described according to the international system for human cytogenetic nomenclature (ISCN) (Figure 2) (88). The presence of a clonal abnormality is defined as two metaphases with the same additional chromosome or the same structural abnormality, or as three metaphases missing the same chromosome. In such instances, an abnormal progenitor has given rise to a clone of abnormal cells, which populate the hematopoietic tissues. Metaphase analysis allows the evaluation of all chromosomes, and is suitable for revealing the presence of multiple and complex changes, as well as clonal progression.
However, it will only demonstrate these abnormalities in dividing (immature) cells. In addition, only a limited number of mitotic cells are usually analyzed and a cytogenetically abnormal clone may be missed. The level of resolution of the analysis is also limited by the quality of the metaphase spreads and the banding techniques used. In order to circumvent these limitations, additional techniques have been introduced in recent years, which may be used along side conventional chromosome analysis.

In situ hybridization

In situ hybridization (ISH) allows analyzing metaphase and interphase cells for the presence of specific chromosomal abnormalities (89-92). This technique is based on base pairing of labeled DNA or RNA probes to complementary sequences (Figure 3,4). Probe DNA and cellular DNA are denatured by heating, after which the probe is allowed to hybridize with the cellular DNA.
A large variety of probes cloned in cosmids or P1 bacteriophage vectors, yeast artificial chromosomes (YACs), bacterial artificial chromosomes (BACs), and P1-derived artificial chromosomes (PACs), are available for demonstrating specific chromosomal aberrations. Oligonucleotide probes can be chemically synthesized (92,93). Peptide nucleic acid (PNA) probes and padlock probes are used for quantitative and high specificity ISH, respectively (94,95). Currently, enzymatic incorporation with modified nucleotides (nicktranslation) is commonly used to label the probes with reporter molecules like biotin or digoxigenin, fluorochromes like fluorescein isothiocyanate (FITC) (green), tetramethylrhodamine (TMR) (red), amino-methylcoumarin-acetic acid (AMCA) (blue)

or Cyanin 5 (Cy5) (far red), or enzymes like alkaline phosphatase or horseradish peroxidase (96). When fluorochromes are used for the detection of the hybridized probe, the ISH technique is usually referred to as FISH. The high sensitivity and high signal resolution, the possibility to analyze multiple different sequences on the cellular DNA and to quantify signal intensity, makes FISH a powerful technique. DNA sequences of 1 kb or more, but also whole chromosomes can be visualized with FISH. Fluorescence microscopy with dual or triple bandpass filter sets, confocal laser scanning microscopy, digital cameras and imaging systems contribute to the visualization of chromosome abnormalities at the single cell level. Enzyme labeled probes are visualized by precipitation reactions and are mainly used when ISH is applied to tissue sections.

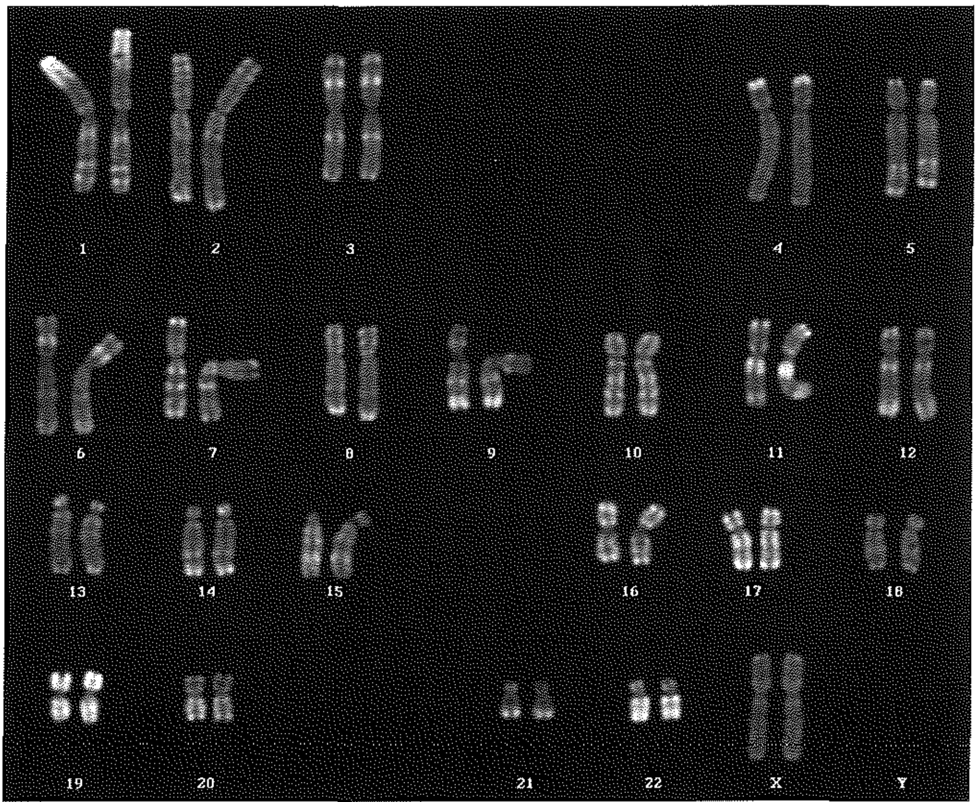

Figure 2.
A normal human karyotype with 22 pairs of chromosomes and two sex chromosomes.

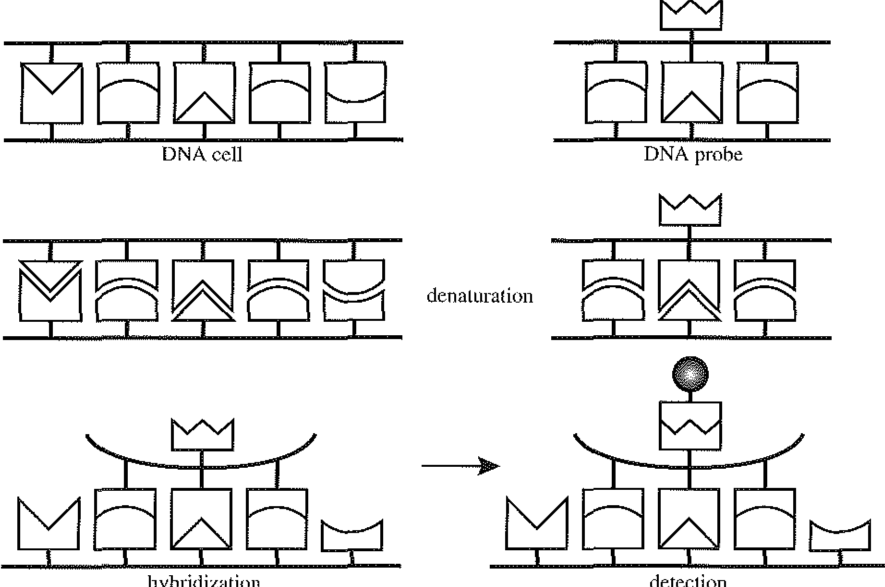

Figure 3.
Schematic representation of the fluorescence in situ hybridization technique.
The probe is labeled with a reporter molecule
After hybridization the probe is detected with a fluorochrome

Various variants of the ISH technique have been developed. In primed ISH (PRINS), an oligonucleotide probe is applied which is hybridized to the cells and serves as a primer for DNA polymerase. The incorporated-labeled nucleotides can then be detected (97-99). This method is much faster than traditional ISH.

Multicolor, even 24-color FISH (M-FISH) or spectral karyotyping (SKY) is based on the individual painting of each pair of chromosomes in metaphase cells. Probes carrying two or more fluorochromes at different ratios are applied. With image analysis systems, all chromosomes in a single metaphase can be studied. These techniques have led to the detection of cryptic chromosome abnormalities and to the identification of complex

chromosome rearrangements (100,101). An interesting approach to multi-color FISH has been introduced: the combined binary ratio labeling (COBRA) technique which is based on labeling of the probes using combinations of three fluorochromes, each combination in either the presence or absence of a fourth label (102).

In the absence of metaphases, comparative genomic hybridization (CGH) can give information on genetic gains and losses in tumor cells. In CGH, normal DNA and tumor DNA are labeled with two different fluorochromes. Then, equal amounts of DNA are mixed. Following competitive hybridization onto metaphase preparations of cytogenetically normal cells, the intensity of the two fluorochromes on each chromosome is measured by digital image analysis. If there are no changes in tumor DNA, the ratio between the two fluorochromes will be one. In case of amplification or deletion, the ratio will shift. Gains, losses and high level amplifications can be detected when clonal aberrations are present in at least 50% of the cells (103-107).

In fiber FISH, naked DNA fibers immobilized to microscope slides are used. The technique is especially useful for assessing gene rearrangements (108-110).

Southern blotting

When gene rearrangements are present in at least 5-10% of the cells, the specific DNA sequences can be detected by Southern blotting (111). After digesting with restriction enzymes and electrophoresis, the DNA fragments are transferred to a membrane and hybridized with a probe representing the DNA sequence of interest. The labeled, hybridized probe can then be used to detect rearrangements with autoradiography.

Reverse transcriptase-polymerase chain reaction

The reverse transcriptase-polymerase chain reaction (RT-PCR) is particularly useful for demonstrating specific structural gene rearrangements in RNA transcripts (112-114). After reverse transcription and repeated cycles of cDNA denaturation, primer attachment and chain amplification, the concentration of the altered cDNA is exponentially augmented and can then be analyzed following electrophoresis, blotting and hybridization. The RT-PCR technique is sensitive: one cytogenetically aberrant cell can be detected among 10^4 - 10^6 normal cells. The technique thus provides a powerful approach for analyzing rare cells, sorted subpopulations, colony cells. Southern blotting and RT-PCR may be useful for detecting the presence of specific genetic abnormalities in conditions where banding techniques reveal no aberrations.

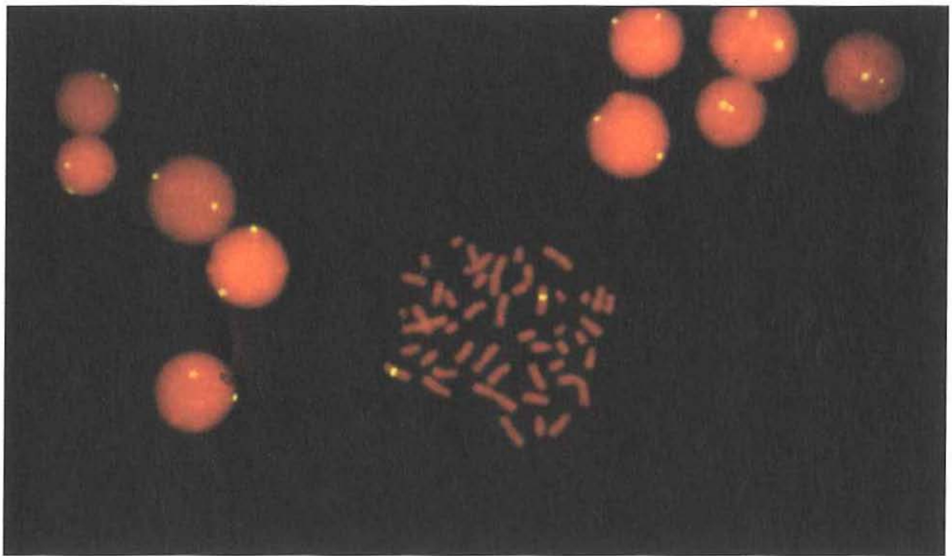

Figure 4.
FISH applied to a metaphase cell and interphase cells using a probe specific for the centromeric region on chromosome 7. In the metaphase two chromosomes 7 show the fluorescent centromere. In interphase cells two FISH spots represent the chromosome 7.

In the context of clonality studies, the analysis of chromosome aberrations does have a limitation since it may only reveal secondary hits and the primary genetic change may remaine undetected (179). Cells without a detectable chromosomal aberration may still be clonally derived but may not be part of the cytogenetically abnormal clone.

1.6 Chromosome abnormalities and FAB subtypes

Specific cytogenetic abnormalities are found in distinct AML and MDS FAB types and have been reported in the cytological, immunologic, and cytogenetic working classification (115-117). In illustration, certain selected examples will be mentioned here (Table 4).

In the t(8;21), the *AML1* gene (also known as core binding factor-α, *CBF-α* or *CBFA2* gene) at 21q22 is fused to the Eight Twenty-One (*ETO*) gene at 8q22 (118,119). The t(8;21) is predominantly associated with AML type M2, although it may be seen in other FAB types (*e.g.* M1) as well (120,121). Dysplastic granulocytic precursors and an increase of eosinophils are often seen in this type of AML.

The t(15;17)(q22;q21), involves the promyelocytic (*PML*) gene at 15q22 and the retinoic acid receptor α (*RARα*) gene at 17q21 (122-128). The latter translocation is mainly seen in AML type M3 (122,125-130). In about 18% of cases with t(15;17) an additional chromosome 8 (trisomy 8) is present (129,130). In rare cases of acute promyelocytic leukemia a translocation t(11;17)(q23;q21) is found which results in a fusion of the promyelocytic leukemia zinc finger (*PLZF*) to the *RARα* gene (131,132).

The inv(16)(p13q22) and t(16;16)(p13;q22) result in the fusion of core binding factor ß (*CBFβ*) gene at 16q22 to the smooth muscle myosin heavy chain (*MYH11*) gene at 16p13 (133,134). The latter aberrations correlate with AML type M4 with atypical eosinophils, as do deletions of chromosome band 16q22 (135,136). A trisomy 4 is also associated with AML type M4 (137,138).

Table 4. Some examples of chromosomal aberrations associated with distinct acute myeloid leukemia FAB types

FAB	Chromosomal aberration	Genes involved	Reference
AML-M2	t(8;21)(q22;q22)	*ETO;AML1*	118-121
AML-M3	t(15;17)(q22;q21)	*PML;RARα*	122-128
AML-M3	t(11;17)(q23;q21)	*PLZF;RARα*	131,132
AML-M4eo	inv(16)(p13;q22) or		
	t(16;16)(p13;q22)	*MYH11;CBFβ*	133-135
	del(16)(q22)		136
AML-M4	+ 4		137,138
AML-M2/M4 baso	t(6;9)(p23;q34)	*DEK;CAN*	139-141
AML-M4/M5	t(4;11)(q21;q23)	*AF4;MLL*	144,146,148
	t(9;11)(p22;q23)	*AF9;MLL*	146,148,151
	t(10;11) (p11~p15;q23)	*AF10;MLL*	147
	t(11;19)(q23;p13)	*MLL;ENL*	146,148

The t(6;9)(p23;q34) involves fusion of the *CAN* gene at chromosome 9q34 to the *DEK* gene at chromosome 6p23. It is mainly seen in AML type M2 or M4 with basophilia but the translocation may also be found in MDS (139-141).

Translocations of chromosome 11 at the breakpoint 11q23 involve the mixed lineage leukemia or myeloid/lymphoid leukemia (*MLL*) gene (142,143). This gene is also known

as the acute lymphoblastic leukemia (*ALL*) gene or the human trithorax (*HRX* or *HTRX*) gene. Common partner genes are *AF4* (for *ALL-1* fused gene from chromosome 4) in t(4;11)(q21;q23), *AF6* in t(6;11)(q27;q23), *AF9* in t(9;11)(p22;q23), *AF10* in t(10;11)(p11~p15;q23) and *ENL* in t(11;19)(q23;p13) but numerous other partner genes have been identified in hematological malignancies involving MLL (142-148). Recently, self fusion rearrangements of the *MLL* gene have been documented in patients with AML (subtype M1 or M2) and a normal karyotype, or trisomy 11 as a sole abnormality (149,150). Translocations involving 11q23 are associated with AML types M4 or M5 and especially the poorly differentiated form of monoblastic leukemia, AML subtype M5a (147,148,151-153). Secondary AML with rearrangement of the *MLL* gene are frequently seen in patients previously treated with topoisomerase-II-inhibitors (148,152-154,158). Translocation (8;21)(q22;q22) and inv(16)(p13q22) have also been observed in patients previously treated with topoisomerase-II-inhibitors and/or alkylating agents, but the numbers of cases are small (155,158). Translocations involving the *MLL* gene have also been reported in acute lymphoblastic leukemia (ALL) (148,152).
Trisomy 8 does not correlate with a specific AML or MDS subtype (116,117). Deletions of parts of the chromosomes 5 and 7, or monosomy 5 and 7, are more frequently seen in patients previously exposed to toxic agents (29,33,154,156-158). Loss of chromosome 7 or del(7q) as a sole abnormality is quite common in juvenile MDS and in leukemic progression of congenital disorders *e.g.* severe congenital neutropenia (17-19). Del(5)(q12~13, q31~33), as a sole abnormality, is associated with the MDS type RA in older women (159-161). Macrocytic anemia, thrombocytosis and typical mononuclear megakaryocytes are characteristic of the 5q- syndrome. The multiple chromosome abnormalities that are so frequently seen in patients with MDS and AML, are indicative of complex molecular changes and suggest successive stages of clonal evolution (21,22,29,33,156-158).

1.7 Clonality assays

In AML and MDS, the clonal expansion of a transformed progenitor cell will generate a population of malignant cells sharing identical genetic aberrations or, in females, the same inactivated X chromosome. To study cell lineage involvement in MDS and AML, assays have been based on disease specific, genetic markers or on X chromosome polymorphisms in females. These techniques may be applied to subpopulations of cells or cultured cell populations to establish the clonal involvement of distinct cell lineages.

In females, X chromosome inactivation occurs by methylation of either the paternal or the maternal X chromosome (162,163). According to Lyon's hypothesis, this is a random process that takes place during embryogenesis and results in a balanced distribution of cells containing the active paternal and cells containing the active maternal X chromosome. Theoretically this should give a ratio 1:1. A clonal population on the other hand, derived from a single cell, i.e. with the same X chromosome inactive, will show a theoretical 1:0 ratio. In reality, in normal tissues, skewing towards one or the other allele is often seen. In practice, this may hamper the interpretation of the analysis with regard to clonality. Different levels of skewing may be seen in specific tissues, and the levels may also increase with age (164-166). In addition, the differential methylation patterns, which distinguish active from inactive X chromosomes, can complicate the interpretation of these assays (167). Therefore, the value of X chromosome analysis depends on the choice of the cut-off level for clonality (taking skewing into account) and the use of proper control cells.

