Acute myeloid leukaemia in the elderly:

Clinical management and the application of molecular cytogenetic techniques

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Abstract

In Western Europe and North America, acute myeloid leukaemia (AML) is predominantly a disease of the elderly, with a median age at the time of presentation in excess of 60 years. However, many clinical trials in AML fail to recruit elderly adults due to a combination of strict entry criteria, or physician or patient bias. Thus, clinical outcome data from many trials may not be readily applicable to older patients with the disease. Furthermore, because the clinical outcome for many older patients with AML is frequently poor, elderly patients who receive intensive chemotherapy with curative intent are frequently selected for treatment on clinical criteria rather than on objective prognostic criteria that may define clinical outcome.

The karyotype at the time of presentation may be considered one of the most important prognostic factors in adult AML. Therefore, the aim of this thesis were firstly to analyse the clinical outcome data from a cohort of elderly patients managed at a single centre in order to document the cytogenetic features of AML in an elderly population, to define the prognostic importance of presentation karyotype in the elderly, and to identify other prognostic factors. Retrospective analysis clearly demonstrated improved clinical outcome for older patients with AML over time, primarily as a consequence of improved supportive care and the delivery of more intensive chemotherapy. In addition, 'unfavourable' presentation karyotype, increasing age and raised serum LDH were found to correlate with poor clinical outcome

Molecular cytogenetic techniques based upon fluorescence *in-situ* hybridisation technology offer the chance to detect and analyse cytogenetic aberrations at a higher resolution than can be achieved with conventional techniques. The cytogenetic data provided by comparative genomic hybridisation and mulitplex fluorescence *in-situ* hybridisation when used in the analysis of elderly patients with AML were found to correlate well with results obtained by conventional methods. Importantly, additive cytogenetic data were more likely to be provided if multiplex-fluorescence *in-situ* hybridisation was used in the analysis of cases with marker chromosomes or in cases with complex karyotype, although the technique was limited by an inability to reliably detect telomeric translocations. In addition, although both techniques can be used to complement conventional

G-banding analysis, conventional FISH methods are often required to confirm the results.

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I would like to say a special thank you to the many patients who provided biopsy material and blood samples, and allowed permission for them to be used for research. It is my sincere hope that their fundamental contribution will result in an improved understanding of the disease and clinical benefit for those patients with acute myeloid leukaemia.

To the members of the Clincial Cytogenetics Laboratory: Debra Lillington, Michael Neat, Nicola Foot, Margaret Burridge, Loretta Byrne, Carla Consoli and Emily Carpenter, I thank you all, for providing much needed help, and humour, particularly whilst navigating the perilous waters of comparative genomic hybridisation! I would also like to say thank you to all other members of the Laboratory staff, who gave of their time, provided advice, and allowed me to win the World cup during my studies.

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Finally, to Henri Charrière, whose autobiography 'Papillion' provided me with much needed self-belief and determination, both of which were required in order for me to complete this body of work.

Cytogenetic symbols and abbreviated terms

amp	Amplified signal
arrow (→)	From - to, in detailed system
cgh	Comparative genomic hybridisation
colon, single (:)	Break, in detailed syste
comma (,)	Separates chromosome numbers, sex chromosomes, and chromosome abnormalities
dim	Diminished signal intensity
del	Deletion
der	Derivative chromosome
dic	Dicentric chromosome
dim	diminished signal
dup	Duplication
enh	Enhanced signal intensity
fish	Fluorescence *in situ* hybridisation
hsr	Homogeneously staining region
i	Isochromosome
idem	Denotes the stemline karyotype in subclones
idic	Isodicentric chromosome
ins	Insertion
inv	Inversion
mar	Marker chromosome
mfish	multiplex *in situ* hybridisation
minus sign (-)	Loss
p	Short arm chromosome
parentheses ( )	Surround structurally altered chromosomes and breakpoint
plus sign (+)	Gain
rev ish	Reverse *in situ* hybridisation including comparative genomic hybridisation
q	Long arm of chromosome
question mark (?)	Questionable identification of a chromosome or chromosome structure
r	ring chromosome
semicolon (;)	Separates altered chromosomes and breakpoints in structural rearrangements involving more than one chromosome
t	Transloation
tel	Telomere
wcp	Whole chromosome paint

Statement of work undertaken

The work presented was carried out during my time as a Clinical research Fellow at the ICRF Department of Medical Oncology at St Bartholomew's Hospital between October 1997 and November 2000. The work was supervised by Professor T.A.Lister.

The laboratory work was carried out in the ICRF Medical Oncology Laboratory, St Bartholomew's Hospital, Charterhouse Square. I identified patient material for the CGH and M-FISH studies, and retrieved samples with the assistance of Karin Summers. DNA extraction, CGH slide preparation, nick translation, hybridisation, and preliminary karyotyping was performed by myself. Michael Neat and Debra Lillington performed confirmatory karyotyping and CGH analysis. Metaphase preparations for the M-FISH assay were identified and retrieved by myself. Debra Lillington and myself prepared the slides used for the M-FISH assay. I performed the M-FISH analysis for all patients and Debra Lillington verified the results. I performed conventional FISH assays under the guidance of Emily Carpenter, and performed FISH analysis myself.

The clinical data was obtained from the medical oncology data base managed by Maxine Carter, and checked by Maxine and myself. I retrieved, and checked patient records. The statistical analysis and methods was performed and written by Mike Bradburn from the ICRF Medical Statistics Group in Oxford.

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Chapter 1

Historical overview

Acute myeloid leukaemia (AML) is the name given to a group of neoplastic disorders characterised by the uncontrolled rapid proliferation and accumulation of malignant myeloid blasts in the bone marrow and other haemopoietic tissues. If the patient is untreated and unsupported the disease has a median survival of less than three months (Southam *et al.*, 1951). Death frequently results from infection, haemorrhage or both, as a direct consequence of bone marrow failure.

The first documented cases of leukaemia were described independently in 1845. The published reports were of a series of cases in which patients died having presented with fever, lethargy, bleeding, abdominal swelling together with changes in the colour and consistency of the blood (Bennett, 1845; Craigie, 1845; Virchow, 1846). Virchow noted at post mortem a reversal in the normal ratio of red to white cells in the blood. He subsequently used the term "Weisses Blut", and later introduced the term leukaemia in his description of similar cases. Virchow later reported that splenic and lymphatic types of leukaemia existed, and that they could be distinguished from each other on the basis of morphological similarities between the leukaemic cells and the normal cells of the splenic and lymphatic tissues. Whilst Bennett and Craigie assumed that the changes in the blood were as a result of a suppurative process, Virchow believed that a disease specific to the splenic and lymphatic tissues was responsible for an increase in the number of white cells in the blood. Subsequent history has shown that Virchow was fundamentally correct in this interpretation.

Although the first documented case of acute leukaemia was reported in 1857 (Friedrick, 1857), the term acute leukaemia was introduced in 1889 when it was used in the description of a rapidly fatal case of leukaemia (Ebstein, 1889). Advances in the histological examination of blood occurred in the late nineteenth century with the introduction of polychromatic stains, and an appreciation of the common origin of red and white cell precursors following the description of myeloblasts and myelocytes. These findings ultimately led to the distinction between myeloid and lymphoid leukaemia.

Once established as a clinical entity a treatment for leukaemia was soon discovered. In the late nineteenth century the arsenicals in the form of Fowler's tonic were found to induce transient but unpredictable 'remission' in patients with chronic myeloid leukaemia (CML). By the beginning of the twentieth century radiotherapy was shown to be beneficial in the clinical management of chronic leukaemia, with only marginal benefit in acute leukaemia. Definitive cures for acute leukaemia remained anecdotal. In 1930 a case in which a patient with AML achieved a durable remission following therapy with arsenic, mesothorium, and finally two whole blood transfusions from a sibling was reported (Gloor, 1930). The leukaemic blasts cleared from the blood soon after transfusion and the author attributed part of the success to blood transfusion, and although dismissed at the time, it seems likely that this was the first documented case of a graft versus leukaemia effect. The introduction of blood transfusion for the correction of anaemia in the leukaemic patient was noted to induce remission by others (Dreyfus 1948), and led to the rationale for exsanguination transfusions in the early 1950's. Unfortunately the response to transfusion were at best temporary and often unpredictable.

The foundations for the chemotherapeutic treatment of leukaemia were established in the 1940s with the discovery and exploitation of the alkylating agents, and the introduction of antimetabolite therapy. Post mortem examinations of soldiers who died from mustard gas exposure during World War I and II revealed that nitrogen mustards produced bone marrow hypoplasia and peripheral cytopenia in those exposed (Zubrod, 1979). Therapeutic alkylators were developed as a result, one such drug, mechloromethamine was subsequently introduced and used in the treatment of lymphoma and leukaemia. Initial studies demonstrated encouraging results in patients with chronic leukaemia, with less impressive results in patients with acute leukaemia (Goodman *et al.*, 1946). Subsequent development led to the introduction of newer agents, namely busulfan, cyclophosphamide and chlorambucil, which are used in the treatment of CML and chronic lymphocytic leukaemia today.