Polymorphic X-linked loci like the hypoxanthine phospho-ribosyl transferase (*hprt*), the phospho-glycerate kinase (*pgk*), the non-expressed *m27β* gene or the human androgen receptor gene (*humara*) can be used for clonality studies (81,168-176). Assays may also be based on RT-PCR of the X-linked glucose-6-phosphate dehydrogenase (G6PD) mRNA, palmitotylated erythrocyte membrane protein (p55) mRNA and iduronate-2 sulphatase (IDS) mRNA (177,178). Cells without a nucleus, *e.g.* reticulocytes and platelets, can then also be studied. In early clonality studies, iso-enzymes of G6PD have been used (179,180). However, heterozygosity of G6PD is less frequent in the Caucasian population and therefore not generally applicable. This test has mainly been used in African-American female patients (175).

Mutations of the *ras* gene family are found in 10-40% of cases with MDS. By applying allele specific restriction analysis (ASRA) and single strand conformation polymorphism (SSCP) these mutations can be used as clonal markers (40,168).

Another approach uses polymorphic, short repeated DNA sequences, known as microsatellites, which exist throughout the genome. Deletion of the DNA repeats on one allele results in loss of heterozygosity (LOH). PCR amplification of the repeats allows assessment for LOH and can be used in clonality assay (181).

Cytogenetic abnormalities, when present, can also be used as markers in clonality studies (182,183). The various methods available for analysis have been introduced above (Paragraph 1.5). To establish the involvement of individual cell lineages in hematological

malignancies, FISH on interphase cells is a useful assay especially when used in combination with cytology, cytochemistry or immunology and cell sorting (184-188).

1.8 Introduction to the experimental work and aims of this study

Clonality studies have shown that in MDS and AML, blasts, granulocytic cells, erythroblasts and platelets are clonally derived. The involvement of lymphocytes is less consistent in myeloid malignancies. In most studies, techniques based on X chromosome inactivation have been used. This implies that whole cell populations collected after cell culture or cell separation were analyzed. In these studies individual cells could not be evaluated.

In this thesis a series of investigations are presented in which clonality was systematically analyzed at the single cell level in patients with MDS, LT-MDS and *de novo* AML. A standardized definition of *de novo* AML was applied. FISH was used to detect the presence of a specific chromosomal marker in cells stained using cytological or immunocytological techniques. This allowed us to determine in which of the maturing cells and cell lineages the chromosome abnormality was present (chapter 2 , 3, 4 and 5). Furthermore, the presence of clonal and non-clonal cells within one cell lineage was investigated.

Chapter 3 presents a detailed analysis of the marrow and blood cells from three patients with MDS and monosomy 7 with regard to cell lineage involvement, using a combined immunocytochemistry and FISH technique.

In chapter 4, we evaluated and compared cell lineage involvement of a chromosomal aberration in MDS, LT-MDS and *de novo* AML as representative evolutionary stages of leukemia progression.

In chapter 5, lineage involvement was studied in bone marrow cells from a limited cohort of patients with AML and t(8;21).

In chapter 6, experiments were conducted to establish the involvement of megakaryocytes in the malignant clone in MDS and LT-MDS using an adapted FISH technique and digital image analysis.

Finally, in chapter 7, the experimental findings of this thesis are summarized and discussed.

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CHAPTER 2

In situ hybridization on May-Grünwald-Giemsa stained bone marrow and blood smears of patients with hematological disorders allows detection of cell lineage specific cytogenetic abnormalities

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Abstract

Bone marrow and blood from patients with acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) were studied by simultaneous analysis of cell morphology and karyotype. A combined technique of May-Grünwald Giemsa (MGG) for cell morphology and fluorescence in situ hybridization (FISH) with chromosome specific DNA probes for detection of cytogenetic aberrations allowed us to investigate cell-lineage-specific chromosomal abnormalities. We introduced video recordings to examine large numbers of cells. Briefly, evaluation was first performed on MGG slides, during which cell position and morphology were recorded on an S-VHS recorder. Subsequently, the same slides were used for FISH. This resulted in the identification of MGG-stained cells on the video screen, and at the same, time the interpretation of FISH signals in the fluorescence microscope. Specimens of bone marrow or blood samples from four patients with different hematologic malignancies were studied. One of these patients was studied before and after cytotoxic treatment. Gain or loss of chromosomes could be detected easily and morphologically assigned to the blasts in all patients, and to a variable proportion of the myelomonocytic lineage in two patients, but not to the lymphocytes. Thus, this method provides new possibilities for investigating the clonality of hematologic malignancies.

Introduction

Diagnosis and classification of the myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML) according to the French-American-British (FAB) criteria are based on cytomorphologic examination of peripheral blood (PB) and bone marrow (BM) smears (1-4). These may show an increased percentage of blasts and/or typical abnormalities of cell morphology (dysplasia). Cytogenetic abnormalities, such as translocations and numerical changes, are shown by banding techniques of BM and PB cells in metaphase and may provide important prognostic and diagnostic information (5,6). These techniques are routinely used as independent methods and provide complementory information. A combined technique of cytogenetic analysis and cytologic (or immunologic) identification of cells would be useful for relating the chromosomal aberrations directly to the abnormal cells and assessing cell-lineage involvement of AML and MDS. Furthermore, an integrated cytogenetic/hematologic technique may allow for the specific characterization of morphologically atypical cells suspected of belonging to the neoplastic clone. It may also be useful for the detection of minimal residual numbers of malignant cells after cytotoxic therapy. Approaches based on cytochemistry, immunology, and/or progenitor assays

combined with metaphase cytogenetics have been examined previously (7-9) and were shown to be applicable to BM from patients with various hematologic disorders. Previously, the use of these combined techniques was restricted because the number of analyzable cells is relatively small. Furthermore, cells of interest have to be able to divide in vitro so that cytogenetic analysis can be applied to the cells during metaphases. The development of interphase cytogenetics using repeat DNA probes that are chromosome specific offers new possibilities for examining cells exhibiting numerical chromosomal changes. Monosomy 7 and trisomy 8, as well as the loss of a sex chromosome, are among the most frequent nonrandom cytogenetic aberrations seen in hematologic disorders. Studies of patients using combined interphase cytogenetics and immunologic or morphologic analysis have recently been published (10,11).

We report here a fluorescence in situ hybridization (FISH) technique applied to May-Grünwald Giemsa (MGG)-stained slides that permits screening of large numbers of cells relatively easily. The use of FISH using chromosome-specific DNA probes in adjunct to the recording of the MGG-stained cells on an S-VHS videotaperecorder allows us to relate karyotype to morphologically identifiable cells. PB and BM from four patients with AML or MDS and from cytogenetically normal controls are described.

Materials and methods

Patients' material

BM and PB smears were made during standard diagnostic procedures in patients with MDS and AML. Four patients with numerical cytogenetic abnormalities were selected. Hematologic and cytogenetic characteristics are summarized in Table 1. In addition, BM smears from cytogenetically normal subjects were prepared. All smears were made without the use of anticoagulants and routinely stained with MGG. Extra MGG slides were kept for later FISH study; they were wrapped in foil and stored at -20 C until the time of hybridization. The smears were stored for up to 6 months in this study.

Morphologic study and video recording

The smears were thawed slowly, ie, at 4 C for 30 minutes and subsequently at room temperature for another 30 minutes. The foil was then removed. Slides were mounted in immersion oil (Merck, Darmstadt, Germany). Three or four markings were made at the back of the slides with a diamond glass pen to indicate the start of the recorded tracks and thus allow relocation of the cells after hybridization. Separate light and fluorescent microscopes

were used. Cells were examined with a Standard Universal microscope equipped for light microscopy (Zeiss, Oberkochen, Germany) and coupled to a camera (Panasonic WV-CD 130; Panasonic, Tokyo, Japan) plus an S-VHS video recorder (JVC HR-S4700E; JVC, Tokyo, Japan) and a colour video monitor (Panasonic BT-D2000 PSN). The MGG-stained cells were recorded on an S-VHS video tape (Fuji Magnetics SE-180; Fuji, Tokyo, Japan), after which the coverslip was removed from the slide. The slides were then rinsed for 10 seconds in a mixture of ethanol 100% (Nedalco, Bergen op Zoom, Netherlands) and methanol 100% (Merck) in a proportion of 95:5. The slides were air-dried.

Table 1. Patient diagnosis and cytogenetic and morphologic data

Patient No.	Diagnosis (FAB)	Karyotype	No. of Metaphases (%)	Material	Differential Count (%)	
1.	M3	46,XY 47,XY,+8, der(9), t(9;11) (q34;q13 or q14), t(15;17) (q22;q21)	21 (27) 57 (73)	BM	LPM NEU ERY LYM	90.0 2.0 7.4 0.6
2.*	M2	45,X-Y, t(8;21) (q22;q22)	20 (100)	BM	Blast NEU ERY LYM Other	65.4 18.6 6.4 1.6 8.0
2.†	CR	NT		BM	Blast NEU ERY LYM	0.6 78.4 17.8 3.2
3.	RAEB	46,XX 48,XX,+8,+9	2 (8) 23 (92)	PB	Blast NEU BASO MONO LYM Other	4.8 55.8 7.6 3.2 24.0 4.6
4.	M7	45,XY,-7	27 (100)	PB	Blast Neu ERY LYM Other	40.8 17.6 33.4 6.6 1.6

* at diagnosis; † after treatment. NT, not tested; LPM, leukemic promyelocytes; NEU, neutrophils, including promyelocytes, myelocytes, metamyelocytes, stab cells, and segmented neutrophils; ERY, erythroblasts; LYM, lymphocytes; BASO, basophilic granulocytes; MONO, monocytes.

Probe labeling and hybridization

The DNA probes that were used, were specific for the alpha-satellite sequences on the centromeric region of chromosome 7 (p7t1) (12) and chromosome 8 (D8Z2) (13) (both kindly provided by Dr. P. Devilee, University of Leiden, The Netherlands), as well as a satellite DNA probe specific for the hetero-chromatic regions of chromosomes 9 (pHUR98) (14) (kindly provided by Dr. A. K. Raap, University of Leiden) and Y (pY3.4) (15) (D. C. Page, Whitehead Institute, Cambridge, USA). The probes were labeled by standard nick translation using biotin-16-dUTP according to the manufacturer's instructions (Bethesda Research Laboratories, Gaithersburg, U.S.A.). The labeled probes were suspended in a hybridization mixture at a concentration of 2-5 ng/µl. The hybridization mixture contained 60% formamide (Merck) in 2 x standard saline citrate and sodium phosphate buffer pH 7.0 (2 x SSCP; 300 mM NaCl, 30 mM sodium citrate, 30 mM sodiumphosphate). The probes were denaturated at 70-72°C for 4 minutes and then cooled on ice. The MGG stained slides were denaturated in 70% formamide 2 x standard saline citrate buffer, pH 7.0 (1 x SSC; 150 mM NaCl, 15 mM sodium citrate) at 70-72°C for 2.5 minutes. Slides were then dehydrated sequentially in 70%, 80%, 95% and 100% ethanol and air-dried. The 70% ethanol was ice-chilled. Thereafter, the hybridization mixture containing the probe was applied to the slides and a coverslip was added. Hybridization was allowed to occur overnight in a moist chamber at 37°C. Subsequently, the slides were washed three times with formamide 50% in 2 x SSC at 39-40°C for 10 minutes and then three times for 5 minutes in 2 x SSC at room temperature. Finally, the slides were incubated in 4 x SSC + 0.05% Tween (Pierce, Rockford, U.S.A.) for 10 minutes. The hybridized probe was detected by using fluorescein isothiocyanate (FITC)-labeled avidin (Vector laboratories, Burlingame, U.S.A.). Cells were counterstained with propidium iodide (Sigma, StLouis, U.S.A.). The slides were analyzed using a standard 14 IV FL fluorescence microscope (Zeiss) equipped with a (FITC) filter combination 09 (BP 450-490, FT 510, LP 520; Zeiss). The previously recorded MGG-stained cells were distinguished on the video screen and in parallel in the fluorescence microscope, permitting a direct comparison of morphologic and cytogenetic characteristics of the cells.

In each experiment normal cells were run in parallel to check the efficiency of the MGG/FISH procedure.

As a control for the hybridization procedure in the case of chromosome loss, double hybridization was performed with a biotin-labeled diagnostic probe and a digoxigenin-labeled control probe resulting in green (FITC) and red (tetra methylrhodamine

isothiocyanate [TRITC]) fluorescent spots. In other experiments the control probe was also biotin labeled and hybridyzed on a separate part of the slide and analyzed independently.

Results

The combined MGG/FISH study with video recording was performed to simultaneously identify cell morphology and hybridization spots. To establish the FISH technique and evaluate its specificity, the distribution of normal values was assessed for all probes in BM smears of cytogenetically normal controls. For each control, 500 cells were scored. Table 2 gives the normal values expressed as percentage of cells with 0, 1, 2, 3 or 4 spots ± standard deviations (SD). To assess the distribution variation of the number of FISH spots in different cell types, MGG/FISH with video recording was applied to BM smears from four cytogenetically normal controls using a probe specific for chromosome 8 (Table 3). FISH spots in erythroblast were easy to detect and, therefore, the percentage of erythroblasts with one spot is low. The compact nucleus structure of lymphocytes explains the relatively high percentage of cells with one spot. MGG-stained smears do respect, to some extent, the tridimensional shape of the cell. Therefore, it required careful exploration of the nucleus with different depths of focus to detect the spots.

Table 2. FISH spots on MGG-stained cells from cytogenetically normal subjects

		FISH spots per cell (mean percentage ± SD)				
Probe (chromosome)	No. of experiments*	0	1	2	3	4
p7t1 (chromosome 7)	8	-	2.4 ± 1.7	97.3 ± 1.7	-	
D8Z2 (chromosome 8)	9	-	3.2 ± 1.1	96.2 ± 1.6	0.4 ± 0.5	-
pHUR 98 (chromosome 9)	5	-	4.7 ± 2.1	95.0 ± 2.3	0.3 ± 0.3	-
pY3.2 (chromosome Y)	9	1.5 ± 1.2	98.2 ± 1.1	-	-	-

* Five hundred cells were scored in each experiment.

Table 3. FISH spots per cell type scored on MGG-stained BM slides. Four cytogenetically normal subjects analyzed using probe D8Z2 specific for chromosome 8

Cytogenetically normal individual	Cell type	No. of cells analyzed	FISH spots per cell type (%)				
			0	1	2	3	4
1.	Blast	6	-	16.7	83.3	-	-
	NEU	83	-	2.4	96.4	1.2	-
	ERY	142	-	2.8	95.8	1.4	-
	LYM	58	1.7	6.9	91.4	-	-
2.	Blast	4	-	-	100	-	-
	NEU	171	-	2.3	97.1	0.6	-
	ERY	177	-	1.7	98.3	-	-
	LYM	99	-	6.1	93.9	-	-
3.	Blast	3	-	-	100	-	-
	NEU	97	-	3.1	96.9	-	-
	ERY	49	-	2.0	98.0	-	-
	LYM	27	-	3.7	92.6	3.7	-
4.	Blast	4	-	-	100	-	-
	NEU	249	0.4	1.2	98.4	-	-
	ERY	187	-	3.2	96.8	-	-
	LYM	81	-	6.2	93.8	-	-

NEU: neutrophils, including promyelocytes, myelocytes, metamyelocytes, stab cells, and segmented neutrophils; ERY, erythroblasts; LYM, lymphocytes.

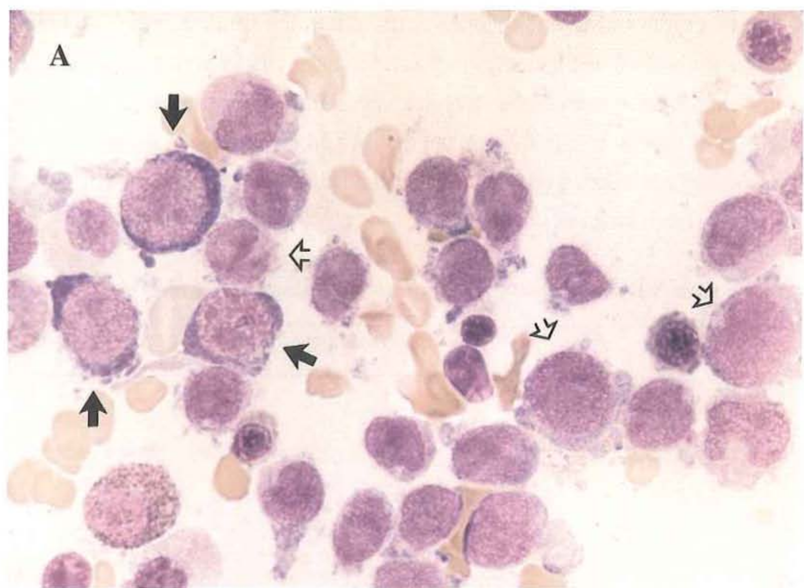
Table 4 shows the compiled results of the analysis of AML and MDS cells with the combined MGG/FISH technique. Hybridization with the chromosome-8-specific probe in patient no. 1 with AML-M3 showed that most of the promyelocytes carried the trisomy 8, but neutrophils, erythroblasts and lymphocytes did not show the +8 cytogenetic marker (Figure 1). The same chromosome probe was applied to the cells of patient no. 3 (refractory anemia with excess of blasts [RAEB]). Here, myeloblasts and significant proportions of basophils, neutrophils and monocytes showed the trisomy 8. Thus, these cells were members of the malignant clone, but the analysis of +8 positive cells also shows the coexistence of normal myeloid cells in addition. Lymphocytes showed the normal background distribution and apparently did not belong to the leukemic cell population. Since patient no. 3 also exhibited a trisomy 9, an analysis with the probe specific for chromosome 9 was conducted as an additional experiment. The results of the latter analysis were in agreement with the chromosome 8 data (Table 4). Cells from patients no. 2 and 4 were used to verify the usefulness of the technique when chromosomes are missing. A loss of the Y chromosome in

patient no. 2 was apparent in myeloblasts as well as the more mature hypogranulated neutrophils but not in erythroblasts and lymphocytes. After the attainment of complete remission (CR) in this individual, FISH analysis of myeloblasts still showed the presence of a cell population missing the Y chromosome. Because metaphase cytogenetics, at diagnosis, showed the association of loss of Y with t(8;21), we interpreted the results as an indicator for residual leukemic myeloblasts: further confirmation of the t(8;21) will have to be performed by polymerase chain reaction (PCR) analysis.