In 1940, the utilisation of sulphonamides in the treatment of bacterial infection led to the realisation that the mode of action of these agents was a direct result of the inhibition of critical biochemical pathways required for bacterial growth. This new theory was applied to cancer chemotherapy in the form of folic acid antagonists or antimetabolites. In 1948, aminopterin and pteropterin were used in clinical trials, and were shown to induce transient remissions in childhood leukaemia (Farber *et*

al., 1948). Antagonists to purine and pyrimidine synthesis were subsequently developed and led to the introduction of 6-mercaptopurine, 6-thioguanine together with allopurinol, and in the 1960s, cytosine arabinoside (ara-C) (Ellison *et al.*, 1968).

The third major source of chemotherapeutic agents for leukaemia came with introduction of steroids, and anthracycline antibiotics. In 1942, adrenocorticoids were shown to induce lymphocyte death, and steroids were subsequently introduced in the therapy for both acute and chronic leukaemia. In the following decades other naturally derived agents were introduced most notably l-asparaginase, the vinca alkaloids and the anthracycline antibiotics (Broome, 1961; Johnson *et al.*, 1963).

Although Farber and others documented remission induction in childhood leukaemia with antimetabolite therapy (Faber, *et al.*, 1948), treatment of adults with AML remained disappointing. However, moderate improvements were noted with the introduction of single agent 6-thioguanine and 6-mercaptopurine, for example, in 1957 complete remission (CR) was achieved in 6 out of 18 adult patients with AML who received single agent 6-mercaptopurine (Bodley Scott, 1957). In the same year, the Medical Research Council set up a steering committee for the evaluation of cancer therapy. On the recommendation of the committee a working party was introduced in order to examine the possibilities of therapeutic trials in leukaemia. The first trial conducted by the working party in 1963 involved the comparison of steroid therapy in conjunction with 6-mercaptopurine (Medical Research Council, 1963).

The treatment of AML with single agent chemotherapy made little impact until the introduction of ara-C. Cytosine arabinoside was first used for the treatment of AML during the late 1960's (Freireich, 1987). It was recognised that the drug had a short half life of 15 minutes when injected intravenously and that optimal responses were achieved when ara-C was administered as a continuous infusion over a 5 to 10 day period. Initial studies in which ara-C was used in AML produced complete remissions ranging from 9 to 33% (reviewed in Rohatiner and Lister, 1996). However, when administered as a continuous infusion, single agent ara-C resulted in CR rates of between 25-55% in AML (Crowther *et al.*, 1973). Further improvement in remission CR rates was not achieved until the introduction of combination chemotherapy in the early 1970's. A regimen consisting of the anthracycline antibiotic daunorubicin (45-60mg/m^2 intravenously) on days 1-3 and ara-C (100mg/m^2 intravenously by continuous infusion) on days 1-7 known as '3+7'

became established as standard remission induction therapy for AML (Rai *et al.*, 1981; Preisler *et al.*, 1987). In comparison to single agent ara-C, the '3+7' regimens typically produce remission rates ranging from 50-75% depending on patient age (Appelbaum *et al.*, 1998). In attempt to improve remission rates further still, various permutations of the basic '3+7' regimen have been investigated. These have included modification of the dose and duration of the drugs, choice of anthracycline, and the addition of additional drugs like etoposide, and 6-thioguanine. Thus in 1986 the 8th MRC AML reported CR rates of 67% and median duration of first remission of 15 months with 'DAT' a combination of daunorubicin, ara-C and 6-thioguanine (Rees *et al.*, 1986). With current chemotherapy, an overall survival of between 10-30%, at 5 years, can be achieved in young adult patients, whilst overall survival falls to less than 10% at 5 years in patients aged 60 years or more, treated similarly (Bennett *et al.*, 1997).

In the early 1950's, laboratory experiments demonstrated that it was possible to 'rescue' animals that had received a lethal dose of radiation by the intravenous administration of bone marrow cells. Subsequently, patients with end stage haematological malignancies were treated with chemo/radiotherapy followed by bone marrow infusion. Although generally unsuccessful, it was possible to demonstrate transient engraftment in some patients. The discovery of the human leucocyte antigen system (HLA) and the development of HLA typing methodologies led to the modern era in bone marrow transplantation. In the early 1970's a number of centres used allogeneic bone marrow transplantation in patients with acute leukaemia in whom conventional chemotherapy had failed, and were able to document long term disease free survival in a significant number of patients (Thomas *et al.*, 1975). The use of autologous bone marrow 'transplants' in the management of haematological malignancy became commonplace in the 1980's. Currently, allogeneic, autologous, matched unrelated and non-myeloablative transplants are used as valid treatment modalities in the management of AML. However, the morbidity and mortality associated with these procedures coupled with the limited availability of suitable donors restricts the practical use of these techniques to patients less than 50 years of age.

The profound pancytopenia that accompanies the use of cytoreductive chemotherapy in AML treatment is responsible for the significant morbidity and mortality associated with remission induction and consolidation chemotherapy. Indeed treatment-related death due to infection is a major determinant of treatment

failure in AML remission induction, accounting for up to 70% of (Bodey *et al.*, 1982). In the elderly, the incidence of severe infection during induction therapy has been estimated at 20% (Stone *et al.*, 1995). Although patients with AML are at risk of bacterial, viral and fungal infection, in practice whilst neutropenic, bacterial infections are the most frequent and often the most serious cause of infection. The risk of bacterial infection increases significantly when the neutrophil count falls below $0.5x10^9$/l and is especially pronounced when below $0.1x10^9$/l. The duration of neutropenia also determines risk, and is particularly important in the elderly who often have prolonged periods of neutropenia following remission induction therapy due to poor bone marrow reserve, or pre-existing myelodysplasia. Thus the empiric use of broad spectrum antibiotics, which cover both gram-negative as well as gram-positive organisms is an established part of supportive care for patients with AML, and has led to a reduction in patient mortality (Hughes *et al.*, 1990). Likewise, since they became routinely available in the mid 1970's, the prophylactic use of platelet concentrates, together with antibacterial and antifungal mouth-care and indwelling venous catheter usage has significantly reduced the morbidity and mortality of AML patients receiving intensive chemotherapy.

Epidemiology and Aetiology of Acute Myeloid Leukaemia

The annual incidence of AML in the United Kingdom is 3/100,000 person years (Cartwright *et al.*, 1997). The incidence rises with increasing age, peaking at 10.5/100,000 person years for patients over the age of 75 years. Similar rates of incidence have been reported in other countries in Northern Europe and North America.

With improvements in the standard of living as well as better health care provision, it has been estimated that there will be a 38% increase in the number of individuals aged over 60 years by 2031. If the incidence of AML remains static during this period, then a 35-40% increase in cases of AML in patients aged over 60 years may occur (Johnson & Liu Yin, 1993). Clearly if these figures are correct, they signal a significant change in the in-patient practice for those involved in the management of AML.

Although the aetiology of AML remains unknown in the vast majority of cases, a number of diseases are known to predispose to the condition including the myeloproliferative disorders (Table 1-1). Specific genetic abnormalities have also been implicated in the pathogenesis of leukaemia. For example, leukaemia is 20 times commoner amongst individuals with Downs syndrome. Furthermore, congenital disorders that result in chromosomal fragility or impaired DNA repair are also associated with an increase risk of AML. These disorders include Fanconi anaemia, Blooms syndrome, and Ataxia telangiectasia.

Epidemiological studies of disease permit the identification of disease related risk factors. In AML three causal environmental factors have been identified, namely: i. Ionising radiation, ii. Chemotherapy, iii. Benzene exposure.

Compelling evidence linking radiation exposure to the development of leukaemia came from the long term follow-up of survivors of the atomic bomb explosions in Hiroshima and Nagasaki. Following the nuclear bombing of these cities the Atomic Bomb Casualty Commission was established by the United States in order to study the health trends of individuals exposed to

Table 1-1

Conditions predisposing to the development of acute myeloid leukaemia

Environmental factors
Radiation
Benzene
Alkylating agents and Topoisomerase II inhibitors
Chloramphenicol
Phenylbutazone
Acquired diseases
Clonal haemopoietic diseases
Chronic myeloid leukaemia
Primary myelofibrosis
Polycythaemia
Paroxysmal nocturnal haemoglobinuria
Aplastic anaemia
Inherited conditions
Downs syndrome
Fanconi anaemia
Blooms syndrome
Identical sibling with AML
Non-identical sibling with AML
Wiskott-Aldrich syndrome
Combined immunodeficiency/Congenital agranulocytosis

radiation. Studies carried out by the agency, and others, demonstrated a higher incidence of AML and CML in individuals exposed to radiation (Doll, 1995). Furthermore the development of leukaemia following exposure to radiation was found to be dose related, with the highest incidence in individuals less than 10 years, or older than 50 years of age at the time of exposure. Similar observations have been found in armed forces personnel who were exposed to nuclear fallout (Caldwell *et al.*, 1983). In the civilian setting landmark studies in the 1960's illustrated an increased incidence of AML amongst patients who received low dose radiation for ankylosing spondylitis and in women who received radiotherapy for cervical cancer (Moloney, 1987).