Table 4. FISH spots scored on MGG-stained cells from patients with hematologic disorders

Patient no. and cytogenetic marker	Cell type	No. of cells analyzed	FISH spots per cell type (%)				
			0	1	2	3	4
1. +8	LPM	269	-	1.5	4.1	94.4	-
	NEU	13	-	-	100	-	-
	ERY	87	-	5.7	94.3	-	-
	LYM	26	-	7.5	92.5	-	-
2. –Y at diagnosis	Blast	717	100	-	-	-	-
	NEU	415	100	-	-	-	-
	ERY	38	5.3	94.7	-	-	-
	LYM	121	2.5	97.5	-	-	-
2. –Y after treatment	Blast	71	11.3	88.7	-	-	-
	NEU	153	3.3	94.8	1.9	-	-
	ERY	33	6.1	93.9	-	-	-
	LYM	32	3.1	96.9	-	-	-
3. +8	Blast	14	-	-	-	100	-
	NEU	189	-	0.5	43.4	54.5	1.6
	BASO	24	-	-	66.7	33.3	-
	MONO	48	-	-	29.2	70.8	-
	LYM	66	-	3.0	94.0	3.0	-
3.+9	Blast	82	1.2	6.1	11.0	81.7	-
	NEU	649	-	3.0	75.8	21.0	0.2
	BASO	54	-	3.7	66.7	29.6	-
	MONO	91	-	1.1	60.4	38.5	-
	LYM	236	-	11.9	88.1	-	-
4. –7	Blast	410	5.8	87.6	4.9	1.7	-
	NEU	148	-	9.4	89.9	0.7	-
	MONO	9	-	67.0	33.0	-	-
	ERY	206	-	11.1	87.9	0.5	0.5
	LYM	33	-	3.0	97.0	-	-

LPM, leukemic promyelocytes; NEU, neutrophils, including promyelocytes, myelocytes, metamyelocytes, stab cells, and segmented neutrophils; ERY, erythroblasts; LYM, lymphocytes; BASO, basophilic granulocytes; MONO, monocytes.



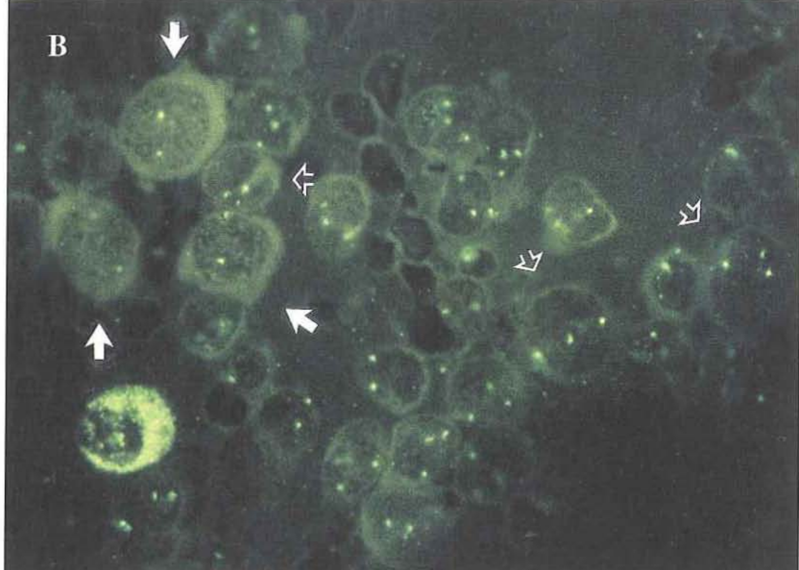


Figure 1.
(A) MGG-stained BM cells from patient no.1 (AML-M3).
(B) FISH on the same cells with a probe specific for chromosome 8. The solid arrows indicate the erythroblasts with two signals and the open arrows indicate the leukemic promyelocytes with three signals representing the trisomy 8.

In patient no. 4, the single chromosome 7 could be traced in myeloblasts and megakaryoblasts most clearly and according to the quantitative distribution as compared with the normal distribution in subpopulations of neutrophils, monocytes and erythroblasts as well. Cytogenetically normal and abnormal myeloid monocytic and erythroid cells apparently coexist. We attributed the relatively high percentage of lymphocytes with one FISH spot in patients no. 1 and 3 to a compact chromatine structure of the nucleus.

Discussion

In this study we show the feasibility of a combined MGG/FISH technique with the use of video tapes. This technique allows a direct comparison of morphologically identified cells and their cytogenetic status without intervention of culturing or immunophenotyping. Furthermore, morphologic abnormalities like hypogranulation in neutrophils can now be coupled to a chromosomal aberration. All our patients showed cytogenetic changes in the blasts. Some patients showed cytogenetic abnormalities in mature myeloid cells, erythroblasts and monocytes, but in lymphocytes the amount of cells with trisomy, monosomy or loss of a sex chromosome was not significantly different from normal controls. Interestingly, the loss of the Y chromosome in patient no. 2 was detected in all hypogranulated neutrophils, indicating that the abnormal myeloid progenitor cell was able to mature, which resulted in abnormal granulated cells. When this patient attained a CR, no hypogranulated cells were detected, although the combined MGG/FISH technique still showed some blasts without a Y chromosome. Megakaryocytes were difficult to interprete because of their variable ploidy, as were eosinophils because of the autofluorescence of granules. We found that the use of a mounting medium after staining with MGG containing xylol reduced the intensity of the hybridization spot. Immersion oil as mounting medium overcomes this technical problem. The combined MGG/FISH technique is a sensitive test for identification of cell-lineage involvement of cytogenetic abnormalities. Selective interphase cytogenetic screening of specific cell types, ie, blasts or mature cells, as in patient no. 2 suggests that the technique can be applied for the detection of residual disease as well as for the study of more than one hematologic malignancy in a single individual. In the future, experiments with cosmid and YAC probes may allow for the identification of translocations and/or partial deletion in MGG stained interphase cells.

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CHAPTER 3

Cytogenetic clonality analysis in myelodysplastic syndrome: monosomy 7 can be demonstrated in the myeloid and in the lymphoid lineage

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Abstract

Bone marrow and blood from three patients with myelodysplastic syndrome (MDS) and monosomy 7 were studied for cell lineage involvement of the chromosomal abnormality. Cytogenetic involvement of the myeloid and erythroid cell lineages in MDS with monosomy 7 has been shown before. Lymphoid subpopulations have also been investigated but generally with negative results. A combined technique of May-Grünwald-Giemsa (MGG) for cell cytology and interphase fluorescence in situ hybridization (FISH) using a chromosome 7 specific DNA probe was applied. Further, immunophenotype and genotype of the cells were simultaneously examined with alkaline phosphatase anti-alkaline phosphatase (APAAP) immunostaining and FISH. The monosomy 7 was found in the blasts and in all or in subpopulations of myeloid and erythroid cells. T-cells (CD3+, CD5+) did not appear to be involved. B-cells (CD19+, CD22+) showed a normal distribution of FISH spots in two patients. In one patient however the loss of a chromosome 7 was found in approximately 70% of the cells positive for B-cell markers including CD79a. The results of this study show that in some cases MDS is a disease arising in a progenitor cell with repopulative abilities restricted to myelopoiesis and erythropoiesis. In other cases, the pluripotent progenitor cells in MDS may show the capacities to differentiate into B-lineage lymphoid cells, as well suggesting that in those instances MDS represents a condition of more primitive transformed hematopoietic ancestor cells.

Introduction

Myelodysplastic syndrome (MDS) is a group of hematologic disorders characterized by ineffective hematopoiesis resulting in a variable degree of anemia, leukopenia or thrombopenia. Eventually approximately 30% of the patients will show progression to acute myeloid leukemia (AML) (1-3). In MDS a hematopoietic progenitor cell is thought to be malignantly transformed. Blood and marrow cells derived from the abnormal progenitor may replace the normal hematopoietic tissue to a variable extent.

Several techniques have been used to study clonality of hematopoiesis in MDS: glucose-6-phosphate dehydrogenase (G6PD) iso-enzyme analysis and studies using restriction fragment length polymorphism (RFLP) of X-linked genes (4-7). Both strategies are based on X chromosome inactivation and therefore only applicable on cells of female patients.

Non-random cytogenetic abnormalities such as del (5q), monosomy 7 and trisomy 8 are common findings in patients with MDS (2). In case of deletions, the absence of an allele can be used as a marker for clonality studies (7,8). When numerical cytogenetic abnormalities

are present, the fluorescence in situ hybridization (FISH) technique using chromosome specific probes has proved useful. FISH combined with cytology or immunophenotyping may provide direct information on the relationship between karyotype and phenotype as well as cell lineage (9-13).

In MDS, studies on cell lineage involvement have demonstrated that cells of myeloid origin are clonally derived. However conflicting results have been published on lymphoid involvement (4-17). We have used FISH in combination with cytology and immunophenotyping to assess the presence of monosomy 7 especially in B- and T- cell lineages in three male patients with MDS and monosomy 7.

Materials and methods

Bone marrow and blood cells

The cells from three patients (two children, one adult) with MDS and a monosomy 7 according to conventional cytogenetic analysis (banding techniques), were examined (18-20).

Patient no. 1 (age 1 year) had refractory anemia with excess of blasts in transformation (RAEB-t) 6 months after a documented phase of juvenile chronic myelomonocytic leukemia (J-CMML). The monosomy 7 was found in all metaphases (n=31). Patient no. 2 (age 1 year) had refractory anemia (RA). Cells were obtained at time of diagnosis, the loss of chromosome 7 was found in 30 of 33 metaphases. Patient no. 3 (age 67 years) developed RA 21 months after treatment for AML-M2. At the time of the RA the monosomy 7 was found in 38 of 44 metaphases. During the AML phase no cytogenetic aberrations were detected.

Bone marrow (BM) smears were made following standard diagnostic procedures and routinely stained with May-Grünwald-Giemsa (MGG). Extra slides were kept for later FISH study; they were wrapped in foil and stored at -20°C until the time of hybridization. In addition, BM or peripheral blood (PB) was collected in heparinized tubes and centrifuged over Lymphoprep (Nycomed, Oslo, Norway; density 1.077 g/ml) to obtain a mononuclear cell fraction (MNC). MNC was stored in liquid nitrogen. MGG stained BM smears of cytogenetically normal subjects were used as controls of the MGG/FISH procedure. MNC of 5 healthy volunteers were centrifuged onto glass slides and used as control slides for the combined immunophenotypic analysis and FISH.

Combined cytomorphology or immunophenotyping and fluorescence in situ hybridization

Cytology of MGG stained cells and karyotype were examined simultaneously as described

previously (13). In short; BM smears were thawed slowly and the MGG stained cells were recorded on a S-VHS video tape using a Standard Universal light microscope (Zeiss, Oberkochen, Germany) coupled to a camera, a S-VHS video recorder and a color monitor. The same slides were used for FISH using a biotinylated probe specific for the α-satellite sequences on the centromeric region of chromosome 7 (p7t1) (21). Following denaturation and hybridization procedures the hybridized probe was detected using fluorescein isothiocyanate (FITC) labeled avidin (Vector Laboratories, Burlingame, CA, USA). The previously recorded MGG stained cells were analyzed on the video screen and in parallel employing the fluorescence microscope (standard 14 IV FL, Zeiss) equipped with a filter combination 09 (BP 450-490, FT 510, LP, 520; Zeiss). The latter parallel analysis permitted a direct comparison of the cytomorphology and cytogenetic characteristics of the cells. In each experiment normal cells were run in parallel to assess the quality of the MGG/FISH procedure.

For alkaline phosphatase anti-alkaline phosphatase (APAAP) immunostaining the cryopreserved MNC were resuspended in phosphate-buffered saline (PBS), cytocentrifuged onto glass slides and air dried. A panel of murine monoclonal antibodies (MoAb) were applied: CD3 (Leu-4; Becton Dickinson, Sunny Vale, CA, USA) and CD5 (Leu-1; Becton Dickinson) for identifying T cells; CD19 (B4; Coulter Clone, Hialeah, FL, USA), CD22 (Leu 14; Becton Dickinson) and CD79a (mb-1 HM57; Dr. Mason DY, Oxford, UK[22]) for B-cells; CD34 (HPCA2; Becton Dickinson) to characterize hematopoietic progenitors, CD14 (My-4; Coulter Clone) for monocytoid cells. Normal mouse serum (1:1000) was used as a control for non-specific binding. A three-stage unlabeled bridge method was used for detecting the MoAb (23). In short, cytospin slides were fixed in acetone, after which a murine MoAb was added. Unlabeled rabbit anti-mouse immunoglobulins (DAKO, High Wycombe, UK) and after that an APAAP complex (mouse) (DAKO) were applied. A substrate of naphtol AS-MX phosphat (Sigma, St Louis, USA) plus Fast red TR (Sigma) was used to obtain a bright red colored and fluorescent complex which is visible in the light and in the fluorescence microscope. Positive cells were scored using a light microscope (Zeiss).

Following the APAAP staining the slides were subjected to the FISH protocol (13). To obtain a blue nucleus, cells were counterstained with '4,6-diamidino-2-phenylindole (DAPI) (Sigma). A fluorescent microscope (Zeiss) equipped with a filter combination 09 (Zeiss) permitted simultaneous analysis of the immunophenotype (APAAP: red fluorescence) and the genotype (FISH: green fluorescent spots). For photography cells were exposed twice to a 400 ASA Kodak film first using an excitation filter for FITC (Zeiss) and again using an

excitation filter for DAPI (Zeiss).

Fresh MNC from 5 healthy volunteers were also examined according to the APAAP/FISH procedure to assess the false-negative background (one or no spot per cell) for the chromosome 7 probe per MoAb.

Table 1. FISH spots scored on MGG-stained bone marrow cells from patients with MDS and monosomy 7 using probe p7t1

Patient no.	Cell-type	Differential Count (%)	No.of cells analyzed with FISH	FISH spots per cell type (%) 0	1	2	3	4
1.	Blast	14	37	-	100	-	-	-
	NEU	34	53	-	100	-	-	-
	MONO	13	27	-	100	-	-	-
	ERY	14	55	-	91	9	-	-
	LYM	25	12	-	42	58	-	-
3.	Blast	1	7	-	86	14	-	-
	NEU	39	74	-	57	43	-	-
	ERY	45	77	-	47	53	-	-
	LYM	4	43	-	6	94	-	-

NEU, neutrophils, including promyelocytes, myelocytes, metamyelocytes, stab cells, segmented neutrophils; MONO, monocytes; ERY, erythroblasts; LYM, lymphoid cells.

Results and discussion

The distribution of normal values for chromosome 7 FISH was assessed in eight MGG stained BM smears of cytogenetically normal controls and in each case 500 cells were scored. The mean percentages (± standard deviation) of cells with one fluorescent spot were 2.4 ± 1.7% and for cells with two spots 97.3 ± 1.7%.

The results of simultaneous analysis of morphology and hybridization spots in BM cells of patients no. 1 and 3 with MDS are shown in Table 1. BM slides of patient no. 2 were not available. In patient no. 1 the monosomy 7 is found in almost all the blast cells, neutrophils (promyelocyte to segment), erythroblasts and monocytes. A subpopulation of the lymphoid cells (42%) showed the monosomy 7 as well.

In patient no. 3 the monosomy 7 was apparent in large proportions of blast cells, neutrophils and erythroblasts, suggesting that both myelopoiesis and erythropoiesis were partly normal, partly abnormal. The one fluorescent-two fluorescent spot distribution among lymphoid cells

was within the normal range.

APAAP/FISH was applied to peripheral blood MNC of 5 healthy volunteers. For each MoAb 80-1000 cells were scored. The normal background of false-positive monosomy 7 cells by APAAP/FISH was between 5.5% and 2.0%.

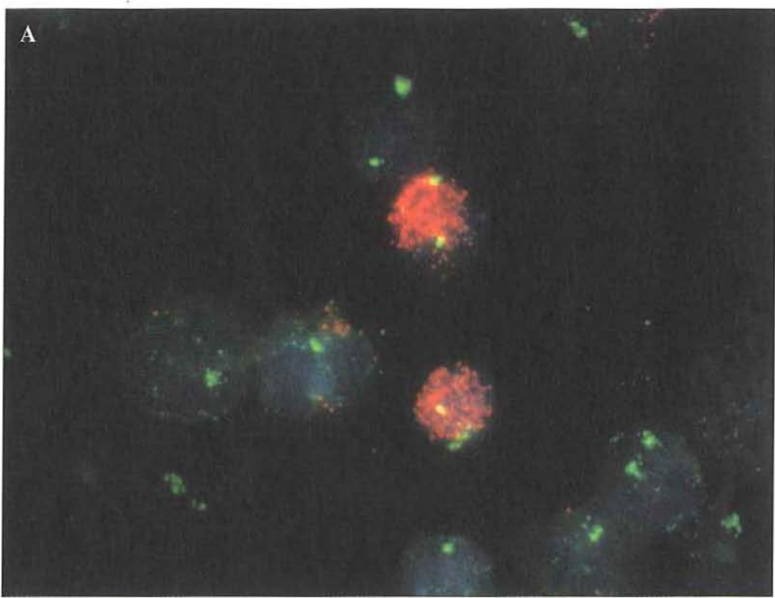
Results of the combined chromosome 7 FISH and immunophenotyping in patients no. 1, 2 and 3 are given in Table 2. Increased values indicative of monosomy 7 were seen in high percentages of CD34 and CD14 positive cells of all patients: precursor cells and monocytes were part of the malignant clone.

Table 2. Percentages of monosomy 7 cells among immunophenotypically distinct subpopulations in controle samples and in three patients with MDS

	CD3	CD5	CD19	CD22	CD79a	CD34.	CD14
Patient 1 PB	6	4	68	69	74	99	100
Patient 2 PB	3	6	9	2	NT	90	93
Patient 3 BM	5	3	2	3	NT	90	97
controle samples	5.5 ± 3.3	2.7 ± 3.8	4.9 ± 2.8	3.9 ± 2.8	4.4 ± 1.2	2.0 ± 3.0	2.7 ± 1.0

PB, peripheral blood; BM, bone marrow; NT, not tested. Patient material: 40-400 APAAP positive cells were analyzed per phenotype. Control samples: 80-1000 cells were scored for each MoAb on peripheral blood mononuclear cells from five cytogenetically normal subjects.