The incidence of myelodysplasia (MDS) and AML in individuals who have received chemotherapy for malignant and non-malignant disorders has been found to be higher than in the general population (Devereux, 1991; Hoyle *et al.*, 1989). Commonly used alkylating agents, which include cyclophosphamide, chlorambucil and busulphan, have been associated with an increased risk of AML. In Hodgkin's disease, the cumulative risk of AML in patients who received alkylating agents is 13% at 7 years, in contrast to less than 1% in patients who receive non-alkylating agents. Therapy-related AML (t-AML) induced by this group of drugs typically occurs some 2 to 8 years after exposure, and is often associated with karyotypic abnormalities such as complete or partial loss of chromosome 5 or 7, and or complex karyotype. The second group of chemotherapeutic agents commonly associated with t-AML are the topoisomerase II inhibitors. Acute myeloid leukaemia arising as a direct result of exposure to these agents is characterised by a short latency period of between 2 to 3 years, (typically without preceding MDS), and with characteristic cytogenetic aberrations involving chromosome sub-region 11q23. In both instances the risk of t-AML increases with the cumulative dose of the chemotherapeutic agent received, and with the combination of chemotherapy and radiotherapy.

Chronic exposure to benzene is associated with an increased incidence of AML (Infante *et al.*, 1977; Rinsky *et al.*, 1987). Other environmental agents, including electromagnetic fields, metal fumes, polychlorinated biphenyls, and cigarette smoking have been linked with leukaemogenesis. However the link between AML and these leukaemogens is less pronounced.

Classification of Acute Myeloid Leukaemia

Classification systems for AML rely upon the morphological, cytogenetic and immunophenotypic features of the leukaemic blast cells. In practice all three modalities are used in conjunction with each other, but historically the classification of AML has been based upon the morphology of leukaemic blast cells.

French American British (FAB) Classification

In 1976 the French American British (FAB) working party proposed a classification system for acute leukaemia based upon the morphological appearance of Romanosky stained blood and bone marrow smears (Bennett *et al.*, 1976). In order to establish the diagnosis of AML, the FAB classification requires that at least 30% of nucleated cells in the bone marrow must be leukaemic blasts. Since its inception a number of modifications to the FAB classification have been made incorporating additional histochemical stains as well as immunophenoyping, in an attempt to recognise all existing AML variants (Bennett *et al.*, 1985a; Bennett *et al.*, 1985b; Bennett *et al.*, 1991). Currently eight subtypes of AML are recognised by the FAB system (Table 1-2). The diagnostic recommendations proposed by the FAB group have been widely accepted and are now used by most groups involved in clinical trials and studies of AML, allowing direct comparison between groups in terms of disease.

World Health Organization (WHO) Classification

The World Health Organization (WHO) classification of haematological malignancy was introduced in 1999 with aim of producing a more clinically relevant classification system for leukaemia by utilising morphological, cytogenetic, immunophenotypic and aetiological criteria (Harris *et al.*, 1999) (Table1-3).

Table 1 Subtypes of acute myeloid leukaemia according to the French American British (FAB) classification

FAB subtype	Microscopy	Cytochemistry/Immunology
M0	Blasts ≥30% of bone marrow nucleated cells <3% of blasts positive for Sudan Black B or myeloperoxidase-posisitivity by ultrastructural cytochemistry	Myeloid immunological markers (e.g CD13,CD33,myeloperoxidase)
M1	Blasts ≥90% of bone marrow non erythroid cells (excluding lymphocytes, plasma cells, macrophages, basophils) ≥3% blasts positive for Sudan Black B or peroxidase maturing granulocytic cells≤10% nonerythroid cells	
M2	Blasts 30-89% bone marrow non erythroid cells Maturing granulocytic cells >10% of non erythroid cells Monocytic cells <20% of non erythroid cells	
M3/ M3 variant	Promyelocytes (hypergranular/ hypogranular or microgranular) >30% of bone marrow nucleated cells	
M4	Blast cells ≥30% of bone marrow nucleated cells Granulocytic cells ≥20% of non erythroid cells Monocytic component ≥20% of nonerythroid cells	Positive Sudan Black B, peroxidase chloroesterase (granulocytic lineage) plus α-napthyl esterase
M5A	Blast cells ≥30% of bone marrow nucleated cells Bone marrow monocytic component ≥ 80% of non erythroid cells Monoblasts <80% of bone marrow monocytic component	
M5B	Blast cells ≥30% of bone marrow nucleated cells Bone marrow monocytic component ≥80% of non erythroid component	
M6	Erythroblast ≥50% of bone marrow nucleated cells Blasts ≥30% of bone marrow non erythroid cells	
M7	Blasts≥30% of bone marrow nucleated cells	Blasts demonstrated to be megakaryoblasts immunologically (CD61+, CD41+) or by ultrastructural morphology

Table 1-3

WHO classification of acute myeloid leukaemia

AML with recurrent cytogenetic translocations
AML with t(8;21)(q22;q22), AML1(CBF-alpha)/ETO
Acute promyelocytic leukaemia (AML with t(15;17)(q22;q11-12) and variants, PML/RAR alpha
AML with abnormal bone marrow eosinophils inv(16)(p13q22) or t(16;16)(p13;q11), CBFβ/MYH11X)
AML with 11q23 (MLL) abnormalities
AML with multilineage dysplasia
With prior myelodysplastic syndrome
Without prior myelodysplastic syndrome
AML and myelodysplastic syndromes, therapy-related
Alkylating agent-related
Epipodophyllotoxin-related (some may be lymphoid)
Other types
AML not otherwise categorised
AML minimally differentiated
AML without maturation
AML with maturation
Acute myelomonocytic leukaemia
Acute monocytic leukaemia
Acute erythroid leukaemia
Acute megakaryocytic leukaemia
Acute basophilic leukaemia
Acute panmyelosis with fibrosis
Acute biphenotypic leukaemias

Unlike the FAB system, the percentage of bone marrow blast infiltration required for the diagnosis of AML was reduced to 20% in order to reflect the similarity in clinical outcome between patients with refractory anaemia with excess blasts in transformation, and patients with AML and more than 30% blast cell infiltration. The classification system also makes the distinction between subtypes of AML based upon distinct non-random primary cytogenetic aberrations which influence leukaemia biology, including translocations which involve core binding factors and retinoic acid receptors. Furthermore, therapy related AML and myelodysplastic syndromes are classified according to the chemotherapeutic agents that have been identified as aetiological factors.

Prognostic Factors in Acute Myeloid Leukaemia

A number of clinical and biological parameters have been identified as reliable predictors of patient response to curative therapy as well as relapse risk. These factors include patient performance status, and tumour burden, manifest by high white count or hepatosplenomegaly. Other more reliable co-variates are currently used to guide appropriate therapy in a 'risk adapted' approach, and include patient age, karyotype and morphological response to therapy.

Patient age

The chronological and biological age of an adult patient may differ considerably, particularly in the elderly. However, despite an association with other prognostic factors whose incidence increase with age, chronological age has been shown to be a major determinant of clinical response, and relapse risk (Sorensen *et al.*, 1993; Walters *et al.*, 1987). The highest CR rates and longest disease free survival are seen in children and adolescents between the ages of 1 and 15 years. By comparison, patients over the age of 60 years have poor CR rates and overall survival (Copplestone & Prentice, 1988; Hiddemann *et al.*, 1999).

Presentation karyotype

Cytogenetic aberrations can be documented in the majority of AML patients at the time of diagnosis, and have been shown to be independently predictive of treatment response and relapse risk (Bloomfield *et al.*, 1984; Grimwade *et al.*, 1998; Mrozek *et al.*, 1997). The cytogenetic aberrations associated with high CR rates and favourable outcome occur more frequently in younger patients and include t(15;17), inv(16), and t(8;21). Conversely, monosomy 5, and 7 as well as

complex karyotype (characterised by three or more distinct chromosomal abnormalities) are associated with reduced CR rates and are more frequent in the elderly at the time of presentation.

Initial morphological response to therapy

Failure to clear blast cells from the marrow after the first course of chemotherapy, particularly if blast cells number more than 20%, is associated with drug resistance and a diminished response to subsequent courses of chemotherapy (Estey, 2000a). Indeed, such failure to respond to standard chemotherapy can considered an indication to try investigational therapies (O'Donnell *et al.*, 1996). Drug resistance has been shown to correlate with the expression of a 170 KD membrane glycoprotein coded by the MDR-1 gene. The immunological and functional expression of this protein, and other functionally related proteins such as lung resistance protein, occur more frequently in elderly patients with AML, and correlate with disease resistance and reduced CR rate (Leith *et al.*, 1997; Lowenberg & Sonneveld, 1998). Modulators of drug resistance such as PSC833 are currently under evaluation, as a means of overcoming drug resistance and improving clinical outcome.

Acute Myeloid Leukaemia in the Elderly

Although AML is generally perceived to be a disease of the young, it is in reality a disease that predominantly occurs in the elderly, with a median age at presentation of 64 years (Brinker, 1982). Frequently the clinical trials investigating curative therapy in AML have low rates of recruitment for patients over the age of 60 years often as a result of misconceptions about the benefits of such trials for older patients on the part of physicians, patients and their families (Hutchins *et al.*, 1999; UKCCCR, 2000). In addition the stringent eligibility criteria imposed by many trials, coupled with logistic barriers, also contribute to the reduced recruitment of older patients. For these reasons the results from clinical trials may not always be strictly applicable to the elderly. Clearly, with an ageing population the number of elderly patients referred to tertiary centres for management of AML is set to rise as mentioned above. Therefore it is important not only to understand the nature of the disease in the older patient, but to also develop improved management strategies for these patients.