The high percentage of CD34 positive cells with monosomy 7 in patient no. 3 and the high percentage of cytogenetically normal erythroid and neutrophils in the MGG/FISH staining suggests that the normal clone had a maturation advantage at that time. In this patient the loss of chromosome 7 is found in a high percentage of CD14 positive cells; apparently there is a preference of the malignant clone to mature into monocytes. CD19 and CD22 positive cells (B cells) showed normal two-spot distributions in patients no. 2 and 3. In contrast, in patient no. 1 the loss of chromosome 7 was also apparent in B cells as high percentages (68-74%) of CD19, CD22 and CD79a positive cells showed one fluorescent spot. The percentages of FISH spots among T-cells (CD3+, CD5+) were not significantly different from normal (Figure 1).



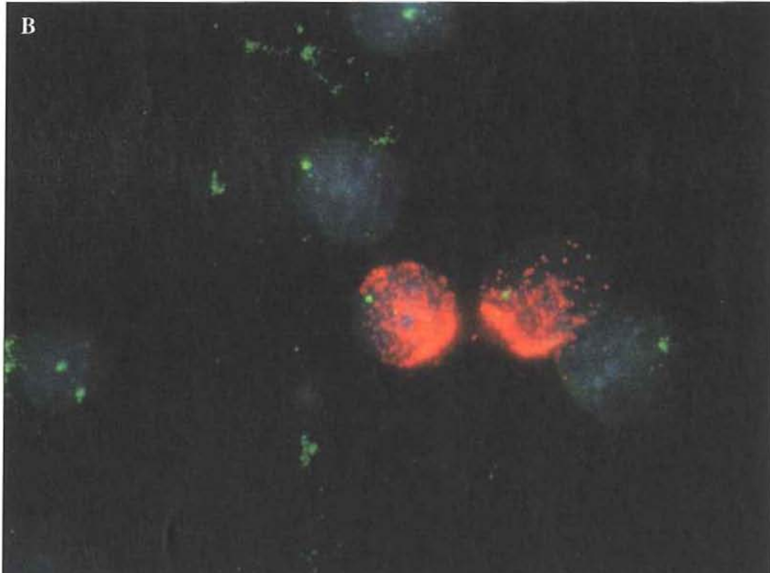


Figure 1.
(A) Patient 1: two cells positive for CD5 (red fluorescence) showing two FISH spots (green fluorescence) indicating two chromosomes 7. (B) Patient 1: two cells positive for CD19 (red fluorescence) showing one FISH spot (green fluorescence) representing monosomy 7.

The results in patients no. 2 and 3 are consistent with previous reports on cytogenetic cell-lineage involvement in MDS. Several authors found chromosomal aberrations in the myeloid lineage, but lymphoid cells did not differ from normal controls. Knuutila et al.[9] used the morphology-antibody-chromosome (MAC) technique, Kibbelaar et al.[11] combined immunophenotyping and FISH. Gerritsen et al.[10] separated peripheral blood cells according to surface markers specific for the lymphoid and myeloid cell lineage with a cell sorter and analyzed for the loss of chromosome 7 by FISH (10). Kroef et al.[6] analyzed patients with MDS and del 5q (8). Cells were separated into fractions of different cell types by cell sorting after which they were analyzed for loss of heterozygosity using highly polymorphic mini-repeat sequences from the 5q31 to 5q33 region. Anastasi et al.[12] studied MDS patients before and after growth factor therapy using MGG/FISH (12). Our data and those of others indicate that in MDS the chromosomal abnormality arise in a precursor cell of both myeloid and erythroid lineage which is capable of maturing.

In patient no. 1 the MGG/FISH on bone marrow cells revealed a high percentage (42%) of lymphocytes showing one chromosome 7 however the number of lymphocytes investigated this way was very low (12). MGG/FISH on the peripheral blood MNC of the patient gave similar results: 300 lymphocytes were analyzed, in 29% of the lymphocytes the monosomy 7 was apparent. FISH was also applied to the PB MNC of patient no. 1 using a probe specific for chromosome 8 (D8Z2). Of all the lymphoid cells, 95% showed two spots indicating that two chromosomes 8 were present and that the structure of the lymphoid nucleus allowed the analysis of more than one FISH spot (data not shown) (13). A high percentage (±70%) of the B cells identified according to CD19, CD22 or CD79a surface marker positivity exhibited a loss of chromosome 7. In the APAAP immunostaining these cells resembled small to medium sized mononuclear cells with round nuclei.

Until now two studies have presented evidence for chromosomal involvement of the lymphoid lineage in MDS (16,17). In one study, cytogenetic analysis in Epstein-Barr virus (EBV) transformed B lymphoblastoid cell lines contained del (13q) in two patients with RARS. Only normal metaphases were found in phytohemagglutinin (PHA) stimulated lymphocytes. White et al.[17] used metaphase analysis and microsatellite PCR to detect a del (20q) in purified populations of granulocytes and monocytes of a MDS patient (17). EBV transformed B cell lines carried the deletion but there was no evidence of the del 20q in PHA stimulated T cells. These results were found using stimulated or transformed lymphoid cells, not in cells directly derived from the patients. In our study, the monosomy 7 was

demonstrated in considerable proportions of untreated B-lymphocytes. This would suggest that in some cases of MDS or in some variants of MDS (juvenile-CMML) with monosomy 7 a pluripotent hematopoietic progenitor cell is transformed that gives rise to a clone of abnormal myeloid, erythroid as well as B lymphoid progenitors. We and others were not able to detect the monosomy 7 in T cells. This would indicate that hematopoietic progenitor cells that are also potential to T cells are generally not involved in MDS.
FISH applied to immunophenotypically distinct cells can establish clonal involvement of specific cell subsets. These studies have not yet been done in significant numbers of patients and therefore the frequency of involvement of the B cell lineage as shown here remains unclear at the present time.

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CHAPTER 4

Cytogenetic clonality analysis : typical patterns in myelodysplastic syndrome and acute myeloid leukaemia

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Summary

The cell morphology and karyotype of bone marrow samples from 24 patients with myelodysplastic syndrome (MDS) and acute myeloid leukaemia (AML) were studied simultaneously with a combined technique of May-Grünwald-Giemsa (MGG) staining and fluorescence in situ hybridisation (FISH) with chromosome-specific DNA probes. This enabled us to investigate cell lineage involvement in three malignant conditions: MDS (n=12), leukaemia-transformed MDS (LT-MDS) (n=5) and *de novo* AML (n=7). In MDS we found blasts and often also significant proportions of mature granulocytic and erythroid cells to be cytogenetically abnormal. Percentages of granulocytic and erythroid cells with cytogenetic aberrations were generally less than those of blasts.

These data support the involvement of a transformed pluripotent stem cell that has retained maturation abilities. In two patients with chronic myelomonocytic leukaemia (CMML), the clonal involvement of monocytes was predominant. Results in the five patients with LT-MDS were similar to those in MDS. In the bone marrow of 5 of the 7 *de novo* AML patients the cytogenetic abnormalities were restricted to the blasts and did not include the more mature granulocytic or erythroid populations. In the other two patients with AML, both with a t(8;21) and a loss of the Y chromosome, high percentages of mature neutrophils were cytogenetically abnormal. These patterns of clonal lineage involvement in MDS, LT-MDS, t(8;21) AML and AML appear typical and may be of clinical use, for example, for distinguishing LT-MDS from *de novo* AML in newly presenting patients.

Introduction

The myelodysplastic syndromes (MDS) comprise haematological disorders characterised by ineffective haematopoiesis resulting in variable degrees of anaemia, leucopenia or thrombocytopenia, and cytological signs of abnormal maturation (dyshaematopoiesis) in one or more cell lineages. Generally, these disorders are thought to result from the malignant transformation of a primitive haematopoietic progenitor cell that has lost the ability of normal proliferation and differentiation. The abnormal haematopoietic cells in MDS have been shown to be clonally derived. Eventually, approximately 30% of the patients will show progression to acute myeloid leukaemia (AML), designated sometimes as leukaemia-transformed MDS (LT-MDS) (1,2). LT-MDS shows a different response to treatment than *de novo* AML presenting without an antecedent history of MDS.

Table 1. Patient diagnosis and cytogenetic Data

Patient No. age(yr)/sex (Ref.)*	Diagnosis (FAB)	% blasts in BM	Karyotype	% (No.) of metaphases
1. 67/M (25)	RA	0.2	45,XY,-7 46,XY	86 (38) 14 (6)
2. 70/M	RARS	1.2	47,XY,+19 48,XY,+8,+19 46,XY	12 (4) 64 (21) 24 (8)
3. 64/F	RAEB	13.4	48,XX,+8,+11	100 (67)
4. 67/M	RAEB	13.8	47,XY,+8 46,XY	62 (22) 38 (18)
5. 70/M	RAEB	7.0	complex abnormalities, including -7	100 (41)
6. 81/M	RAEB	6.6	47,XY,+8	100 (32)
7. 56/F	RAEB-t	26.2	47,XX,+8 46,XX	84 (27) 16 (5)
8. 53/M	RAEB-t	15.8 14.0 in PB	complex abnormalities, including -Y	100 (32)
9. 43/M	RAEB-t	22.0 EO 6.6	45,XY,-7 46,XY,-7,+der (1) t(1;7) (q10;p10)	94 (30) 6 (2)
10. 77/M	CMML	17.6	47,XY,+8	100 (24)
11. 85/M	CMML	3.8	47,XY,+8 46,XY	9 (4) 91 (42)
12. 40/F	CMML	19.0	45,XX,-7	100 (32)
13. 63/M	M2 post RAEB-t	31.2	complex abnormalities, including +9	75 (24)
14. 27/M (27)	M2 post RAEB	32.6	complex abnormalities, including +i(12p)	100 (8)
15. 62/M	M2 post RAEB-t	37.6	complex abnormalities, including -7,	83 (19)
16. 56/M	M2 post RAEB	40.5	45,XY,t(2;3) (p22 or 23;q27 or 28), -7 46,XY, t(2;3)	94 (29) 6 (2)
17. 69/F	M2 post RA	35.2	47,XX, add (3) (q12), +8 46,XX	88 (35) 12 (5)
18. 64/M	M1	96.2	45,XY,-7 46,XY	49 (19) 51 (20)
19. 31/M (7)	M3	90.0**	47,XY,+8,der (9), t(9;11) (q34;q13 or q14), t(15;17) (q22;q21) 46,XY	73 (57) 27 (21)

20. 39/M (7)	M2	65.4	45,X,-Y,t(8;21)(q22;q22)	100 (20)
21. 62/M	M5a	86.0	91,XXYY,mar (2q+),4q-, -5,7q+,+mar (5?) 46,XY	 79 (11) 21 (14)
22. 27/M	M2	75.0	45,X,-Y,t(8;21)(q22;q22) 46,XY	94 (30) 6 (2)
23. 62/F	M2	65.4	48,XX,+4,+14,+mar 46,XX	50 (12) 50 (12)
24. 63/M	M2	62.0	48,XYY,+8 46,XY	52 (17) 48 (16)

RA, refractory anaemia; RARS, refractory anaemia with ringed sideroblasts; RAEB, refractory anaemia with excess of blasts; RAEB-t, refractory anaemia with excess of blasts in transformation; CMML, chronic myelomonocytic leukaemia; BM, bone marrow; PB, peripheral blood; EO, eosinophils. The time interval between diagnosis of MDS and that of AML in patients no. 13 to no. 17 was 2 and 27 months. *Patients also reported in other study.
** Leukaemic promyelocytes instead of blasts.

Cytogenetic abnormalities, *e.g.* numerical chromosome changes, are found both in MDS and AML (1,3,4) and can be used as karyotypic markers to establish blood cell lineage involvement. In MDS, myeloid cells (neutrophils, monocytes) and erythroblasts are frequently involved in the malignant clone (5-8). Less is known of the lineage involvement pattern and maturation in LT-MDS and in *de novo* AML (8-10).

We have performed fluorescence in situ hybridisation (FISH) with chromosome-specific probes and directly combined FISH with cytology in order to relate the cytogenetically abnormal clone with cell lineage in MDS, LT-MDS and *de novo* AML.

Materials and methods

Patients

MDS and AML were diagnosed according to the criteria of the French-American-British (FAB) classification (11-13). Cases of AML following a documented history of MDS of at least 2 months duration were defined as LT-MDS. The diagnosis of (*de novo*) AML was made when there were no clinical suspicion of MDS nor features of trilineage myelodysplasia (TMDS) in the bone marrow smears (14-16).

Patients with numerical chromosome abnormalities as shown by cytogenetic examination of bone marrow cells in metaphase were included in this study. The haematological and cytogenetical characteristics are summarised in Table 1. For comparison, BM smears from cytogenetically normal subjects were prepared.

Combined cytomorphology and fluorescence in situ hybridisation

Cytological examination of MGG stained cells was followed by FISH as described previously (7). All smears were made without the use of anticoagulants and routinely stained with MGG. Extra MGG slides were kept for later FISH study; they were wrapped in foil and stored at -20°C until the time of hybridisation. Briefly, cryopreserved BM smears were thawed slowly and the morphology of the MGG stained cells recorded on a S-VHS videotape using a standard Universal Light Microscope (Zeiss, Oberkochen, Germany) coupled to a camera, a S-VHS video recorder and a colour monitor. The same slides were then used for FISH using a biotinylated probe specific for the α-satellite sequences of chromosome 4 (pYAM11.39) (17), chromosome 7 (p7t1) (18), chromosome 8 (D8Z1) (19) and chromosome 12 (pα12H8) (20). For chromosome 1, 9 and Y we used probes specific for the heterochromatic regions: PUC1.77 (21), pHuR98 (22) and pY3.4 (23) respectively.

Following denaturation and hybridisation procedures, the hybridised probe was detected using fluorescence isothiocyanate (FITC) labelled avidin (Vector laboratories, Burlingame, CA, USA). The previously recorded MGG-stained cells were analysed in parallel on the video screen and in the fluorescence microscope (standard 14 IV FL, Zeiss) equipped with a filter combination 09 (BP 450-490, FT 510, LP 520; Zeiss). The parallel analysis permitted a direct comparison of the cytological and cytogenetical characteristics of the cells. In the MGG-stained smears, only well-preserved cells were evaluated. Bare nuclei or partly damaged cells were excluded. The FISH spots had to be clear and distinct.

Results

To assess normal values of FISH for chromosomes 1, 4, 7, 8, 9, 12 and Y, MGG-stained BM smears were prepared from at least four cytogenetically normal subjects and in each case 500 cells were scored. The mean percentages (± standard deviations) are given in Table 2.

The detailed results obtained in the 12 patients with MDS, five patients with LT-MDS and eight patients with AML are summarised in Table 3.

With regard to the quantitative involvement of the distinct cell lineages, certain points are notable. Blasts and, to some extent, mature neutrophils including promyelocytes, myelocytes, metamyelocytes, stab cells and segmented neutrophils, were cytogenetically abnormal in most MDS and LT-MDS patients (Figure 1).

Erythroblasts were karyotypically abnormal in 9/12 MDS and 3/5 LT-MDS patients (no. 13, 14 and 17). In one patient (no. 8) blasts were the only cytogenetically aberrant cells.

Table 2. FISH spots on MGG- stained bone marrow cells from cytogenetically normal subjects

Probe (chromosome number)	No. of samples *	FISH spots per cell (mean percentage ± SD)				
		0	1	2	3	4
PUC1.77 (1)	8	-	2.5 ± 1.7	96.8 ± 1.7	0.3 ± 0.2	-
pYAM11.39 (4)	4	-	1.9 ± 0.1	97.2 ± 0.2	0.9 ± 0.3	-
p7t1 (7)	8	-	2.4 ± 1.7	97.3 ± 1.7	-	-
D8Z2 (8)	9	-	3.2 ± 1.1	96.2 ± 1.6	0.4 ± 0.5	-
pHuR98 (9)	5	-	4.7 ± 2.1	95.0 ± 2.3	0.3 ± 0.3	-
pα12H8 (12)	8	0.2 ± 0.2	3.1 ± 2.4	96.4 ± 2.7	0.3 ± 0.3	-
pY3.4 (Y)	9	1.5 ± 1.2	98.2 ± 1.1	-	-	-

*Five hundred cells were scored in each experiment. SD, standard deviation.

Therefore the results in MDS and LT-MDS are indicative of the multilineage involvement of the cytogenetically abnormal clone. However, it is of interest that the percentages of erythroid involvement were significantly less than the percentages of abnormal granulocytic cells (*e.g.* patients no. 2, 3, 7, 9, 13, 15 and 16). This was most striking in patient no. 3 in whom none of the erythroid cells showed the trisomy 8. In two CMML-MDS patients (no. 10 and 11) the percentages of cytogenetically abnormal monocytes were significantly greater than those of neutrophils, indicating that the cytogenetically abnormal clone had preferentially matured along the monocytic lineage. In patient no. 9 with RAEB-t, an increased percentage of eosinophils (6.6%) was found in the bone marrow differential count. Almost all eosinophils showed a loss of a chromosome 7 indicating that the cytogenetically abnormal clone had mainly developed towards neutrophils as well as eosinophils. Although the results in MDS and LT-MDS showed multilineage involvement of the clonal abnormality, the results of AML were often consistent with selective blastic involvement. In 5/7 cases of AML both granulocytic and erythroid involvement were minimal or absent,

except in M2 cases no. 20 and 22 where a significant fraction of neutrophils cells carried the abnormal karyotypic marker. Finally, the lymphoid cells in none of the cases showed cytogenetic abnormalities.

Table 3. Percentage of bone marrow cells with abnormal number of FISH spots

Patient diagnosis	Cytogenetic marker	% (number of cells analysed)				
		Blast	NEU	MONO	ERY	LYM
1. RA	-7	100 (16)	66 (129)	NT	54 (206)	4 (54)
2. RARS	+8	NT[1]	73 (131)	NT	54 (50)	0 (58)
3. RAEB	+8	100 (11)	51 (41)	NT	0 (12)	0 (7)
4. RAEB	+8	65 (57)	33 (82)	NT	22 (92)	0 (42)
5. RAEB	-7	97 (38)	28 (149)	NT	32 (87)	0 (46)
6. RAEB	+8	85 (107)	45 (98)	NT	47 (128)	0 (24)
7. RAEB-t	+8	92 (76)	36 (46)	NT	17 (117)	0 (37)
8. RAEB-t	-Y[2]	88 (58)	6 (34)	NT	3 (33)	2 (43)
9. RAEB-t	-7	98 (124)	89 (85) EO: 95 (41)	NT	7 (57)	3 (58)
10. CMML	+8	91 (55)	37 (51)	78 (23)	66 (76)	0 (38)
11. CMML	+8	NT[1]	21 (91)	87 (31)	13 (30)	0 (28)
12. CMML	-7	100 (16)	74 (105)	NT[3]	24 (58)	2 (49)
13. M2 post RAEB-t	+9	59 (74)	50 (66)	0 (9)	12 (17)	0 (19)
14. M2 post RAEB	+12	97 (38)	38 (8)	NT	56 (43)	0 (8)

15. M2 post RAEB-t	-7	80 (129)	34 (53)	NT	3 (87)	6 (47)
16. M2 post RAEB	-7	97 (119)	38 (37)	NT	2 (42)	4 (47)
17. M2 post RA	+8	93 (114)	35 (79)	NT	44 (97)	0 (37)
18. M1	-7	100 (114)	8 (12)	NT	NT	2 (49)
19. M3	+8	94 (269)[4]	0 (13)	NT	0 (87)	0 (26)
20. M2	-Y	100 (717)	100 (415)	NT	5 (38)	3 (121)
21. M5a	+1	100 (113)	NT	NT	0 (91)	0 (29)
22. M2	-Y	99 (97)	40 (10)	NT	2 (42)	0 (13)
23. M2	+4	72 (65)	3 (121)	NT	0 (58)	0 (20)
24. M2	+8	90 (83)	0 (56)	NT	0 (81)	0 (56)

NEU, neutrophils, including promyelocytes, myelocytes, metamyelocytes, stab cells, and segmented neutrophils; ERY, erythroblasts; MONO, monocytes; LYM, lymphocytes; NT, not tested; EO, eosinophils.
[1] Blast cell count too low for adequate MGG/FISH analysis.
[2] Loss of chromosome Y in bone marrow cells, not in PHA stimulated lymphocytes.
[3] Bone marrow cells were of lesser quality and did not allow cytological differentiation between neutrophils and monocytes.
[4] In case no. 19 with AML M3 promyelocytes were analysed instead of blasts; no promyelocytes were analysed under NEU.

Discussion

We found a difference as to the lineage involvement between *de novo* AML on one hand and MDS and AML following progression from MDS (LT-MDS) on the other. Generally, in *de novo* AML, only minor populations of mature neutrophils or erythroblasts were identified as cytogenetically abnormal. The fact that blast cell counts are generally high in *de novo* AML (14) may complicate the investigations of the other cell lineages for dysplastic features and cytogenetic abnormalities and the interpretation of the data. Nevertheless, the results of the FISH analysis indicate that in primary AML leukaemic blasts are selective representatives of

the cytogenetically abnormal clone. This also indicates that these blasts do not exhibit abilities for maturation.

In this study a strict definition of *de novo* AML was used. Only patients without a prior history of MDS and without prior chemotherapy or radiotherapy were selected to represent *de novo* AML. Cases with morphological features of trilineage dysplasia (TMDS) were also excluded, because the presence of TMDS may indicate a clinically silent MDS phase and may predict the reappearance of myelodysplasia after chemotherapy (15).

In two of the selected *de novo* AML patients, metaphase cytogenetics revealed a t(8;21). Together with the t(8;21) there was a loss of a sex chromosome which we used as a cytogenetic marker in interphase analysis. The deletion of the Y chromosome was apparent in high percentages of blasts and mature neutrophils. Apparently t(8;21) AML as a specific entity frequently retains the ability of granulocytic maturation and the leukaemia contributes significantly to repopulate the maturing granulocytic compartment.

The involvement of different cell lineages in the leukaemic clone in AML has been studied before using simultaneous analysis of karyotype and phenotype. (8-10). In some AML cases the cytogenetic abnormality was restricted to the granulocytic lineage, but in other cases erythroblasts and megakaryocytic cells were also involved. However, it was not always clear whether these cases represented *de novo* AML (without trilineage dysplasia) or LT-MDS.

In MDS and LT-MDS we found blasts and variable proportions of mature granulocytic cells and erythroblasts to be cytogenetically abnormal, suggesting the transformation of a more primitive multipotent progenitor cell still able to express some capacities of maturation. Thus the cytogenetically abnormal clone in MDS and LT-MDS often involves more than one cell lineage, which is in contrast to the observations in primary AML. Further, it is to be noted that the percentages of karyotypically abnormal mature granulocytic cells were usually less than the corresponding values of blasts, suggesting that a proportion of maturing myeloid cells had descended from cytogenetically normal haematopoietic progenitors. One explanation is that not all cytogenetically abnormal blasts are capable of maturing, leading to a maturation advantage of the cytogenetically normal clone.

These results confirm earlier reports that in MDS multiple haematopoietic cell lines are involved (5, 6, 8, 24, 25). LT-MDS showed similar patterns as compared to MDS. The mean percentages of cell line involvement in cases of MDS and LT-MDS were not significantly different, i.e. 46.6% versus 39% for granulocytic cells, and 28.2% versus 23.4% for erythroid cells, respectively. Patient no. 17 developed LT-MDS after a 29- months period of stable refractory anaemia. Patient no 15 with RAEB-t progressed to LT-MDS in 3 months. In the

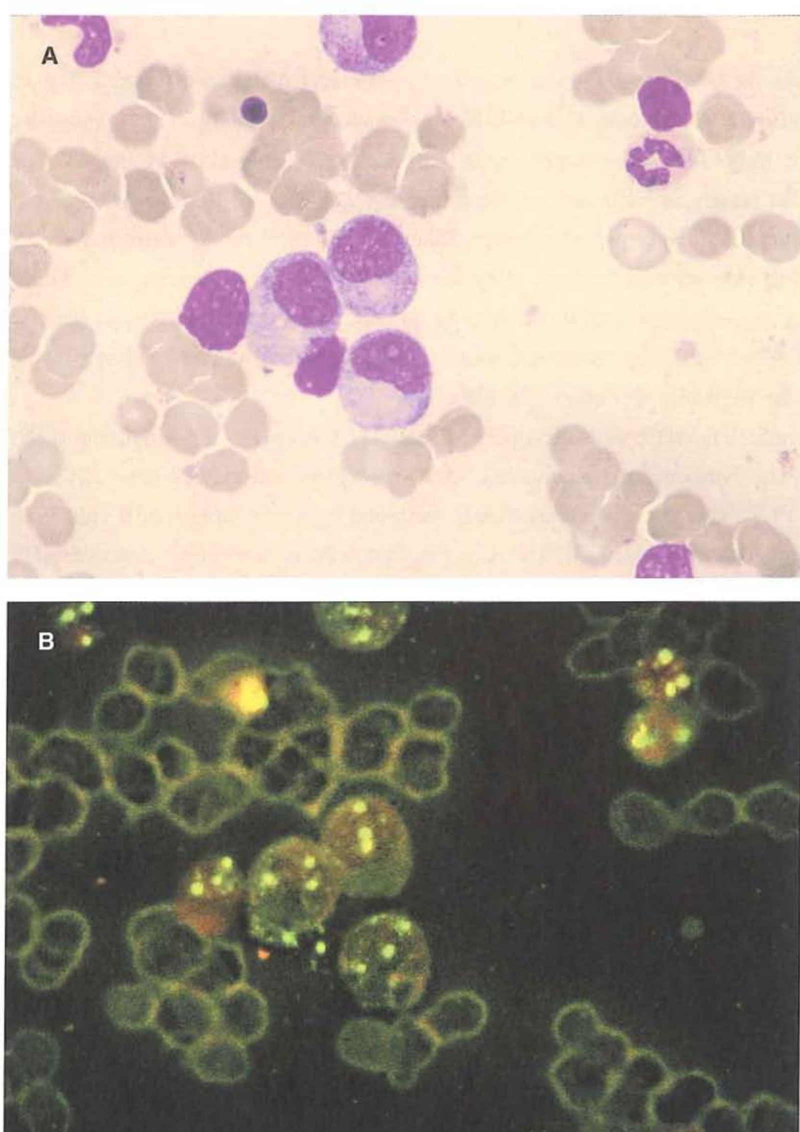

Figure 1.
(A) MGG-stained BM cells from patient 6 (RAEB).
(B) FISH on the same cells with a probe for chromosome 8. Three spots representing the trisomy 8 in three myelocytes.

latter two patients, blasts, neutrophils and erythroblasts were all karyotypically abnormal as well.

In several patients the percentages of cytogenetically abnormal metaphases were less than the values of abnormal blasts in the MGG/FISH. For instance in patient no. 18 a monosomy 7 was found in only 49% of metaphases but all blasts (n=114) showed the loss of chromosome 7. In patient no. 19, 94% of the promyelocytes showed the +8, whereas the trisomy 8 was found in 73% of the metaphases. Likewise in patient no. 24 a trisomy 8 was found in 52% of the metaphases but 90% of the blasts (n=83) revealed the trisomy 8. These differences are most likely explained by the fact that in bone marrow cultures for cytogenetic analysis, nonmalignant cells may be brought into mitosis, resulting in an underestimate of the percentages of karyotypically abnormal cells (26).

In AML, MDS and LT-MDS we were unable to detect cytogenetic abnormalities in the lymphoid cells. The lymphoid population has been investigated before, but generally with negative results (5-7). However, in a recent study we found high percentages of B cells with monosomy 7 in one MDS patient (25). This suggests that only in exceptional cases of MDS or in certain variants of MDS the haematopoietic cell is able to mature along the lymphoid pathway.

LT-MDS and *de novo* AML are sometimes difficult to recognise in newly presenting patients. The typical differences of cell lineage involvement and maturation in (LT-) MDS and *de novo* AML as evidenced by combined cytomorphological and FISH analysis, appear quite specific, and may be of diagnostic use for distinguishing the two conditions.

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CHAPTER 5

Clonality analysis of hematopoietic cell lineages in acute myeloid leukemia and translocation (8;21): only myeloid cells are part of the malignant clone

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Abstract

Bone marrow from six patients with acute myeloid leukemia (AML) and t(8;21) (q22;q22) or a variant t(8;13;21) was studied by simultaneous analysis of cell morphology and karyotype. Combination of May-Grünwald-Giemsa (MGG) and fluorescence in situ hybridization (FISH) using probes specific for the breakpoint regions of chromosome 8 and 21 allowed us to establish the extent of cell-lineage involvement of the translocation. The translocation was found in all myeloid blasts and in high percentages of the more mature neutrophilic cells. In one patient we could demonstrate the translocation in the eosinophils as well. Erythroblasts and lymphocytes did not show the t(8;21) abnormality. These results indicate that the t(8;21) in AML is restricted to the myeloid (granulocytic) lineage.

Introduction

The t(8;21) (q22;q22) is a balanced reciprocal translocation found in approximately 6% of all adult cases with acute myeloid leukemia (AML) (1). Most of these cases are classified as French-American-British (FAB) subtype M2 (2,3). The cytology of the bone marrow cells is very characteristic: blasts often contain Auer rods. The more mature granulocytic elements often show salmon to orange coloration of the cytoplasm and lack of granulation. Bone marrow eosinophilia can be found and the number of erythroblasts is low as is the number of megakaryocytes (2,4).

The breakpoint on chromosome 21 has been shown between exons 5 and 6 of the *AML1* gene and on chromosome 8 the *ETO* gene is involved (5,6). As a result of the translocation an identical *AML1/ETO* fusion transcript can be detected irrespective of the exact breakpoint location within the *AML1* intron (7). This chimeric gene is thought to generate a novel protein that probably contributes to the development of the leukemia (8).

Blasts and the more mature myeloid cells very likely belong to the malignant clone in AML with t(8;21). Few studies have addressed the question of the extent of lineage involvement (4,9). Metaphase cytogenetics was used which limits these studies to cells able to divide. It was suggested that the translocation was found in myeloblasts only and not in erythroblasts or T-lymphocytes. To investigate cell-lineage involvement of the leukemia the combination of cytology and interphase cytogenetics using centromeric specific DNA probes has proved to be a powerful technique. May-Grünwald-Giemsa combined with fluorescence in situ hybridization (MGG/FISH) has been used to detect numerical cytogenetic changes in different cell types (10). We have adapted the MGG/FISH technique using a plasmid and a cosmid probe specific for the breakpoint regions of chromosome 8 and 21, ie a P1.164 probe

containing the entire *ETO* coding sequence of chromosome 8 and a cosmid CO.664 probe containing the first five exons of *AML1* (11).

Material and methods

Patients

The cells from six patients with *de novo* AML-M2 and t(8;21) were examined. The diagnosis *de novo* AML was made while there was no clinical suspicion of a previous MDS phase nor features of tri-lineage dysplasia. The morphological diagnosis was made according to the French-American-British (FAB) criteria (3). Cytogenetic analysis was performed using standard banding techniques. Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed as described previously using an antisense primer based on the sequence of the *ETO* gene and a sense primer based on the sequence of the *AML1* gene (7). Hematological and cytogenetical characteristics of all patients are summarized in Table 1. We considered patient no. 5 as having AML-M2 although at the time of the bone marrow sampling the percentages of blasts were less then 30% (12,13). However after one week a second marrow aspirate showed that the percentage of blasts had increased to 35.8%. In one patient (no. 6) chromosome 13 was also involved in the translocation. The *AML1/ETO* fusion gene however was still located on the derivative 8 chromosome.
Bone marrow slides were made according to standard diagnostic procedures and stained with MGG. Slides were wrapped in foil and stored at -20°C until time of hybridization. In addition, bone marrow slides of control patients without cytogenetic abnormalities were treated in a similar way. Video recordings of the bone marrow cells were made as described previously (10).

Fluorescent in situ hybridization and probes

One hundred and seventy-five nanograms of a biotin-labeled plasmid P1.164 specific for the q22 region of chromosome 8 and 175 ng of a digoxigenin-labeled cosmid CO.664 specific for the q22 region of chromosome 21 were mixed with 10 ng of Cot-1 DNA (Gibco BRL, Gaithersburg, MD, USA) (11). This mixture was then denaturated at 70°C for 4 min and allowed to preanneal at 37°C for 15 min to suppress repetitive DNA sequences. The MGG-stained bone marrow slides were thawed slowly and denaturated in 70% formamide 2 x SSC for 2.5 min after which a mixture of the probes was applied. After overnight incubation in a moist chamber at 37°C, a post hybridization wash was performed. The biotin-labeled probe was detected by fluorescein isothiocyanate (FITC)-labeled avidine (Vector Laboratories,

Burlingame, CA, USA) and amplified with anti-avidine (Vector Laboratories) followed by avidine-FITC. The digoxigenin-labeled probe was detected by sheep-anti-digoxigenin-Rhodamine (Boehringer Mannheim) after which a second layer of Texas Red-labeled donkey anti-sheep antibody (Jackson Immunoresearch Laboratories) was applied. Cells were counterstained with DAPI (Serva) which was solved in the antifade solution Vectashield (Vector Laboratories). The slides were covered with glass coverslips.

Table 1. Patients with AML-M2: hematological and cytogenetical data

	Differential count						
Patient no. age/sex	PB % blasts	BM % blasts	BM % NEU	BM % EO	BM % ERY	Karyotype [no. of metaphases]	*AML1/ETO* RT-PCR
1. 35/F	85.0	88.8	2.6	0	2.0	45,X,-X, t(8;21)(q22;q22) [29]/ 46,XX [1]	+
2. 48/M	78.5	80.0	18.0	0.5	1.0	46,XY, t(8;21)(q22;q22) [31]/ 46,XY [2]	+
3. 17/M	63.5	52.6	8.0	30.2	0.4	46,XY,t(8;21) (q22;q22), del(9) (q13q33) [17]/ 45,X,-Y,t(8;21) [20]/ 46,X,-Y,t(8;21),+8 [6]	+
4. 39/M	88.0	65.4	18.6	8.0	6.4	45,X,-Y, t(8;21)(q22;q22) [20]	+
5. 29/F	5.5	26.4	59.9	1.2	1.8	45,X,-X, t(8;21)(q22;q22) [34]/ 46,XX [1]	+
6. 40/M	47.0	74.8	20.4	0.4	0.4	45,X,-Y,t(8;13;21) (q22;q21;q22) [20]	+

NEU, neutrophils including promyelocytes, myelocytes, metamyelocytes, stab cells, segmented neutrophils; EO, eosinophilic granulocytes; ERY, erythroblasts; F, female; M, male; PB, peripheral blood; BM, bone marrow; NT, not tested; RT-PCR, reverse transcriptase- polymerase chain reaction.

Evaluation of the FISH spots and photography

Using simultaneous analysis of the cytology of cells on the video screen and the FISH spots in the fluorescence microscope, we were able to relate the cytogenetic abnormality to specific cell types. Only cells with two red and two green fluorescent spots were evaluated. Cells were considered positive for the translocation when a yellow fusion spot was visible or

when the distance between a green and red spot was less than 1 spot wide. Images of the fluorescent spots were captured on the Probemaster Unit PSI (Chester).

Results

RT-PCR was performed to detect the presence of the fusion transcript: the 298 bp was generated by amplification in all six cases.

MGG-stained bone marrow cells of four patients without cytogenetic aberrations and without major cytological abnormalities revealed two green and two red spots in 97.2% ± 1.5 of the cells (mean ± standard deviation). The fluorescent spots were small but bright and clearly visible against a very low fluorescent background. Per control patient 500 cells were studied, thus including erythroblasts, myeloid cells and lymphoid cells. In 3.0 % of the cells (SD ± 1.1) a green spot was distinguished next to a red spot. In one control patient cytology was combined with FISH (Table 2). In low percentages of erythroblasts, neutrophils and lymphocytes, pairing of a green and red spot was observed. The number of blasts investigated was low (six), no fusion spots were seen in eosinophils.