Characteristics of AML in the elderly

It is important to realise that although the definition of 'elderly' by an arbitrary defined age cut-off point (usually an age of 60 years or more at the time of diagnosis) is common practice particularly for the purposes of clinical trials, it is not always ideal. Variation in cardiovascular, renal and respiratory baseline function, and reserve capacity occur with increasing age, and are often most marked in the elderly, particularly between the ages of 70-80 years (Cohen, 1999). Consequently, biological age may not strictly reflect chronological age.

If the arbitrary definition of elderly is applied then the overall outcome of elderly patients with AML is much worse than similarly treated younger patients with de-novo AML. Conventional remission induction regimens produce CR in approximately 70-80% of young adults (Rees *et al.*, 1986). With consolidation chemotherapy and or bone marrow transplantation between 30-45% of those who achieve remission will be cured (Buchner, 1993). Thus, approximately 20% of young adults achieve long term disease-free survival with current AML therapy (Bishop, 1999). By comparison, CR rates of 40-55% in the elderly are commonplace with similar induction chemotherapy regimens, whilst only 5-10% achieve durable disease free remissions (Hiddemann *et al.*, 1999). Furthermore, the incidence of treatment-related deaths, as a result of induction chemotherapy, increases with age. For example, in reported series treatment-related deaths range from 12-44% in elderly patients (Lowenberg, 1996), compared to 10-15% in younger patients (Bishop, 1999). The relatively poor outcome of elderly AML patients may be explained by a combination of intrinsic disease resistance, as a consequence of distinct biological factors, and patient related factors that result in reduced tolerance to myelosuppressive chemotherapy.

Pathophysiology of AML in the elderly

A number of biological characteristics that are associated with poor response to therapy are found with increased frequency in the elderly and suggest that AML biology in this group of patients may be distinct from the disease biology in *de novo* AML associated with younger patients.

Patient karyotype at the time of presentation has been shown to be an important prognostic factor in AML (Bloomfield *et al.*, 1984; Mrozek *et al.*, 1997), predicting CR rate and overall survival. Compared to younger patients the elderly have a higher frequency of 'unfavourable' cytogenetic aberrations at the time of

diagnosis. These include complex karyotype, monosomy 5 and monosomy 7 (Leith *et al.*, 1997).

A significant number of elderly patients with AML have a history of an antecedent haematological disorder (AHD), defined as an abnormal blood count present for at least one month without requirement of a bone marrow biopsy. Furthermore the incidence of MDS is thought to increase with age, and may be as high as 20 to 30% in elderly AML patients compared to 15% in younger AML patients (Johnson & Liu Yin, 1993; Oscier, 1987). Both preleukaemic MDS and AHD have been found to correlate with reduced CR rates, disease-free survival and overall survival, particularly if present for at least 2 months prior to presentation with AML (Gajewski *et al.*, 1989).

Resistance to chemotherapy is associated with the expression of a number of membrane transporter proteins, which produce a multi-drug resistance phenotype. These proteins include P-glycoprotein encoded by the multi-drug resistance gene (MDR-1), multi-drug associated protein (MRP) and lung resistance protein (LRP). At the time of diagnosis, antigenic and functional P-gp expression can be demonstrated in 71% and 58% of leukaemic blast cells from elderly patients respectively. In contrast, younger patients have antigenic and functional P-gp expression of 35% and 41%, respectively (Leith *et al.*, 1999). The intrinsic drug-resistance associated with elderly AML results in a reduced CR rate (Leith *et al.*, 1997), and may result in an increased treatment-related death rate as a result of delayed normal haemopoiesis following induction chemotherapy.

The leukaemic blasts in elderly AML are thought to arise from a pluripotent CD34+ stem cell, more frequently than in younger patients. Thus as a result, 'clonal remissions', resulting in the persistence of the leukaemic cells, may occur more frequently in the elderly than in younger adults (Fialkow *et al.*, 1987).

Many of the biological characteristics of elderly AML are shared with t-AML, including the high incidence of poor outcome cytogenetics and the association with preleukaemic MDS/AHD and multi-drug resistant phenotype. Both types of AML are characterised by a poor response to conventional induction chemotherapy. For this reason AML in the elderly and t-AML should be considered as being biologically distinct from *de-novo* AML in younger patients.

Management of AML in the elderly (curative treatment strategies)

Early clinical studies of chemotherapy in elderly AML patients demonstrated that CR could be achieved using a combination of an anthracycline and cytarabine (Peterson & Bloomfield, 1977). Furthermore, a comparison between standard induction therapy and a 'watch and wait' approach where mild myelosuppressive chemotherapy is given only to control the symptoms of AML, demonstrated a significant advantage in terms of CR (58% v 0) and overall survival (21 weeks v 11 weeks) for patients who received intensive chemotherapy, whilst the number of days spent in hospital by patients in the two groups was identical (Lowenberg *et al.*, 1989). Thus it can be argued that elderly patients with AML should receive aggressive curative therapy if at all possible.

Presently, typical CR rates using '3+7' anthracycline/cytarabine regimens range from 40-55% in patients 60 years or older (Dombret *et al.*, 1995; Liu Yin *et al.*, 1991; Lowenberg *et al.*, 1998; Lowenberg *et al.*, 1997), compared to 70% or more in younger adults. In an attempt to improve efficacy and reduce treatment-related toxicity, daunorubicin has been substituted with other anthracyclines, or anthracendiones namely idarubicin or mitoxantrone. Higher CR rates with mitoxantrone in combination with cytarabine have been reported in single centre studies (Liu Yin *et al.*, 1991; MacCallum *et al.*, 1995). However, when the Eastern Cooperative Oncology Group (ECOG) prospectively compared three induction regimens in the elderly: daunorubicin 45mg/m^2 v mitoxantrone 12mg/m^2 v idarubicin 12mg/m^2 – all given intravenously for 3 days with identical doses of cytarabine 100mg/m^2 for 7 days, as well as identical consolidation chemotherapy. The three regimens were of identical efficacy, with a median CR rate of 42% and disease free survival of 14 months, furthermore treatment related mortality was lowest in patients who received mitoxantrone at (16%), although it was not statistically significant (Rowe *et al.*, 1998). Thus in older patients with AML, the optimal anthracycline and dose has yet to be determined.

Low dose curative chemotherapy has been investigated in the elderly. Although some authors have reported impressive results, in terms of CR rate with low dose cytarabine regimens (Manoharan, 1998), a large retrospective analysis of 751 patients revealed that CR rates using low dose cytarabine regimens were at best 32%, and that the use of such regimens was associated with prolonged myelosuppression (Cheson *et al.*, 1986). Therefore, current evidence would

suggest that although CR can be achieved with low dose cytarabine, standard dose curative therapy is more efficacious, and should be used in preference.

The precise benefit of post-remission therapy in the elderly has not been documented extensively. In younger patients with AML it is widely accepted that post-remission chemotherapy is required in order to produce durable remissions. Current consolidation therapies entail the administration of chemotherapy at similar doses to those used at induction. In recent years, dose escalation of cytarabine has been investigated. In a trial conducted by the Cancer and Leukaemia Group B a dose-response effect in terms of disease-free and overall survival was observed in patients less than 60 years, with cytarabine at $3g/m^2$ being superior to $400mg/m^2$ and $100mg/m^2$ (Mayer *et al.*, 1994). Unfortunately, in older patients (32% of the cohort) treatment was poorly tolerated, with significant neurotoxicity in those patients receiving $3g/m^2$ cytrabine. Importantly, no discernible difference between the treatment dose of cytarabine was seen in the elderly group studied. Despite the findings some authors suggest that post-remission therapy using lower doses of cytarabine ($1.5g/m^2$) are better tolerated in older patients, with overall survival at 2 years of 30% (Rowe, 2000).

Consolidation chemotherapy in elderly AML patients may seem logical based upon results in younger adults. However, the optimal therapy and number of courses remain unknown. Furthermore, the morbidity and mortality associated with curative therapy frequently preclude the routine use of post-remission therapy in many older patients, particularly those over the age of 70 years. Thus, until extensive randomised studies of consolidation therapy in the elderly are conducted, it would seem sensible to plan consolidation therapy on a case by case basis for older patients.

Complications during remission induction therapy: Patient related factors

With current curative therapy, approximately 30% of elderly patients develop complications associated with induction chemotherapy resulting in treatment-related death (Lowenberg, 1996). The most frequent cause of death in these cases can be attributed infection, often during the period of bone marrow aplasia (Kimmick *et al.*, 1997). The risk of infection is a direct result of quantitative and qualitative neutropenia due to bone marrow infiltration, and bone marrow hypoplasia resulting from chemotherapy.

The normal physiological changes that occur with increasing age, result in reduced cardiac function and vessel wall compliance, as well as a decline in pulmonary function. Furthermore, the presence of co-morbid disease such ischaemic heart disease and hypertension, result in the significant reduction of cardiac physiological reserve (Cohen, 1999). Consequently, the elderly are less able to tolerate the haemodynamic consequences of severe infection. Thus treatment-related deaths as a direct result of infection occur more frequently in elderly patients than in younger adults. In addition to co-morbid disease, physiological age related changes affecting renal and hepatic function result in pharmacokinetic and pharmacodynamic changes that increase the systemic bioavailability and plasma concentrations of antineoplastic agents. When coupled with the reduced haemopoietic reserve of elderly patients, the systemic and haematological toxicity associated with chemotherapy may often be profound (Balducci *et al.*, 1988; Kimmick *et al.*, 1997; Rubin *et al.*, 1992).