Table 2. Percentages (number) of pairing of a green and red FISH spot in different cell types

Patient No.	Blasts	NEU	ERY	LYM	EO
1.	100 (116)	97 (38)	3 (53)	0 (49)	NT
2.	100 (206)	100 (76)	4 (45)	1 (74)	NT
3.	100 (22)	97 (31)	4 (23)	0 (21)	NT
4.	100 (98)	98 (98)	0 (31)	0 (25)	100 (28)
5.	100 (65)	100 (65)	0 (20)	0 (12)	NT
6.	100 (60)	97 (30)	0 (19)	0 (27)	NT
Control patient	0 (6)	2 (125)	4 (88)	3 (33)	0 (20)

NEU, neutrophils including promyelocytes, myelocytes, metamyelocytes, stab cells and segmented neutrophils; ERY, erythroblasts; LYM, lymphocytes; EO, eosinophilic granulocytes; NT, not tested.

Table 2 also contains the results of parallel analysis of cytology and cytogenetics of the patients' bone marrow. The percentages of cells positive for the t(8;21) are expressed per cell type. In all blasts and in high percentages of more mature and often dysplastic myeloid cells including promyelocytes, myelocytes, metamyelocytes, stab cells and segmented neutrophils

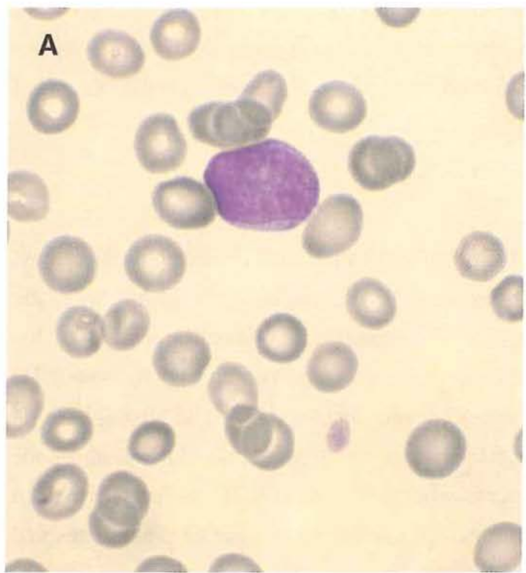
either a fusion spot or a close pairing of a green and a red spot were seen (Figure 1). In one patient (no. 4) the eosinophilic lineage was also studied. All analyzable eosinophils showed the t(8;21). Normal values of pairs of red and green spots were observed in erythroblasts and lymphocytes. Megakaryocytes were not studied.

Discussion

AML with t(8;21) represents a subtype of the acute leukemias with characteristic cytology, immunological phenotype and distinct prognosis. Few studies have dealt with the investigation of cell lineage involvement in AML with t(8;21). Knuutila et al used the morphology- antibody- chromosomes (MAC) technique in one patient and found t(8;21) only in CD13-positive metaphase cells (granulocytic lineage) (9). The glycophorin-A positive (erythroid) and CD3 (T-lymphocytes)-positive metaphase cells did not show the translocation. Berger et al studied 10 patients with AML and t(8;21) by comparing the number of blasts and erythroblasts to the number of normal and abnormal metaphases and suggested that only myeloblasts were cytogenetically abnormal (4).

The strength of the investigations reported here is the use of breakpoint specific-probes for the analysis of the primary cytogenetic event. The combination of MGG and FISH permits a direct comparison of cytology and karyotype. The often dysplastic granulocytic line appears to be an integral part of the abnormal clone. In one patient (no. 4) the percentages of eosinophils was slightly elevated. We found these cells to belong to the malignant clone as well. One of the characteristics of this type of AML is the ability of eosinophilic maturation when blasts are stimulated *in vitro* with IL-5 (14). The percentages of erythroblasts and lymphocytes with pairing of a green and red spot was within the normal range. Lack of sensitivity of the technique used (3% false positive ± 1.1, cut-off 5.2%) is inherent to the technique and best explained by the fact that the volume of the cell is projected and analyzed as a flat surface. Therefor, we cannot exclude the possibility that single cells in the erythroid and lymphoid lineage belong to the cytogenetically abnormal clone.

In addition to the experiments described in this paper, we also performed cytology combined with FISH using the loss of a sex chromosome as a marker (15). The loss of a sex chromosome is often associated with t(8;21), suggesting that this is secondary to the structural rearrangement (4). Only blasts and more mature myeloid cells we found to be cytogenetically abnormal.



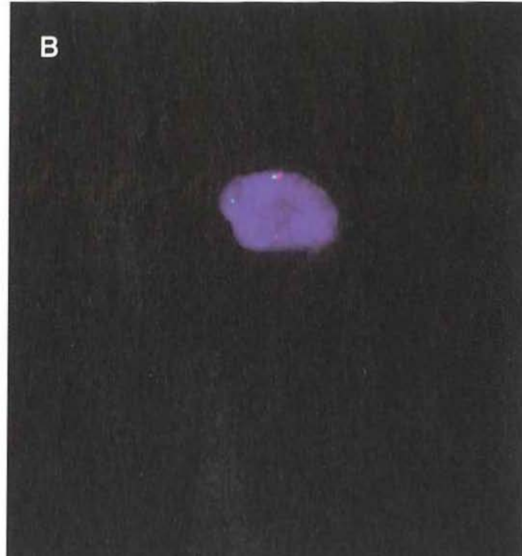


Figure 1.
(A) MGG stained blast (patient no. 5).
(B) FISH on the same cell with probes specific for the breakpoint regions of chromosome 8 (q22) (green spots) and chromosome 21 (q22) (red spots).

Many patients in long term remission still express the *AML1/ETO* transcript. This suggests the presence of a preleukemic population. Recent evidence suggests that this population represents progenitor cells with tri-lineage potential (16). The fact that we did not find involvement of the t(8;21) in erythroblasts and lymphocytes, would therefore suggest that during leukemia the cells with the cytogenetic abnormality are programmed to proliferate mainly into the granulocytic lineage.

In three of six patients the karyogram showed normal metaphases next to metaphases with t(8;21). However, in MGG/FISH almost all blasts express t(8;21). These differences are most likely explained by the fact that bone marrow cultures for cytogenetic analysis include nonmalignant cells that are stimulated *in vitro*.

In conclusion, erythroid and lymphoid cells are not part of the neoplastically transformed hematopoietic cell clone in AML and t(8;21). The entity AML t(8;21) represents a single cell lineage malignancy of the myeloid counterpart which has retained abilities to mature towards terminally differentiated cells.

Acknowledgments

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CHAPTER 6

Cytogenetic clonality analysis of megakaryocytes in myelodysplastic syndrome by dual-color fluorescence in situ hybridization and confocal laser scanning microscopy

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Abstract

In the myelodysplastic syndrome (MDS), cytogenetic abnormalities are often present and can be used as markers in studies for cell lineage involvement. Little is known of the involvement of the megakaryocytic lineage due to the variable ploidy of these cells. We applied dual-color fluorescence in situ hybridization (FISH) to routinely prepared bone marrow (BM) smears of cytogenetically normal patients and 7 patients with MDS and monosomy 7 or trisomy 8. Probes specific for the centromeric regions of chromosomes 7 and 8 were detected with fluorescein isothiocyanate (FITC) and Texas Red, respectively. This enabeled us to assess the ratio between the numbers of chromosomes 7 and 8 in the polyploid cells. We utilized confocal laser scanning microscopy to count the FITC and Texas Red FISH signals in the different focal layers of the megakaryocytes. Fifty-six megakaryocytes in six normal BM smears were analyzed giving a mean ratio of 1.0, a standard deviation (SD) of 0.12, and a range of 0.8-1.33. This ratio was applied to evaluation of clonal involvement of individual megakaryocytes in the patients with MDS. In two patients with monosomy 7, the majority of the megakaryocytes were monosomic. In the five patients with trisomy 8, all or a majority of the analyzed megakaryocytes were trisomic. These results add direct evidence that in MDS megakaryocytes are involved in the malignant clone.

Introduction

The myelodysplastic syndrome (MDS) comprises a group of clonal stem cell disorders characterized by anemia, leukopenia, and/or thrombopenia. Approximately 30% of the patients may eventually show progression to acute myeloid leukemia (AML), sometimes designated as leukemia-transformed MDS (LT-MDS) (1-3). Clonality assays have been used to establish the involvement of the different cell lineages in hematologic malignancies. Methods based on the random inactivation of one of the X chromosomes have been used infrequently to assess the clonality of the thrombopoietic lineage in MDS, AML, and myeloproliferative diseases (MPD) (4-7). X chromosome inactivation techniques however, are limited to female patients and can be difficult to interpret, *e.g.*, due to skewing of the X chromosome pattern, which can be tissue-specific and which can increase with age (8-10).

Cytogenetic abnormalities including numerical chromosome changes are often found in bone marrow cells in patients with MDS (3,11). These abnormalities can be identified with fluorescence in situ hybridization (FISH) and used as karyotypic markers to establish blood cell lineage involvement (12-16). With this technique, all or part of the blasts, granulocytic

cells, monocytes, or erythroblasts have been often found to be cytogenetically abnormal. Megakaryocytes have not been easily accessible to cytogenetic analysis because of their cell size and the difficulties of inducing mitotic cells in culture. In one study in which immunology was combined with metaphase and interphase cytogenetics, two patients with refractory anemia (RA) and trisomy 8 were examined (15). In several cells positive for the platelet/megakaryocyte surface marker CD61 (anti-GpIIIa), trisomy 8 could be identified. However, in one patient with RA and t(1;7), the cytogenetic abnormality was not found in the CD61-positive cells. The large number of chromosomes in the multinuclear megakaryocytes has hampered a broader use of the FISH technique to investigate numerical chromosome abnormalities.

In most cells, the numbers of nuclei are unknown and vary per cell. Therefore, they cannot be related to the number of FISH signals. Moreover, the FISH signals appear at different focal levels and do not permit reliable counting with a conventional fluorescence microscope. Here we report on the use of dual-color FISH with probes specific for the centromeric regions of chromosomes 7 and 8 to establish the ratios between the numbers of chromosomes. The megakaryocytes of cytogenetically normal patients and patients with MDS with monosomy 7 or trisomy 8 were studied. Ratios different from those in cytogenetically normal megakaryocytes were indicative of the loss or gain of a chromosome. We used confocal laser scanning microscopy to image consecutive optical planes of the cells, which allowed more accurate counting of the FISH signals.

Materials and methods

Bone marrow

MDS was diagnosed according to the criteria of the French-American-British (FAB) classification (1). Seven patients with MDS with monosomy 7 or trisomy 8, according to routine cytogenetic analysis, were included in this study. The hematologic and cytogenetic characteristics are summarized in Table 1. One patient (no. 7) was diagnosed as having LT-MDS (AML) after a documented history of MDS. BM smears were made during standard diagnostic procedures. In addition, BM smears from cytogenetically normal subjects were prepared. All smears were routinely stained with May-Grünwald Giemsa (MGG). They were wrapped in foil and stored at -20°C until the time of hybridization.

Fluorescence in situ hybridization

Briefly, cryopreserved BM smears were thawed slowly to room temperature (17). FISH was

applied using a biotinylated probe specific for the α-satellite sequences of chromosome 7 (p7t1) (18) and a digoxigenin- labeled probe specific for the α-satellite sequences of chromosome 8 (D8Z1) (19). After denaturation and hybridization procedures, the biotin-labeled probe was detected by fluorescein isothiocyanate (FITC)-labeled avidin (Vector Laboratories, Burlingame, CA, USA) and amplified with anti-avidin (Vector Laboratories) followed by avidin FITC. The digoxigenin-labeled probe was detected by sheep anti-digoxigenin-rhodamine (Boehringer, Mannheim, Germany), after which a second layer of Texas-Red-labeled donkey anti-sheep antibody (Jackson ImmunoResearch Laboratories, Westgrove, PA, USA) was applied. An antifade solution (Vectashield, Vector Laboratories) was then applied, and the slides were covered with glass coverslips.

Table 1.Patient diagnosis and cytogenetic data

Patient no Age(yrs)/sex	Diagnosis	Karyotype [no. of metaphases]
1.* 67/M	RA	45,XY,-7 [38] 46,XY[6]
2. 44/F	RARS	45,XX,-7 [12] 47,XX,+1,der(1;7)(q10;q10) [2] 46,XX [17]
3.* 67/M	RAEB	47,XY,+8 [22] 46,XY [18]
4. 76/F	RAEB	47,XX,+8 [32]
5. 81/M	RAEB-t	47,XY,+8 [32]
6.* 77/M	CMML	47,XY,+8 [24]
7.* 69/F	LT-MDS	47,XX,add(3)(q12),+8 [35] 46,XX [5]

RA, refractory anemia; RARS, refractory anemia with ringed sideroblasts; RAEB, refractory anemia with excess of blasts; RAEB-t, refractory anemia with excess of blasts in transformation; CMML, chronic myelomonocytic leukemia; LT-MDS, leukemia-transformed myelodysplastic syndrome.
*Patients also reported in another study (van Lom et al.,1996)

Confocal laser scanning microscopy

Three-dimensional image reconstructions of FISH-labeled megakaryocytes were obtained with the aid of a Zeiss LSM 410 confocal laser scanning microscope (Zeiss, Oberkochen,

Germany) equipped with a computer-controlled, motorized scan stage and objective. Two lasers were used for dual-color imaging, an argon laser for FITC excitation at 488 nm and a helium/neon laser for Texas Red excitation at 543 nm. A beam splitter (560 nm) separated the FITC and Texas Red emission signals. A 510-525 nm band-pass filter for the FITC emission signal and a long-pass (>570) nm filter for the Texas Red emission signal were placed in front of the detectors (photomultipliers). For each megakaryocyte, 25 optical planes were scanned, each consisting of 512 x 512 voxels (volume pixels). In each plane, the voxels were spaced at 0.325 μm. Consecutive planes were spaced at 1 μm. To reduce the time spent on image acquisition, the coordinates of the megakaryocytes were indicated and stored on disk. The consecutive planes of each megakaryocyte were recorded automatically. Additionally, a two-dimensional image reconstruction was made by projecting all optical planes in one maximum image.

FISH signal counting

Using the maximum image, the megakaryocyte was outlined on an overlay. The FISH signals present in the first optical plane of the three-dimensional image stack were marked on the overlay. The signals present in the second optical plane were then marked on the same overlay. All subsequent optical planes of a megakaryocyte were analyzed in this way. Finally, all chromosome 7 and chromosome 8 signals were counted, and the ratio was calculated.

To validate the FISH procedure, the numbers of FITC and Texas Red spots were counted in 100-500 nucleated cells in each of the six cytogenetically normal BM smears. In addition, FITC (chromosome 7) signals were counted in 100-500 nucleated cells in the BM smears from the patients with MDS with trisomy 8. Texas Red signals (chromosome 8) were scored in the cases of MDS with monosomy 7. For chromosome 7, the mean percentage of cells with no FITC signals was 0.5%; that with only one signal, 4.6%; with two signals, 94.9%; and with three signals, 0.2%. For chromosome 8 Texas Red signals, the mean percentages of negative cells, cells with one signal, cells with two signals, and cells with three signals were 0.1%, 2.6%, 96.2%, and 1.0%, respectively. These results confirm earlier observations (16).

Statistical methods

For each megakaryocyte analyzed, the ratio of the numbers of chromosomes 7 and 8 was calculated. The nonparametric Mann-Whitney test was applied to test for a difference in the distribution of the ratios for each individual patient, compared to the distribution of the ratios

of the 56 megakaryocytes of 6 cytogenetically normal individuals.

Results

In cytogenetically normal cells with two copies of chromosomes 7 and 8, the theoretical ratio between the numbers of chromosomes is 1. In the case of loss of a chromosome 7 (monosomy 7), the expected ratio would be 0.5, and in the case of gain of one chromosome 8 (trisomy 8), the expected ratio would be 1.5.

In the experiments, we examined the number of cumulative FISH signals per cell for chromosomes 7 or 8 among 56 cytogenetically normal megakaryocytes from six individuals. The signals per megakaryocyte varied from 4 to 38. The mean ratio between the numbers of FITC and Texas Red signals was 1.0 [standard deviation (SD), 0.12; range, 0.8-1.33].

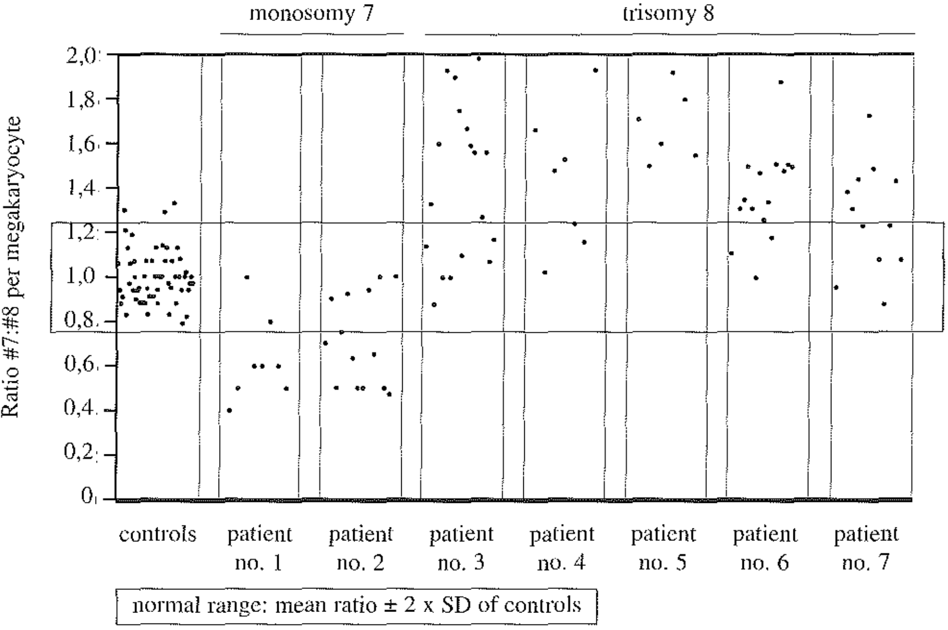

Figure 1.
Ratio of the numbers of chromosome 7 and chromosome 8 per megakaryocyte. A normal range (mean ± 2 x SD: 0.76-1.24) was established by analyzing 56 cytogenetically normal megakaryocytes. The ratios of the individual megakaryocytes (•) in two patients with MDS and monosomy 7 (patient 1 and 2) and five patients with MDS and trisomy 8 (patient 3-7) clearly indicate the involvement of a majority of these cells in the malignant clone.

A

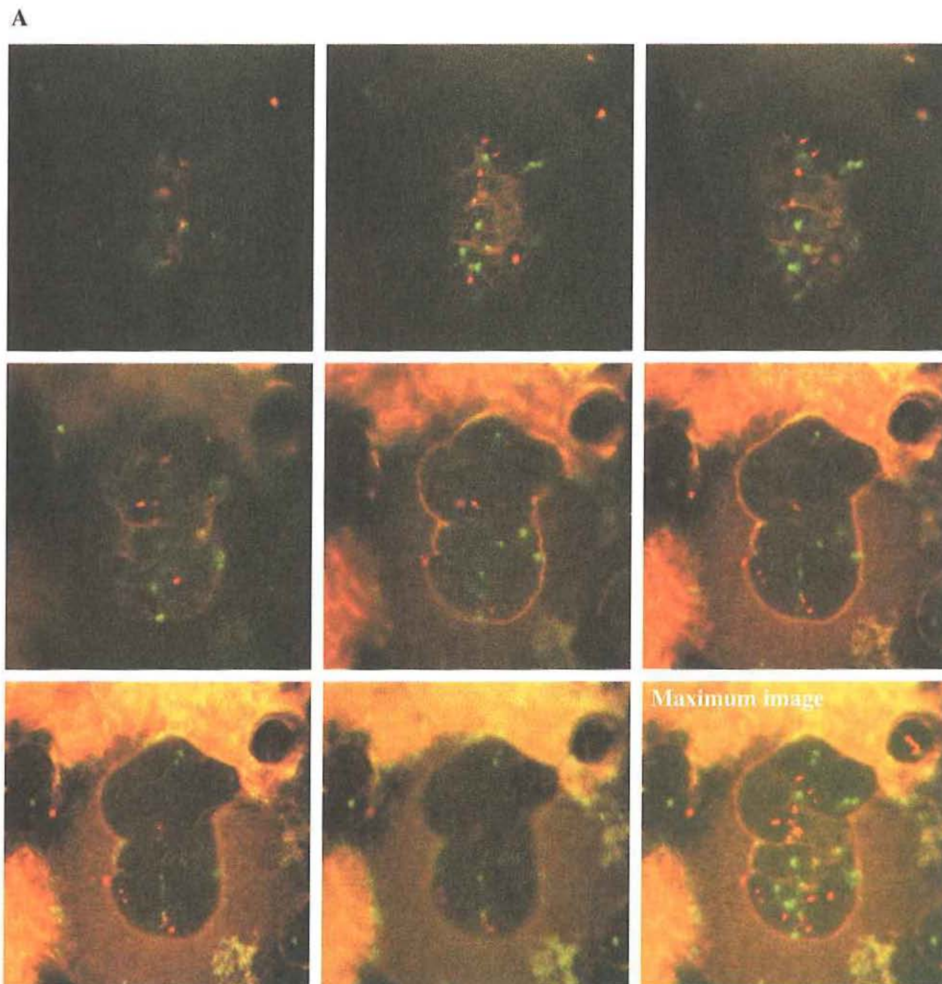

Figure 2. Images of consecutive optical planes of megakaryocytes. The FITC signals represent chromosome 7 (#7), and the Texas Red signals represent chromosome 8 (#8). Per megakaryocyte, 8 of the 25 optical images are shown. All 25 optical planes were projected in one maximum image (M).

A:.A megakaryocyte in a cytogenetically normal BM. Sixteen FITC (#7) and 16 Texas Red (#8) signals were counted providing a ratio of 1.0.

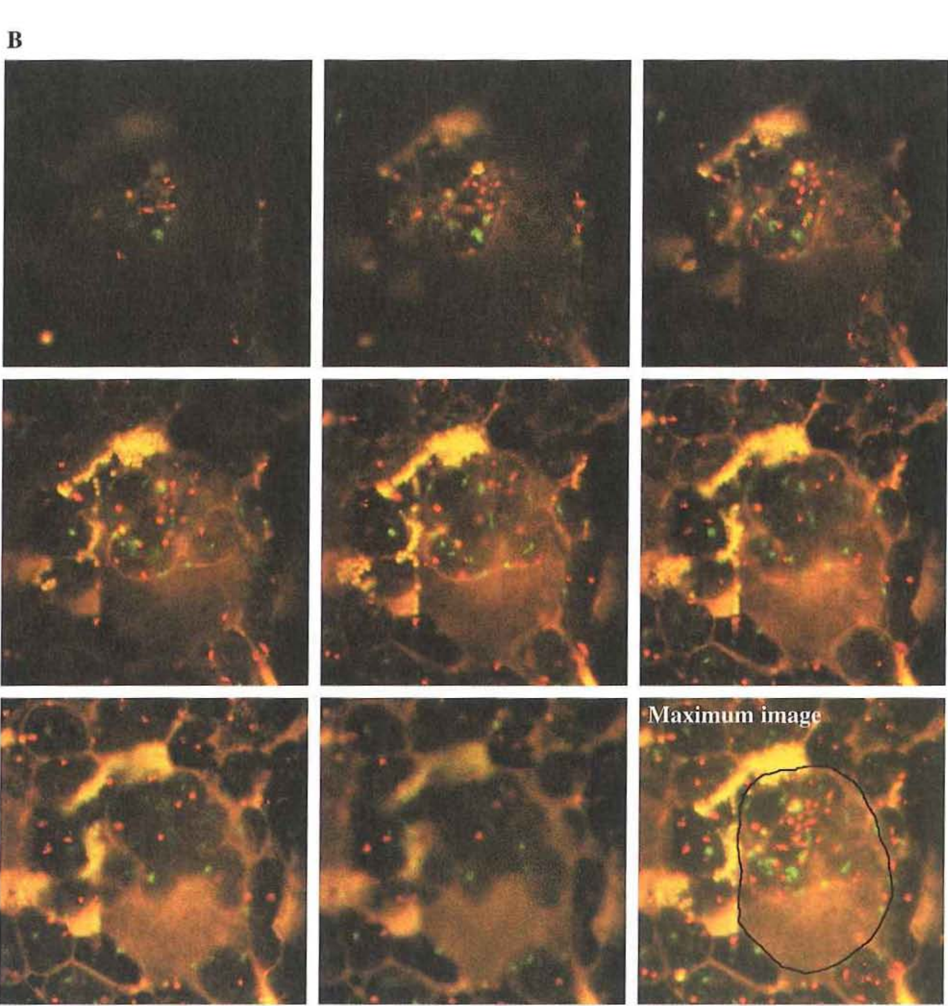

Figure 2.

B: In the BM of patient 3 with MDS and trisomy 8, a megakaryocyte showed 17 FITC signals (#7) and 27 Texas Red signals (#8). The ratio of 1.6 indicates involvement in the cytogenetically abnormal clone. The megakaryocyte in the maximum image is outlined.

We then set out to estimate the cumulative number of FISH signals for both chromosomes in the cases of MDS to assess their ratios. In patients no. 1 and 2 with monosomy 7, the cumulative numbers for chromosome 7 signals per megakaryocyte varied from 2 to 24 and for chromosome 8, from 4 to 30. In patients no. 3 to 7 with trisomy 8, we counted 4 to 93 FISH signals for chromosome 7 per megakaryocyte and 7 to 117 for chromosome 8. We then evaluated the chromosome 7/chromosome 8 index per megakaryocyte. For each patient and the controls, the ratio of chromosome 7 and 8 signals per individual megakaryocyte analyzed is plotted in Figure 1. In the patients, the distributions of the ratios of the individual megakaryocytes were significantly different from the distributions of the ratios of the 56 cytogenetically normal megakaryocytes (all P values < 0.001). In the two patients with MDS with monosomy 7, more than 50% of the megakaryocytes showed a ratio of less than 0.76. This indicates the loss of a chromosome 7 in the majority of the megakaryocytes. In one patient with MDS and trisomy 8, all megakaryocytes analyzed showed a ratio of > 1.24. In the other four patients with trisomy 8, the majority of the megakaryocytes showed a ratio above 1.24. Hence, these findings appear to be consistent with the presence of an extra chromosome 8 in the majority of megakaryocytes in each of these cases of MDS.

Discussion

For the study of cell lineage involvement in hematopoietic malignancies, various techniques have been used. Numerical or structural chromosome abnormalities, when present, can conveniently be used as markers in clonality studies. FISH using chromosome or breakpoint-specific probes has been combined with cytology or immunology to establish cell lineage involvement (20). In this way, megakaryocytes have been found to carry the Philadelphia chromosome in chronic myeloid leukemia (21). In cells positive for the platelet and megakaryocyte antigen CD61, cytogenetic abnormalities have been demonstrated in patients with MDS, MPD, and AML (15,22). However, these techniques have not been readily applicable to the analysis of the megakaryocyte cell population. Technical obstacles have prevented an adequate analysis of the multinucleated interphase megakaryocytes for numerical chromosome changes. In order to overcome the problems of counting the signals of the probes in the FISH technique at different depths of the nucleus, we used confocal laser scanning microscopy. With the latter technique, FISH signals were recorded in consecutive optical planes and subsequently stored on disk. This allowed an accurate cumulative estimate of FISH signals per megakaryocyte (Figure 2). We also tried to overcome the

difficulties intrinsic in the polyploidy of megakaryocytes and to relate the numbers of FISH signals to the variable numbers of nuclei. For this purpose, the numbers of signals of the abnormal chromosome were related to that of a reference chromosome. Therefore, we used dual-color FISH to calculate the ratios of the numbers of chromosomes 7 and 8. The expected ratio in diploid cells is 1; of course, in cells with monosomy 7 or trisomy 8, the respective ratios would theoretically be 0.5 and 1.5. Indeed, we found the estimates to approximate these theoretical values in validation experiments, although limited deviations from the expected ratios were found. Random counting errors, *e.g.*, due to poor visibility of some spots, may account for the latter deviations. The fact that the FISH- stained centromeric region was sometimes present in two or more sequential optical planes may also have contributed to errors. For these reasons the ploidy of the megakaryocytes was not calculated. Because the ratios of signals of chromosomes 7 and 8 found in cytogenetically normal and abnormal megakaryocytes show some overlap, it may not always be possible to verify clonality on a per-cell basis with certainty. This overlap that is apparent in a fraction of the megakaryocytes in patients no. 1, 2, 3, 4, 6 and 7 either may result from the quantitative variations that are intrinsic in the variability of nuclei per cell, or it might be indicative of the coexistence of cytogenetically normal megakaryocytes in the marrow of these patients. Nevertheless, because the majority of the ratios found in MDS megakaryocytes were different, it is possible to determine the involvement of megakaryocytes on a population basis, provided a significant proportion of these cells are indeed abnormal.

Thus, most megakaryocytes in the cases of MDS that we investigated here appear cytogenetically abnormal and represent the cytogenetically abnormal MDS clone. In most BM smears of the MDS patients, small mono- or bi-nuclear megakaryocytes were seen. These cells, which can be indicative of MDS, were not analyzed; they were difficult to locate in the FISH stained smear. Large megakaryocytes, as in patient no. 6, are less common in MDS. These cells showed ratios in and beyond the normal range (Figure 2c).

The method that we report here may be particularly useful in clinical conditions where one would wish to verify the presence of a clonally abnormal megakaryocyte population, if there is an appropiate cytogenetic abnormality for this type of analysis present.

C

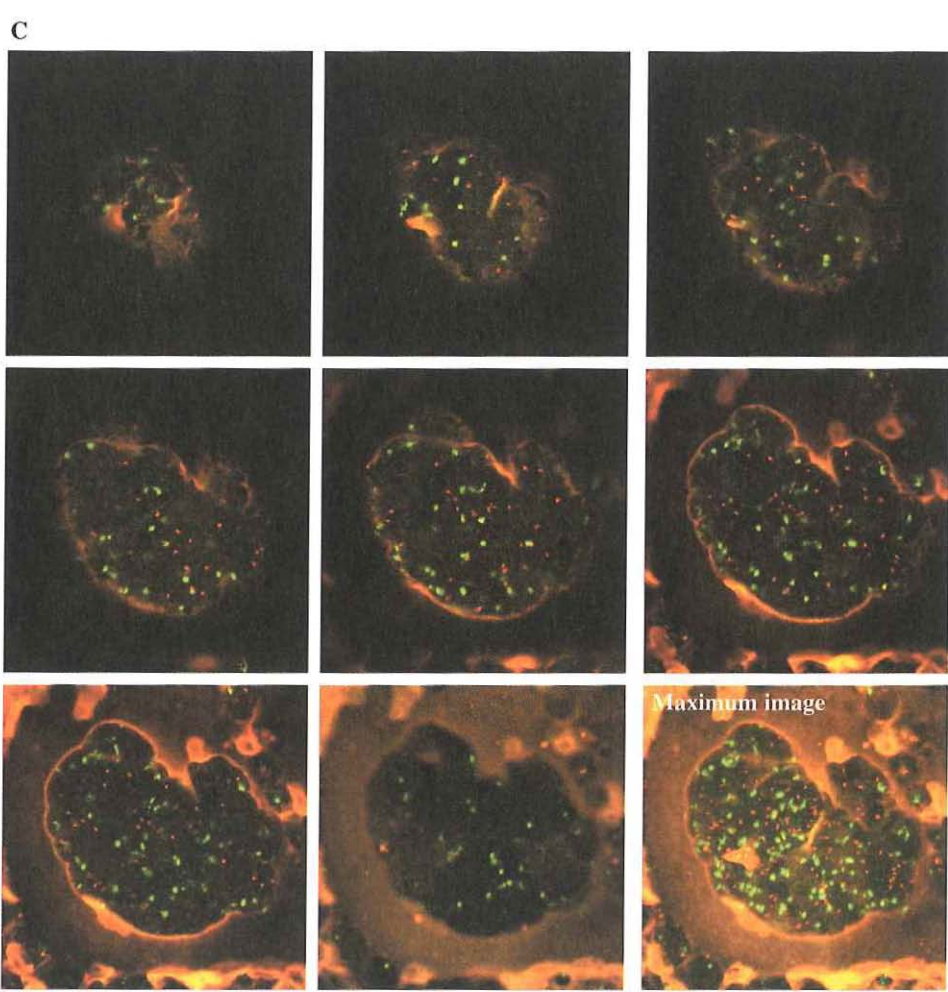

Figure 2.
C: In patient 6, 93 FITC signals (#7) and 117 Texas Red signals (#8) were counted in a megakaryocyte providing a ratio of 1.26.

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CHAPTER 7

General discussion and summary

7.1 Introduction

Myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) represent clonal proliferations of hematopoietic cells. Clonal involvement of the different hematopoietic cell lineages in MDS and AML has not been fully established. In the studies presented in this thesis, we have addressed the following questions in selected patients with MDS or AML. 1). What is the extent of clonal involvement in both diseases? 2). Are the clonal progenitor cells able to mature in blood cells in MDS as well as in *de novo* AML? 3). Do morphologically dysplastic cells always belong to the malignant clone?

7.2 Clonality assays

To address the above mentioned questions, the choice of clonality assay is of critical importance. In earlier clonality studies, assays based on the random X chromosome inactivation in female cells have been widely used. Active and inactive X chromosome alleles are distinguished by their methylation patterns and the X-linked polymorphisms at the paternal or the maternal X chromosome can then be analyzed at the DNA, RNA or protein level. Apart from the fact that these assays are restricted to female patients, a second draw back is that they cannot be applied to individual cells. The technique is also limited because studies on healthy women have revealed unbalanced or skewed distributions in the inactivation rate of paternal and maternal X chromosomes. Consequently, the results of these assays should be interpreted with caution. Clonality can also be determined using cytogenetic and molecular markers but these may represent 'second hits', whereas X-linked polymorphism can provide independent insights into clonality in broader cell populations. RT-PCR on genetic abnormalities offers the potential advantage of the analysis of rare or even single cells, sorted immature progenitors or colony cells.

In this thesis, the fluorescence in situ hybridization (FISH) technique has been used to study the clonal origin of the different cell lineages and individual cells in MDS and AML. Chromosomal abnormalities, as found by conventional cytogenetic analysis, were used as genetic markers. The FISH technique was applied to interphase cells and combined with cytology or immunocytochemistry techniques. Cell type and cell lineage were thus related directly to the presence of the chromosomal aberration without the intervention of cell culturing or cell separation. Routinely made blood and bone marrow smears were used for the studies. Probes specific for centromeric regions were applied to detect numerical chromosome abnormalities, and breakpoint specific probes were used to

detect chromosome translocations. In chapter 2, the applicability of a combined cytology and FISH technique has been evaluated, and in chapter 3 a combined immunocytochemistry and FISH technique is introduced.

7.3 Cytogenetic clonality analysis in MDS and LT-MDS

Using the combined cytology and FISH technique and, in certain cases, the combined immunocytochemistry and FISH technique, the cells from several patients with MDS and leukemia transformed MDS (LT-MDS) have been examined (chapters 3 and 4). In these patients, we demonstrated the presence of karyotypic abnormalities in a high percentages of blasts. The chromosome abnormalities were also present in granulocytic cells (including promyelocytes, myelocytes, metamyelocytes, stab cells and segmented neutrophils), monocytes and in erythroblasts. Apparently the cytogenetically abnormal clone is able to mature in MDS, but this results in ineffective hematopoiesis. Anemia, leucopenia or thrombopenia can occur as well as morphologic dysplasia. Also, deficiency of intracellular enzymes like pyruvate kinase or peroxidase can be found.
Mature blood cells without the karyotypic aberration were also seen. These cells may have descended from cytogenetically normal progenitors, coexisting with the cytogenetically abnormal progenitors. However, as an alternative explanation one cannot exclude the possibility that the cells negative for the chromosomal marker may still be part of the malignant clone, since the karyotypic abnormalities may represent a later step in the development of the disease. Clonality studies of bone marrow stromal cells have yet to be performed.

7.4 Cytogenetic clonality analysis in *de novo* AML

In many cases of secondary AML, an MDS phase precedes AML and the dysplastic features of the more mature cells often remain visible. Patients presenting with *de novo* AML, showing similar cytological dysplastic changes, may have gone through a clinically silent phase of MDS. In our study, a more restrictive definition of *de novo* AML was used, *i.e.*, it included only those patients without prior chemotherapy or radiotherapy, without tri-lineage dysplasia in marrow and blood smears and without clinical evidence for a preceding phase of MDS. The results of the clonality assays in the individuals presented in chapter 4, differ greatly from those in the patients with LT-MDS: in *de novo* AML the cytogenetic aberration was restricted to the blasts and could not be found in the neutrophils or erythroblasts. Thus, cytogenetic in situ analysis produced

results that allowed a distinction between *de novo* AML and LT-MDS as clinical-biological entities.