Supportive care

The empiric use of broad-spectrum antibiotics is central to the supportive care of the neutropenic patient and thus all patients with acute leukaemia (Hughes *et al.*, 1990). The provision of red cell concentrates to febrile neutropenic patients, particularly during severe infection, augments tissue oxygenation and is therefore also an essential part of patient management. Additional supportive care measures aimed at reducing the incidence of infection include systemic antifungal therapy, antiseptic and antifungal mouthwash, nursing patients in rooms fitted with high efficiency particulate air filter (HEPA) to reduce air borne pathogens, and the use of colony stimulating factors during the period of neutropenia. It is important to emphasise that the majority of nosocomial infections require a human vector for transmission. Thus general hygiene procedures for health care workers in contact with patients, including hand washing before and after patient contact, should be considered an integral part of supportive care aimed at reducing infection (Handwashing liason group, 2000).

Haemostatic support for patients receiving curative therapy is also a vital part of supportive care. Until platelet concentrates became routinely available in the mid 1970's, haemorrhagic complications attributable to the use of myelosuppressive therapy was a major cause of patient morbidity and mortality (Lister *et al.*, 1981). Furthermore, although not strictly thought of as supportive care, the inclusion of

all-*trans* retinoic acid in curative regimens for patients with AML M3 has led to a significant reduction in early deaths due to haemorrhage in patients with this sub-type of AML (Barbui *et al.*, 1998).

Growth factor therapy

The therapeutic rationale for the use of haemopoietic growth factors in AML therapy is two fold. Firstly, the use of colony-stimulating factors (CSF) may shorten the duration of neutropenia following chemotherapy with a concomitant reduction in morbidity and mortality from infection. Secondly in vitro studies have shown that certain CSF may sensitise AML blasts to chemotherapy and thus co-administration of growth factors with chemotherapy may enhance CR.

Commercially available recombinant human haemopoietic CSF have been available for over a decade. Two CSF, namely granulocyte colony stimulating factor (G-CSF) and granulocyte-macrophage colony stimulating factor (GM-CSF) have been extensively evaluated in the treatment of AML. Although both GM-CSF and G-CSF have been shown to induce AML blast proliferation in-vitro, their use in AML therapy appears to be safe. Several trials in which both growth factors have been used as supportive agents in AML therapy have now been reported. The use of G-CSF and GM-CSF is associated with a reduction in the number of days of neutropenia during induction therapy (Dombret *et al.*, 1995; Lowenberg *et al.*, 1997; Rowe *et al.*, 1995), although only one trial has reported a significant reduction in treatment-related deaths (Rowe *et al.*, 1995), and in one other a significantly higher CR rate was reported in patients who received G-CSF (Dombret *et al.*, 1995). Thus the results of these studies do not present a convincing argument for the routine prophylactic use of G-CSF or GM-CSF in elderly patients receiving intensive chemotherapy.

Attenuated dose chemotherapy

Attempts to reduce treatment-related deaths in patients receiving intensive chemotherapy has led to the investigation of attenuated dose remission induction chemotherapy. In trials in which standard and attenuated regimens have been compared, results have shown that attenuated regimens result in fewer treatment-related deaths, usually at the expense of reduced CR rates. For example the MRC AML 9 study which included elderly patients, demonstrated reduced CR, and survival, despite less induction deaths for patients receiving

DAT (1+5) compared to DAT (3+10) (Rees *et al.*, 1996). Similarly CALGB trials have shown higher CR rates with intensification of induction (Preisler *et al.*, 1987).

Individualised drug dosing has been shown to improve outcome and reduce treatment-related toxicity in children with acute lymphoblastic leukaemia (Evans *et al.*, 1998; Wall *et al.*, 2000). By applying a similar rationale to older patients with AML it may be possible to compensate for the age-related change in hepatic and renal function, and so reduce treatment related toxicity and also improve CR rate (Desoize & Robert, 1994).

Immunotherapy

The morbidity and mortality associated with myeloablative chemotherapy may be reduced or avoided with the introduction of monoclonal antibody therapy. The CD33 cell membrane antigen, which is highly expressed on leukaemic myeloid blasts cells, has been used as the primary target for monoclonal antibody therapy in AML. Initial studies using unmodified murine antiCD33 demonstrated a transient reduction in the number of circulating blasts in patients with AML (Caron *et al.*, 1998). Humanised antiCD33 conjugated with calicheamicin, a cytotoxic antibiotic, has been evaluated in phase I and II studies (Sievers *et al.*, 1997; Sievers *et al.*, 1998). It has been shown to induce CR, with an overall response rate of 43% and is associated with less toxicity than standard chemotherapy. Common adverse reactions include fever, chills, asymptomatic hypotension and elevation of liver enzymes. Clearly targeted immunotherapy using monoclonal antibody may offer a new modality of treatment that is both effective and less toxic than conventional chemotherapy. However, the optimum role of such agents in AML, particularly in the elderly, will only determined by the results of phase II and III studies.

Cytogenetics of Acute Myeloid Leukaemia

Over the last forty years cytogenetic analysis has led to improvements in the diagnosis of AML, and to a greater understanding of leukaemogenesis. Furthermore, current treatment protocols used in AML now utilise cytogenetic data in order to stratify patients in terms of prognosis, and treatment.

Background

The first consistent chromosomal abnormality in human cancer was documented in 1960 when a shortened G group chromosome, now called the Philadelphia chromosome (Ph), was documented in patients with CML (Nowell & Hungerford, 1960). The true nature of this chromosomal aberration was clarified in 1973 when it was demonstrated that the Ph chromosome arose as a direct result of a translocation between the long arm of chromosome 9 and 22 [t(9;22)(q34;q11.2)] (Rowley, 1973). This work led to a search for cytogenetic aberrations in other haematological malignancies, including AML. The results were initially disappointing; however, with the introduction of chromosome banding techniques in the early 1970's (Caspersson *et al.*, 1970), specific chromosomal aberrations were detectable in up to 50% of patients with AML. Improvements in cell culture techniques and the introduction of high resolution banding protocols led to an improvement in the rate of detection of chromosomal aberrations and the belief that the majority of patients with AML had an abnormal karyotype (Yunis *et al.*, 1981).

In adult patients with AML, clonal chromosomal aberrations can be detected in approximately 60-80% of cases (Heim & Mitelman, 1995). Thus, a significant percentage of patients with AML still present with a normal karyotype. This may be attributable to the existence of non-neoplastic cells dividing preferentially in vitro in a number of cases. However, many patients with normal karyotype at presentation maintain a normal karyotype throughout, including at the time of relapse. This strongly suggests that AML can occur in the absence of recognised microscopic cytogenetic aberrations. Nevertheless with the introduction of molecular cytogenetic techniques sub-microscopic chromosomal aberrations can now be detected in patients with either normal and abnormal karyotype (Caligiuri *et al.*, 1994; Shurtleff *et al.*, 1995; Tosi *et al.*, 1999).

Cytogenetic analysis

Conventional karyotype analysis depends upon the ability to arrest dividing neoplastic cells in metaphase or prophase, and to achieve suitable spreading, fixation and staining of chromosomes prior to microscopic examination. Early cytogenetic studies in leukaemia were performed using bone marrow cells that had been subjected to short-term culture. This was followed by the introduction of more 'direct' methods in which freshly aspirated bone marrow cells were

cultured for up to two hours in the presence of colchicine. The direct method was considered to be the most appropriate technique for the cytogenetic analysis of leukaemic cells, as the distortion of the *in vivo* population, was kept to a minimum by the very short *in vitro* culture time. However, it was frequently recognised that the quality of metaphase chromosomes produced by direct methods was often poor, and commonly associated with a low mitotic index, particularly in cases of ALL. In order to overcome these problems a variety of culture techniques was introduced, with and without cell synchronisation.

The identification of individual chromosomes in cytogenetic analysis is essential and relies upon the use of specific dyes in order to produce transverse bands along the entire length of the chromosome. The first banding methods used quinacrine mustard in order to create fluorescent transverse bands of variable brightness (Q-banding) (Caspersson *et al.*, 1970). Currently the most widely used banding methods use Giemsa dye preceded by denaturing techniques in order to produce chromosome bands (G-banded). Conventional banding techniques provide the most comprehensive assessment of karyotype as both numerical and structural abnormalities, including translocations and inversions can be detected simultaneously. However, these techniques can only be applied to dividing cells. Furthermore, certain chromosomal aberrations may be missed by these techniques as a consequence of limited resolution or reduced contrast between adjacent bands at chromosomal breakpoint regions.

In the 1980's, molecular cytogenetic techniques were developed and addressed a number of the technical limitations of banding methods. Fluorescence *in situ* hybridisation (FISH) techniques employ fluorescently labelled nucleic acid sequences as probes for cellular DNA targets. The most frequently used FISH probes in cytogenetic analysis include (i) repetitive sequence probes (ii) whole chromosome paints and (iii) locus specific probes. Consequently, it is now possible to identify numerical aberrations, components of chromosomal rearrangements, and specific DNA sequences by FISH (Kearney, 1999). Unlike conventional banding analysis FISH can be applied to non-dividing (interphase) cells. Therefore, it is now possible to screen a large number of cells and obtain cytogenetic data from a wide variety of source material, including paraffin-embedded archival sections, blood and bone marrow smears, by utilising interphase FISH.

The *in situ* hybridisation reactions, that form the basis of FISH, are highly specific as a direct consequence of the complementarity of base pairing between homologous DNA sequences. Thus, FISH affords an inherently objective assessment of a cytogenetic aberration, at much higher detection sensitivity (≥ 1kb) than can be achieved with banding analysis, which is inherently subjective. However, unlike banding analysis, it is only possible to obtain cytogenetic information from chromosomes or specific genes that have been probed by FISH. Two new FISH-based molecular cytogenetic techniques, comparative genomic hybridisation and multiplex-FISH have expanded the limitations of conventional FISH methods and allow the screening of the entire genome in one hybridisation experiment.

Comparative genomic hybridisation (CGH) is a molecular cytogenetic technique which provides a global view of chromosomal gains or loses (DNA copy number change) in genomic DNA derived from a tumour sample. It requires no prior knowledge of the karyotype associated with the tumour specimen, and it is not dependent upon the presence of dividing cells. The methodology is based on the simultaneous *in situ* hybridisation of differentially labelled total tumour DNA and normal DNA, in fixed proportion, to normal human metaphase chromosome preparations, under conditions of repeat sequence suppression (Kallioniemi *et al.*, 1992). The technique has mainly been applied to the study of solid tumour cytogenetics, as these tumour types are frequently characterised by a low mitotic index and have poor chromosome morphology. Balanced chromosomal rearrangements do not alter DNA copy number, and therefore can not be detected by CGH.

Multiplex-FISH (M-FISH) allows the identification of individual chromosomes by representing each of the 22 autosomes and the 2 sex chromosomes in a distinct colour (Speicher *et al.*, 1996). The technique, which relies upon specific whole chromosome paint mixture can be used to identify rearrangements such as cryptic translocations, small insertions and marker chromosomes, and can be considered complementary to CGH and conventional cytogenetic analysis.

Primary chromosomal aberrations

It has been estimated that up to 55% of patients with AML present with one specific chromosomal aberration (Heim & Mitelman, 1995). The cytogenetic changes that have been documented include both numerical and structural chromosomal aberrations. However, the majority of these aberrations are non-random translocations that are leukaemia-specific (Table 1-4).

Chromosomal translocations are a consistent cytogenetic feature of many types of haematological malignancy and are thought to play an important role in the initiation of leukaemogenesis (Rabbitts, 1994). The rearrangements that occur as a consequence of chromosomal translocation may either disrupt the transcriptional regulatory elements of genes, or result in the formation of novel chimeric gene(s) as a result of an in-frame fusion of two or more exons either side of the breakpoint junction. Chromosomal translocations that

disrupt the regulatory elements of a gene frequently involve T-cell receptor or immunoglobulin heavy chain genes and therefore are associated with lymphoid malignancy, whilst gene rearrangements resulting in the formation of chimeric genes frequently occur in AML.

The loss of a tumour suppressor gene through the direct loss of chromosomal material or inactivation as a result of mutation has been implicated in the pathogenesis of solid tumours such as Retinoblastoma, Wilms tumour, and colonic cancer. However, role of tumour suppressor genes in leukaemogenesis is less clear. Cytogenetic deletion maps demonstrate that elderly patients with AML, and patients with t-AML or MDS frequently lose genetic material from chromosome 5 and 7 (Johansson *et al.*, 1991; Johansson *et al.*, 1993; Mauritzson *et al.*, 1998), suggesting that the loss of tumour suppressor gene activity may be important in leukaemogenesis. However, putative tumour suppressor gene(s) associated with these chromosomal aberrations have yet to be detected.§§

The molecular mechanisms through which trisomies in AML exert their leukaemogenic potential remains unclear. The *ALL1* gene is partially duplicated in trisomy 11 and has been implicated in leukaemogenesis. However, other genes duplicated in trisomy 11 may also be involved (Schichman *et al.*, 1995).

Table 1-4
Recurrent primary chromosomal translocations in acute myeloid leukaemia

Group	Translocation	Genes
AML1/CBF	t(8;21)(q22;q22)	ETO/AML1
	inv(16)(p13q22)	MYH11/CBFß
	t(16;16)(p13;q22)	MYH11/CBFß
RARα	t(15;17)(q22;q21)	PML/RARα
	t(11;17)(q23;q21)	PLZF/RARα
	t(5;17)(q32;q21)	NPM/RARα
	t(11;17)(q13;q21)	NuMA/RARα
	der(17)	STAT5b/RARα
	t(3;5)(q25;1;q34)	MLF1/NPM
MLL	t(6;11)(q21;q23)	AF6q21/MLL
	t(9;11)(p22;q23)	AF9/MLL
	t(11;22)(q23;q11)	MLL/hCDCrel
	t(11;17)(q23;q21)	MLL/AF17
	t(10;11)(p12;q23)	AF10/MLL
	t(11;19)(q23;p13)	MLL/ELL
	t(1;11)(q21;q23)	AF1q/MLL
	t(11;19)(q23;q25)	MLL/MSF
	t(10;11)(p11.2;q23)	ABI1/MLL
	t(11;19)(q23;p13)	MLL/EEN
	t(6;11)(q27;q23)	AF6/MLL
	t(11;22)(q23;q13)	MLL/p300
	t(8;16)(p11;q13)	MOZ/CBP
	inv(8)(p11;q13)	MOZ/TIF2
Nucleoporin	t(7;11)(p15;p15)	HOXA9/NUP98
	t(1;11)(q23;p15)	PMX1/NUP98
	t(6;9)(p23;q34)	DEK/CAN/NUP214
Tyrosine kinase group	t(9;22)(q34;q11)	ABL/BCR
	t(5;14)(q33;q32)	PDGFRβ/H4/D10S170

The primary cytogenetic aberrations associated with AML may occur with other non-random, secondary chromosomal abnormalities. Such changes may be found in the same clone as the primary aberration, or in other side line clones. These abnormalities are thought to represent later genetic events, which may contribute to disease progression. A number of these secondary chromosomal abnormalities associate with particular primary aberrations; trisomy 8 is frequently associated with t(15;17) and t(9;22), loss of sex chromosomes or del(9q) frequently occur in association with t(8;21) whilst trisomy 22 is associated with inv(16). Although the clinical and biological significance of these associations remains unknown in AML, the secondary cytogenetic changes associated with CML frequently predict for acceleration or impending blast crisis.

Core Binding Factor (CBF) translocations

Clinically, young adult patients with AML associated with core binding factor (CBF) rearrangements are highly responsive to remission induction therapy and high-dose cytarabine containing post-remission therapy. Complete remission rates of 80-90% and median disease-free survival in excess of 5 years are not uncommon (Bloomfield *et al.*, 1998; Haferlach, 1996). The chromosomal aberrations associated with these responses include t(8;21)(q22;q22), inv(16)(p13q22) and t(16;16)(p13;q22).

The t(8;21)(q22;q22) translocation is found in approximately 10% of patients with *de novo* AML, and is strongly associated with M2 FAB subtype, the morphological features associated with this rearrangement include dysgranulopoiesis, eosinophilia, prominent Auer rods, and cytoplasmic vacuolation. The molecular consequence of t(8;21) involve the disruption of *AML1* gene on 21q22 and the *ETO* gene on 8q22, resulting in the formation of the *AML1-ETO* chimeric fusion gene on the derivative chromosome 8. The *AML1* gene codes for the α subunit of CBF, a heterodimeric transcription factor that binds to the enhancer core motif TGT/cGGT. Transcriptional regulation by AML1 is thought to regulate the expression of several myeloid and T-cell specific genes, including GM-CSF, M-CSF, IL-3, and the T-cell receptor α, β, γ and δ subunits. The t(8;21) translocation results in loss of a C terminal transcriptional activation domain on AML1 which results in differentiation block, and or leukaemic transformation of normal myeloid differentiation. Indeed knock-in murine embryos heterozygous for AML1-ETO have impaired foetal liver

haemopoiesis, and die in utero from severe CNS and soft tissue haemorrhage as a consequence of thrombocytopenia (Okuda *et al.*, 1998). This suggests that *AML1-ETO* fusion gene inhibits normal haemopoiesis through the dominant negative repression of AML1 mediated transcriptional activation.

The inv(16)(p13q22) and t(16;16)(p13;q22) chromosomal aberrations occur in approximately 8% of *de novo* AML cases and are strongly associated with AML $M4_{EO.}$ This FAB subtype is characterised by myeloblastic/monoblastic infiltration of the bone marrow, together with atypical eosinophil infiltration. The inv(16)/t(16;16) results in the disruption of the β subunit of CBF at 16q22, and the smooth muscle myosin heavy chain gene at 16p13. The inversion results in the formation of a chimeric *CBFB-MYH11* fusion gene, which dominantly represses CBF-mediated transcriptional activity, resulting in a block in myeloid cell differentiation.