In early reports, a distinction between *de novo* AML, LT-MDS or therapy related AML was generally not made. This confounds previous analysis of the prognostic differences between *de novo* AML and secondary AML, and complicates the direct comparisons between our studies and those of others. In a study by Fialkow et al, clonality was shown to involve blasts, erythroid cells and platelets in six elderly patients (age > 60 years) (1). Two of them had previously received radiotherapy and/or chemotherapy before the diagnosis of AML was made, and one was known to have LT-MDS. In the same study, 16 adults and children of age < 30 years, were found to have polyclonal erythroid cells, platelets or both. Since the leukemia of the younger patients are most likely to be of *de novo* origin, these data seem to be consistent with our findings.

Until now, no clinical distinction is being made between LT-MDS and *de novo* AML. However, the clonality patterns as described above, clearly emphasize differences in pathogenesis. These results may support the importance of developing different therapies for both diseases.

In chapters 4 and 5 clonality studies on seven patients with *de novo* AML and t(8;21) (q22;q22) are described. In some metaphases, loss of a sex chromosome accompanied the translocation. The corresponding *AML1/ETO* gene fusion was confirmed by RT-PCR. Dysplastic neutrophils and dysplastic eosinophils are characteristic of this type of AML, and may suggest involvement of the neutrophils and eosinophils in the malignant process. Indeed we found that high percentages of the neutrophilic lineage were cytogenetically abnormal. In contrast, erythroblasts did not show the fusion gene or the loss of a sex chromosome. These data are consistent with the results of a study by Berger et al., who compared the numbers of blasts and erythroblasts to the numbers of normal metaphases and metaphases with t(8;21), and suggested that only myeloblasts were cytogenetically abnormal (2). In one patient, we found eosinophils to be positive for the (8;21) translocation. Similarly in AML M4eo and inv(16) (p13q22) eosinophils have been shown to harbor the chromosomal aberration (3). Although the monocytic component is more abundant in AML M4eo, bone marrow and blood cytology reveal dysplastic morphological abnormalities similar to those seen in AML M2 and t(8;21). In AML with t(8;21) and inv(16), genes encoding subunits of the same transcription factor complex, core binding factors (CBF), are involved: the *CBFα* or *AML1* gene at 21q22 in case of translocation t(8;21)(q22;q22) and the *CBFβ* gene in case of inv(16)(p13q22). The CBF

complex activates transcription of several myeloid genes, *e.g.* the *GM-CSF* and *M-CSF*-receptor. The similarities of morphological features and corresponding clonality patterns likely reflect the involvement of common pathogenic pathways in these cytogenetic subtypes of AML.

Thus, *de novo* leukemia and t(8;21) is able to mature towards the granulocytic lineage. Perhaps this could provide support for new strategies in AML therapy. Maturation of the malignant clone may be stimulated relatively early with one or a combination of growth factors. Induction of maturation has proved to be an effective therapy strategy in patients with promyelocytic leukemia and t(15;17) where the administration of all-trans-retinoic acid has been added to chemotherapy (4). Recently, further differentiation of blasts in AML and t(8;21) has been achieved after in vivo administration of G-CSF: the numbers of blasts expressing differentiating antigens like CD11b, CD13 or CD15 increased (C. Chomienne and L. Degos, Hôpital St. Louis, Paris; personal communication).

7.5 Cytogenetic clonality analysis of lymphocytes

The question of a clonal derivation of lymphocytes in myeloid malignancies has been addressed in several studies, generally using X chromosome inactivation assays or chromosome analysis. In most studies, Epstein-Barr virus transformed B-cells or phytohemagglutinin stimulated T-cells were analyzed. Only in rare instances, clonal involvement of B and/or T lymphocytes have been demonstrated in MDS and AML. In this thesis, lymphocytes were not found to carry the karyotypic abnormalities of the leukemia (chapter 2-5). Apparently, the malignant clone does not frequently extend to the lymphoid lineages in MDS and AML. The "malignant hit" may have taken place after the lymphoid and non-lymphoid lineage diverge in the hematopoietic scheme. Alternatively, the chromosome aberration may have been present early in the lymphoid differentiation lineage, but these progenitor cells may have had a survival disadvantage (Figure 1).

However, in a child with MDS and monosomy 7 (chapter 3), the loss of chromosome 7 was apparent in the B-lymphocytes, whilst the T-cells were disomic for chromosome 7. In this patient, the MDS type RAEB-t had been preceded by MDS type J-CMML. This type of MDS bears some similarities of a myeloproliferative disease while white blood cell counts may be elevated and tissue infiltration of monocytes may be present. Clonality studies, mostly using X chromosome inactivation assays, on a myeloproliferative disease, the chronic myeloid leukemia (CML), have shown clonal B-cells and in some cases clonal T-cells on infrequent occasions. Recently, a cytogenetic clonality study has shown

that the Philadelphia chromosome, t(9;22)(q34;q11), associated with CML, was present in unmanipulated or unsorted B-cells and T-cells (5). This supports the hypothesis that in CML the "malignant hit" takes place early in hematopoiesis. Our findings of B-lymphocyte involvement in a young patient with (J-)CMML underline the resemblance of this type of MDS with a myeloproliferative disease. The absence of monosomy 7 in T-cells is probably due to the fact that T-cells are long living cells; the analyzed T-cells might have been produced before the loss of one of the chromosomes 7 had occurred.

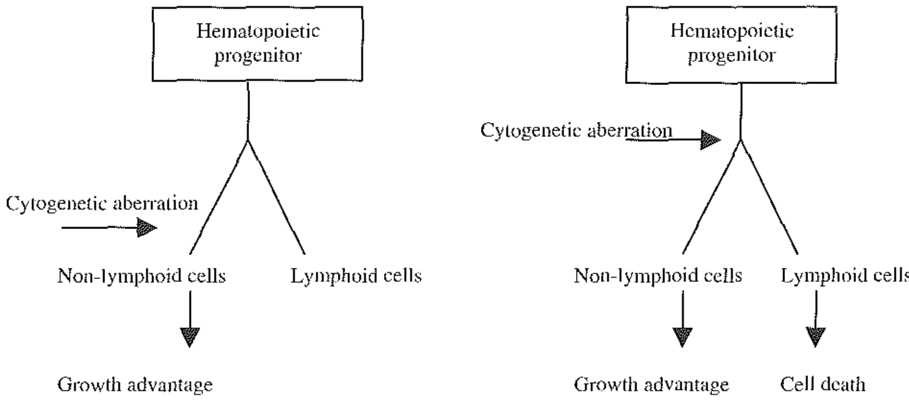

Figure 1.
Two models explaining the lack of cytogenetically abnormal lymphoid cells in MDS and AML.

7.6 Clonality of megakaryocytes

Using X chromosome inactivation techniques, platelets have been found to be clonal in MDS, which indicates that megakaryocytes are also clonally derived. Megakaryocytes have been rarely studied directly for clonal involvement due to two major problems. Firstly, the numbers of nuclei per cell are unknown and it is therefore difficult to determine the numbers of FISH spots per nucleus. Secondly, when a conventional fluorescence microscope is used, the FISH spots are present at different focal levels and therefore difficult to count. To resolve the first problem, two probes were applied, one probe for the chromosome of interest, and a second probe for a reference chromosome. In cytogenetically normal megakaryocytes the ratio between the numbers of the two

chromosomes is expected to be 1 :1. In case of monosomy the theoretical ratio will be 1 : 0.5 and in case of trisomy 1.5 :1. By applying confocal laser scanning microscopy it was possible to count the FISH spots at different focal levels. This approach is described in chapter 6. We were able to show that in the patients with MDS, a mixture of clonal and non-clonal megakaryocytes was present, indicating that cytogenetically normal progenitors persist in measurable numbers.

7.7 Conclusions and prospectives

It has been demonstrated in this thesis that in MDS and LT-MDS, blasts, neutrophils, eosinophils, monocytes, erythroblasts and megakaryocytes can be clonally derived. Lymphocytes are clonally derived only in rare cases and, if so, these conditions may represent specific subtypes of MDS (Figure 2).

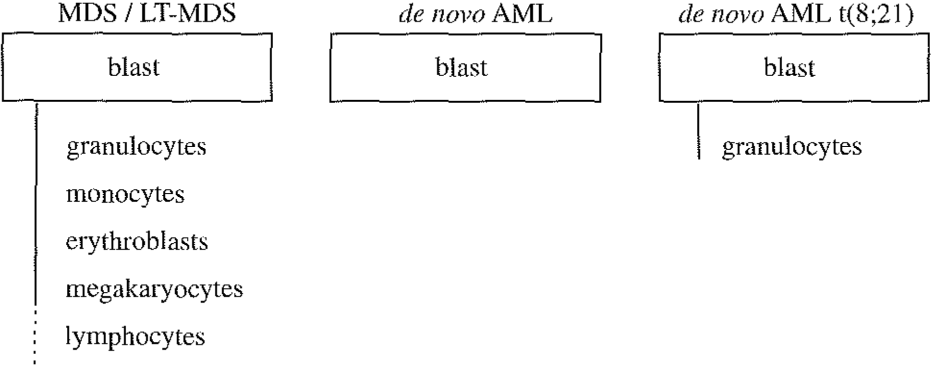

Figure 2.
Maturation abilities of the malignant clone in MDS and LT-MDS, *de novo* AML and *de novo* AML with t(8;21).

In *de novo* AML, no or minor maturation of the malignant clone is observed. The granulocytic lineage, erythroblasts and lymphocytes are generally cytogenetically normal. Megakaryocyte involvement has yet to be studied.

In *de novo* AML with t(8;21) blasts, neutrophils and eosinophils are clonally derived. In contrast, erythroblasts and lymphocytes do not show the chromosome abnormality.

Several topics still have to be studied. For instance, little is still known of the lineage involvement of the malignant clone in therapy related AML (t-AML). Some balanced chromosome translocations like t(8;21), inv(16) and t(15;17) are usually found in *de novo* AML but can be seen occasionally in t-AML and are then associated with the use of

epipodophyllotoxins. Comparison of the clonality results of t-AML and *de novo* AML is especially of interest when the balanced translocations are accompanied by loss or deletion of chromosome 5 and/or 7. The latter chromosome aberrations are associated with therapy related MDS and the use of alkylating agents. Furthermore, the behaviour of the MDS clone in time has not yet been established. For instance, is the percentage of cytogenetically abnormal erythroblasts stable during a certain period? What happens when MDS progresses to LT-MDS? Further technical developments, even automation of the FISH assays will contribute to the study of clonality in hematological malignancies.

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Samenvatting

Het myelodysplastisch syndroom (MDS) en acute myeloïde leukemie (AML) zijn kwaadaardige ziekten van het bloedvormend (hematopoïetisch) systeem. Bij beide ziekten is de normale bloedcelvorming in het beenmerg grotendeels verdrongen door een maligne kloon van cellen. Met een "kloon" wordt een groep cellen bedoeld die allen afstammen van één en dezelfde, in dit geval kwaadaardig veranderde voorlopercel. Bij AML is er in het beenmerg een toename van onrijpe cellen (blasten) te zien; bij MDS kunnen de rijpere cellen diverse uiterlijke afwijkingen vertonen. Ook de chromosomen van beenmerg- of bloedcellen van patiënten met MDS of AML kunnen afwijkingen vertonen. Deze afwijkingen kunnen betrekking hebben op een verandering van het aantal chromosomen (numerieke afwijkingen) of op structurele afwijkingen (deleties, translocaties). In de inleiding van dit proefschrift wordt een beknopt overzicht gegeven van de normale bloedcelvorming en enkele aspecten van de pathogenese van MDS en AML. Bovendien worden technieken besproken waarmee chromosoomafwijkingen kunnen worden aangetoond.

Het thema van het onderzoek in dit proefschrift betreft de vraag of, en zo ja, in hoeverre, bij patiënten met MDS of AML, de maligne kloon uitrijpt langs de verschillende paden tot rijpe bloedcellen. Bovendien wordt geprobeerd een verband te leggen tussen de morfologische afwijkingen van de beenmerg- en bloedcellen enerzijds, en de chromosoomafwijkingen anderzijds.

In de hoofdstukken 2 en 3 worden kleuringmethodes (cytologie/immunocytochemie) behandeld. Experimenten worden beschreven om cellen te karakteriseren en om tegelijkertijd, met behulp van interphase cytogenetica, de chromosomale afwijkingen te herkennen. Met behulp van fluorescentie in situ hybridisatie (FISH) en chromosoom specifieke probes is zo onderzocht of per individuele, geïdentificeerde cel de chromosoomafwijking aanwezig was.

In hoofdstuk 3 en 4 worden klonaliteitsanalyses beschreven bij patiënten met MDS en bij patiënten bij wie de MDS is overgegaan in een AML (leukemisch getransformeerde MDS, LT-MDS). Per bloedceltype is nagegaan welke cellen tot de kloon van de kwaadaardige beenmergcellen behoren. Uit de resultaten blijkt dat bij alle patiënten de blasten cytogenetisch afwijkend zijn en dus deel uit maken van de maligne kloon. Daarnaast blijkt dat de maligne kloon in verschillende richtingen uitrijpt tot erythroblasten en bloedcellen zoals granulocyten en monocyten. Overigens worden ook

cytogenetisch normale cellen gezien. Er lijken dus ook bloedvormende cellen aanwezig zonder chromosoomafwijkingen.

Zoals hierboven beschreven, is bij sommige patiënten bij wie de diagnose AML wordt gesteld, de ziekte ontstaan vanuit een voorafgaande myelodysplasie. Zo'n leukemie wordt leukemisch getransformeerde MDS (LT-MDS) genoemd ter onderscheiding van de zogenaamde *de novo* AML. In hoofdstuk 4 worden de resultaten van de klonaliteitsanalyses bij LT-MDS en *de novo* AML vergeleken, waarbij een strikte definitie is gehanteerd voor patiënten met *de novo* AML: zij zijn tevoren nimmer behandeld met chemotherapie of radiotherapie, er ontbreken dysplastische kenmerken van de beenmergcellen (die passen bij MDS) en er zijn geen klinische aanwijzingen voor een voorafgaande MDS fase. De resultaten van de uitgevoerde klonaliteitsanalyses bij *de novo* AML en LT-MDS tonen opmerkelijke verschillen. Bij AML zijn uitsluitend de blasten cytogenetisch afwijkend. Blijkbaar is de rijpingsblokkade bij *de novo* AML zo absoluut, dat er geen rijpere bloed cellen worden aangetroffen die de chromosomale afwijkingen hebben.

De chromosoomtranslocatie t(8;21), waarbij gedeelten van chromosoom 8 en 21 zijn verwisseld, is de meest voorkomende cytogenetische afwijking bij AML. In de hoofdstukken 4 en 5 worden klonaliteitsanalyses beschreven bij patiënten met zowel *de novo* AML als deze chromosoomtranslocatie. Dit type leukemie is morfologisch herkenbaar aan de sterk afwijkende granulopoïese. Uit onze bevindingen blijkt dat bij alle geanalyseerde patiënten zowel de blasten als de uitrijpende granulocyten in meerderheid de chromosoomtranslocatie dragen.

Lymfocyten worden beschouwd als behorend tot een ontwikkelingslijn binnen de hematopoïese die vroeg aftakt en apart staat van de myeloïde cellen. Wij hebben in de lymfocyten de chromosoomafwijking niet gevonden. AML of MDS ontstaan waarschijnlijk in een stadium voorbij de aftakking van de lymfocytenlijn in de hematopoïese. Echter, in hoofdstuk 3 wordt een kind beschreven met MDS en verlies van een van de twee chromosomen 7 (monosomie 7). Een hoog percentage blasten, granulocyten, monocyten, erythroblasten maar ook B lymfocyten lieten de monosomie 7 zien. Blijkbaar kunnen bij uitzondering de lymfocyten betrokken zijn bij de maligne kloon. In dat geval zijn zij net als de granulocyten, monocyten en erythroïde cellen, afkomstig van een gemeenschappelijke kwaadaardige vroege voorlopercel.

In hoofdstuk 6 tenslotte, wordt het onderzoek naar chromosoomafwijkingen in megakaryocyten beschreven. Deze voorlopercel van de thrombocyten (bloedplaatjes) is

meestal meerkernig. Vanwege het grote aantal kernen en het grote aantal FISH spots zijn deze cellen moeilijk met een normale microscoop te analyseren. Uit de analyse van driedimensionale beelden verkregen met confocale laser scanning microscopie bleek dat bij patiënten met een MDS en monosomie 7 of trisomie 8 het merendeel van de megakaryocyten deel is van de maligne kloon.

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Curriculum Vitae

Kirsten van Lom is op 11 mei 1952 geboren te Rotterdam. In 1969 verliet zij de HBS van het Libanon Lyceum te Rotterdam. In het zelfde jaar werd begonnen met de opleiding tot klinisch chemisch analiste, (Stichting ter behartiging van de vorming tot Assisterend Laboratorium personeel, SAL) op de afdeling Centraal Klinisch Chemisch Laboratorium van het Academisch Ziekenhuis Rotterdam-Dijkzigt (Prof. dr. B. Leijnse). Het diploma werd in 1972 behaald. In 1974 werkte zij als analiste in het laboratorium van het Phalombe ziekenhuis, Phalombe, Malawi. In 1975 begon zij als analiste op de afdeling Hematologie, Academisch Ziekenhuis Rotterdam-Dijkzigt (Prof. dr. J. Abels, nu Prof. dr. B. Löwenberg) in het laboratorium hematologische cytologie en biochemie. Sinds 1977 is zij hoofdanaliste van dit laboratorium. In 1990 behaalde zij alsnog het VWO diploma (staatsexamen). In 1993 werd aangevangen met het in dit proefschrift beschreven wetenschappelijk onderzoek bij de afdeling Hematologie onder begeleiding van Prof. dr. B. Löwenberg en Prof. dr. A. Hagemeijer (afdeling Celbiologie en Genetica van de Erasmus Universiteit Rotterdam, nu Centrum voor Menselijke Erfelijkheid, Katholieke Universiteit Leuven, België).

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