The RARα translocations and Acute Promyelocytic Leukaemia (APL)

The t(15;17)(q22;q21) translocation is specific to AML M3 and $M3_v$, which account for 7-10% of all AML cases. In association with above translocation, these FAB subtypes are characterised by high complete remission rates and prolonged disease-free survival if managed with curative therapy that includes all-*trans* retinoic acid (ATRA). The translocation disrupts the *RARA* and *PML* genes at 17q21 and 15q22 respectively, resulting in *PML-RARA* and *RARA-PML* chimeric fusion genes. The *RARA* gene encodes RARα, a ligand activated nuclear transcription factor, and member of the retinoic acid receptor family (RARs). The retinoic acid receptor family consists of two distinct protein groups, the RARs and RXRs. These proteins form RAR-RXR heterodimers that bind to retinoid regulatory elements (RARE) on target genes. In the absence of specific ligand, the RAR-RXR heterodimer bind a nuclear protein (N-CoR) which induces gene repression through its interaction with other proteins, including histone deacetylase (HDAC) and Sin3. Under normal physiological conditions, specific ligand (retinoic acid) produces conformational change in the RAR-RXR complex with resultant dissociation of N-CoR/co-repressor complex, allowing recruitment of transcriptional coactivators and gene expression at RARE sites. Although it remains unclear how expression of PML-RARA chimeric protein leads to leukaemogenesis, it has been suggested that PML-RARA may interfere with RAR-RXR heterodimer formation in a dominant negative manner. This

inhibition can be overcome by pharmacological levels of retinoic acid in patients with the t(15;17) translocation (Collins,1998; Melnick and Licht 1999).

A variant of APL, morphologically identical to AML (M3), is characterised by a t(11;17)(q23;q21) translocation which results in the fusion of RARα to a gene at 11q23 termed promyelocytic leukaemia zinc finger (PLZF), with the formation of a chimeric protein *PLZF-RARAα*. PLZF-RARAα forms a stable complex with N-CoR, which remains associated at pharmacological concentrations of ATRA. Thus PLZF-RARAα APL, unlike PML-RARAα APL, is unresponsive to ATRA and is characterised by a poor clinical outcome.

11q23 translocations

There are at least 30 translocations in acute leukaemia that involve rearrangement of 11q23 (Table 1-4). Although 11q23 rearrangements have been documented in adult AML and ALL, the highest frequency is found in childhood leukaemia, particularly in infants where it accounts for 60-80% of cytogenetic aberrations (Rubnitz & Look, 1998). The 11q23 translocations frequently involve the disruption and in-frame fusion of the *MLL* gene (mixed lineage leukaemia gene) at 11q23, with distinct genes located at specific breakpoints on partner chromosomes. The *MLL* gene codes for a 4000 amino acid protein which contains several important functional domains including three adjacent "AT hooks" which bind DNA, a cysteine rich region which shares homology with DNA methyltransferase and a SET domain implicated in DNA repair and telomere function. The partner genes of MLL may determine leukaemic phenotype, for example, t(9;11)(p22;q23), t(11;19)(q23;p13.1) and t(6;11)(q27;q23) are associated with AML with M4/M5 FAB type, whereas t(4;11)(q21;q23) and t(11;19)(q23;p13.3) are associated with ALL.

The prognostic importance of 11q23 aberrations in AML is varied. In secondary AML associated with topoisomerase II exposure, 11q23 aberrations are associated with a poor outcome to therapy, whereas in *de novo* AML 11q23 translocations carry an intermediate risk (Grimwade *et al.*, 1998). This variation in prognosis may partly reflect the inherent heterogeneity of 11q23 translocations, and the direct influence of MLL partner genes on disease biology.

t(9;22)(q34;q11.2) in de novo AML

The t(9;22)(q34;q11.2) translocation occurs in over 90% of cases with CML, but occurs infrequently in *de novo* AML where only 2% of patients present with the Philadelphia chromosome. The translocation results in a fusion between the *BCR* gene at 22q11.2 and *ABL* at 9q34. The resultant *BCR-ABL* chimeric gene encodes an activated tyrosine kinase, which leads to leukaemic transformation. Philadelphia positive AML commonly co-express myeloid and lymphoid marker antigens, and is associated with a poor outcome to chemotherapy.

Chromosomal deletions in AML

Structural chromosomal aberrations resulting in the deletion of genetic material occur in leukaemia and myelodysplasia. The loss of tumour suppressor genes in a number of non-random deletions is thought to be critical to the initiation of leukaemogenesis. In order to localise these genes investigators have defined consistently deleted regions in clinical material, and screened candidate genes in these regions for loss of heterozygosity using molecular based methods.

Complete and partial deletions of chromosome 5

Chromosomal deletions involving 5q-/-5 frequently occur in malignant myeloid disorders, and are especially prevalent in t(AML) arising as a result of radiation, or alkylating agent exposure. In this group of disorders, chromosome 5 deletions occur in up to 40% of cases (Pedersen-Bjergaard *et al.*, 1990). Chromosome 5 deletions also occur with increased frequency in the elderly, compared to younger patients with *de novo* AML (Leith *et al.*, 1997). Acute myeloid leukaemia with these these cytogenetic aberrations are characterised by a poor prognosis, with low rates of complete remission and median survival times of less than 4 months (Schiffer *et al.*, 1989). Candidate tumour suppressor genes associated with 5q-/-5 AML are thought to be located in region spanning 700bp at 5q31 (Horrigan *et al.*, 2000), at least 9 potential candidate genes are located at this locus.

Complete and partial deletions of chromosome 7

The complete and partial loss of chromosome 7 (7q-/-7) are the most frequent chromosomal aberrations associated with t(AML) and like 5q-/-5 aberrations, commonly occur in elderly AML patients. With current intensive chemotherapy, AML associated with monsomy 7, like monosomy 5, is characterised by a low CR

rate and poor disease-free survival (Grimwade *et al.*, 1998; Swansbury *et al.*, 1994). Although a definitive tumour suppressor gene associated with loss of material from chromosome 7 in patients with myeloid malignancy has yet to be identified, chromosomal banding studies suggest that regions, 7q22 and 7q32-36 may contain a possible tumour suppressor gene(s) (Dohner *et al.*, 1998; Liang *et al.*, 1998).

Trisomies in AML

Trisomy is commonly seen in AML, and other myeloid malignancies, either as a single chromosomal aberration, or in conjunction with primary cytogenetic aberrations, where the trisomy usually represents cytogenetic evolution of the primary clone. The most frequently observed numerical chromosomal aberration in AML is trisomy 8, which is associated with a intermediate prognosis (Grimwade *et al.*, 1998; Schoch *et al.*, 1997). However, some studies have documented an unfavourable prognosis in AML associated with trisomy 8 (Byrd *et al.*, 1998).

Presentation karyotype and clinical characteristics

The overall frequency of recurrent chromosomal aberrations in AML does not differ between men and women (Mauritzson *et al.*, 1998). However, a number of less common aberrations have been associated with sex of the patient, for example, idic(X)(q13) in AML is found more frequently in females, whilst t(11;19)(q23;p13.3) is associated with male infants (Mrozek *et al.*, 1997). Furthermore, if numerical aberrations involving sex chromosomes are ignored, an association between hyperdiploidy and male sex and hypodiploidy and female sex can be demostrated (Fourth International Workshop on Chromosomes in Leukaemia, 1984).

A number of cytogenetic changes occur more frequently among different age groups. Structural rearrangements such as t(8;21), inv(16), t(15;17) commonly occur in patients less than 60 years, whilst numerical aberrations such as –5/5q-, -7/7q-, and complex aberrations have been found to occur preferentially in older patients with AML (Dastugue *et al.*, 1995; Leith *et al.*, 1997). Other chromosomal aberrations that have been reported to occur more frequently in the elderly include trisomy 8, 11, and 13.

Clinical correlations between presentation karyotype and haematological parameters have been shown to exist. Acute myeloid leukaemia presenting with high leucocyte count may occur in association with rearrangement of 16q22, whilst relatively low leucocyte counts occur most frequently in association with t(15;17). In addition, AML with t(15;17) and t(8;16) aberrations are often associated with coagulopathy (Barbui *et al.*, 1998; Stark *et al.*, 1995). The extramedullary presentation of AML occurs with increased frequency with specific karyotypic abnormalities, for example granulocytic sarcoma is associated with the t(8;21) translocation, and may occur in up to 25% of patients with this cytogenetic aberration, whilst intracerebral myeloblastomas have been associated with $M4_{EO}$ and inv(16)(p13q22) (Tallman *et al.*, 1993; Vidal *et al.*, 1999).

Cytogenetics and AML morphology

Although several associations between AML FAB type and specific recurrent chromosomal aberrations have been established, only two karyotypic abnormalities have a strong association with specific AML FAB subtypes. These chromosome rearrangements include t(15;17) and its molecular equivalent *PML-RARα* which are found exclusively in AML M3/$M3_v$ only, and 16q22 which invariably associates with AML $M4_{EO}$.

A weaker association exists between t(8;21) and AML M2. Although 90% of cases with t(8;21) occur in association with AML M2, the t(8;21) translocation accounts for one third of M2 cases with a karyotypic abnormality. If patients with a normal karyotype are included then the frequency of microscopically detectable t(8;21) is nearer 17% (Fourth International Workshop on Chromosomes in Leukemia, 1984). However, if the molecular rearrangement of t(8;21), namely *AML1-ETO* is screened for in patients with AML M2, it can be found in up to 25% of cases (Andrieu *et al.*, 1996). Rearrangements of 11q frequently occur at breakpoint 11q23, and commonly associate with AML M5a. The numerical aberrations monosomy 5, monosomy 7, trisomy 8, trisomy 21, and AML associated with normal karyotype show no specific correlation with AML FAB types.

Correlations exist between bone marrow morphology in AML, and presentation karyotype. For example, bone marrow eosinophilia (defined as ≥5% bone marrow eosinophils) is associated with 16q22 rearrangements irrespective of whether the FAB type is $M4_{EO}$, and to a lesser extent with t(8;21). Bone marrow

basophilia is a common feature of AML with t(6;9) or 12p deletion. The presence of Auer rods is associated with t(8;21), t(15;17), and t(9;22) as well as complete or partial deletions of chromosomes 5 or 7, whilst the presence of micromegakaryocytes and trilineage dysplasia commonly occurs in *de novo* AML with abnormalities of 3q21 and 3q26.

Patient karyotype: an independent prognostic factor in AML

Presentation karyotype was shown to be an independent prognostic factor in AML for the first time in a multicentre prospective study reported by the Fourth International Chromosomes in Leukemia Workshop (Bloomfield *et al.*, 1984). This study, which included 716 patients with newly diagnosed AML, of which 305 received intensive induction chemotherapy, demonstrated that patients with a completely normal karyotype (NN) had a better overall survival than patients with a mixture of abnormal and normal metaphases (AN) or exclusively abnormal metaphases (AA). There was no significant difference in overall survival between AN and AA patients. These findings were substantiated in a follow up study in 1989 (Arthur *et al.*, 1989), which demonstrated that patient karyotype remained an independent prognostic factor in predicting clinical outcome when patient age, FAB type and sex were also considered in multivariate analysis. This study also demonstrated that patients with a complex karyotype had a poor overall survival. Other studies, using different classification criteria for patient karyotype, have also demonstrated that presentation karyotype is an independent prognostic determinant for complete remission, remission duration and overall survival (Grimwade *et al.*, 1998; Stasi *et al.*, 1996).

Complete remission rates

The clinical studies in AML where cytogenetic data has correlated with outcome have typically involved younger patients. In the majority of these studies, the highest rates of complete remission, typically (80-98%), where attained in patients with t(8;21) or inv(16)/t(16;16) (Bloomfield *et al.*, 1984; Grimwade *et al.*, 1998). Less favourable CR rates have been associated with complete and partial deletions of chromosome 5, and 7 as well as complex karyotype (40-67%). However, although the partial deletion of chromosome 7 (7q-) is commonly associated with low CR rates, relatively high CR rates have been observed in some series (Grimwade *et al.*, 1998), suggesting that 7q- may be a cytogenetic

marker of standard risk.

Following the use of ATRA, AML M3 characterised by the t(15;17) translocation has been associated with favourable CR rates of nearly 90%. In contrast, patients with AML M3 and the t(11;17) (characterised by the fusion of *PLZF* and *RARα* genes), have low rates of CR irrespective of whether all-*trans*-retinoic acid is used as part of induction therapy (Licht *et al.*, 1995).

The complete remission rates associated with structural rearrangements affecting 11q23 have varied between series (Mrozek *et al.*, 1997) and may reflect differences in induction therapy, size of the patient cohort, and or the heterogeneity of the translocations, for example adults with t(9;11)(q22;q23) have better CR rates than adult patients with other 11q23 translocations (79% vs 57%) (Mrozek *et al.*, 1997).

Duration of remission

The duration of remission in AML often correlates with the cytogenetic aberrations present at the time of diagnosis. Thus patients with t(8;21), inv(16), and t(15;17) have the highest probability of remaining in CR at five years. By way of contrast patients with complex karyotype, monosomy 7, and complete or partial loss of chromosome 5 are characterised by a high relapse risk (Grimwade *et al.*, 1998).

Overall survival

Superior overall survival in adult patients with AML have been associated with t(8;21), 16q22 rearrangements and t(15;17), with five year survivals ranging from 50-70% in some studies (Bloomfield *et al.*, 1998; Grimwade *et al.*, 1998; Mrozek *et al.*, 1997). Interestingly although a favourable outcome is associated with t(8;21) translocation a subset of patients with granulocytic sarcoma and t(8;21) have a relatively poor outcome (Byrd *et al.*, 1995). Likewise in children, AML associated with t(8;21) confers a standard or poor prognosis (Martinez-Climent *et al.*, 1995).

In contrast to the chromosomal aberrations associated with favourable outcome, cytogenetic aberrations repeatedly associated with poor survival included rearrangements of 3q21, -5/5q-,-7, and complex karyotype (Grimwade *et al.*, 1998; Leith *et al.*, 1997).

The prognostic significance of cytogenetic data and indeed other prognostic factors are in part dependent upon the treatment protocols used to treat AML.

Clearly the advent of ATRA therapy in AML M3, has resulted in a significant improvement in CR and overall survival for patients with this subtype of AML. Whilst favourable outcome associated with AML characterised by rearrangements involving CBF partly reflect their sensitivity to high dose cytarabine containing regimens (Bloomfield *et al.*, 1998). Thus cytogenetic aberrations currently predictive of unfavourable outcome may in the future predict for standard or favourable prognosis if significant improvements in therapy can be found. Furthermore, by uncovering novel cryptic aberration it may be possible to obtain important prognostic data that influence clinical management. This principle has been clearly illustrated in childhood leukaemia with the discovery of the cryptic t(12;21)(p13;q22) translocation resulting in *TEL/AML1* fusion. Children with this translocation are characterised by an age between 1 and 10 years, B-lineage ALL and an extremely good prognosis with standard curative therapy (Faderl *et al.*, 1998; Shurtleff *et al.*, 1995).

At the present time cytogenetic data allows for a risk-adapted approach to therapy in AML. For example, young AML patients with 'unfavourable' cytogenetic abnormalities unlikely to benefit form standard induction regimens are often treated with 'high-risk' experimental protocols in an attempt to improve CR and survival. Similarly, patients with 'favourable' karyotype may not necessarily require (or be offered) consolidation with high dose chemotherapy and stem cell rescue in first CR, due to the superior results achieved with consolidation regimens containing high-dose cytarabine

'Minimal residual disease'

At the time of presentation patients with acute leukaemia may have up to 10^{12} leukaemic blasts. However, as a consequence of the limited resolution of morphological analysis, which at best may detect up to 1 leukaemic cell in 100, significant residual leukaemic infiltration (10^{9}cells) may still persist in patients who are considered to be in complete remission, which may ultimately lead to relapse.

Assays used to detect minimal residual disease (MRD) rely upon the ability to detect leukaemic cells using a variety of techniques including, cytogenetic and molecular cytogenetic analysis, immunophenotyping and cell growth

characteristics. Minimal residual disease detection using conventional cytogenetic techniques allows the detection of leukaemia based upon the chromosomal aberrations present at the time of diagnosis. The detection of abnormal clones after therapy may predict relapse (Burnett *et al.*, 1999), although the failure to detect abnormal clones does not necessarily preclude the possibility of relapse. Similarly, the attainment of a complete or major cytogenetic response in CML (<35%, t(9;22) +ve metaphases) equate with an improved long term survival in patients (Kantarjian *et al.*, 1996). Molecular cytogenetic techniques such as FISH, and the molecular detection of gene rearrangements using polymerase chain reaction (PCR) have further refined MRD detection in leukaemia (Campana & Pui, 1995). In AML the aim of therapy in patients with AML M3 is the establishment of PCR negativity for *PML-RARα*. Patients who are persistently positive for the transcript after the completion of therapy have a higher rate of relapse (Campana & Pui, 1995). In the case of t(8;21) AML a number of studies have reported positive reverse transcriptase-PCR results in patients in long term remission (Burnett *et al.*, 1999). However, at least one multicentre study has demonstrated a correlation between PCR negative results for *AML-ETO* after induction therapy, and absence of relapse (Morschhauser *et al.*, 2000).

The Scope of This Thesis

Acute myeloid leukaemia primarily occurs in individuals 60 years or older. However, the results from clinical trials frequently reflect outcome in younger patients due to patient selection. Thus clinical outcome data from many trials may not be applicable to elderly adults with AML.

Prognostic factors such as presentation karyotype influence therapeutic decision making in AML. Furthermore, new molecular cytogenetic technologies capable of refining cytogenetic analysis now exist. In this thesis, the therapeutic management of AML in patients 60 years or older at a single centre will be reviewed and analysed in order to provide a historical overview of the disease in the elderly. Factors influencing clinical outcome in this group will also be examined, along with cytogenetic data. This will include an appraisal of two new FISH technologies, namely CGH and M-FISH in order to determine the relevance of these techniques in elderly patients with AML.

Chapter 2

Introduction

The distinct pathophysiology of AML in older patients frequently results in poor outcome in those patients in whom aggressive curative therapy is attempted. Thus for many physicians the decision to treat an elderly patient with AML using intensive chemotherapy is often controversial. Frequently, clinical criteria and the perceived ability to withstand aggressive chemotherapy, are the limiting factors when curative therapy is contemplated in older patients with the disease.

It can be argued that factors predictive of clinical outcome should be used to direct therapeutic decisions in AML regardless of age. However, clinical studies limited to elderly patients with AML are uncommon. Thus it can be deduced that prognostic factors which influence clinical outcome in older patients with the disease have been poorly investigated and under utilised.

Aims of this Analysis

In Western Europe and North America AML is predominantly a disease of the elderly (age>60years) with a median age at the time of presentation of 64 years (Brinker, 1982; Cartwright *et al.*, 1997). Unfortunately many elderly patients are excluded from clinical trials due to strict entry criteria, and or patient or physician bias (Hutchins *et al.*, 1999; UKCCCR, 2000). Thus the results of clinical trials in AML can not be readily extrapolated to older patients, many of whom are characterised by poor clinical outcome due to patient and disease-related factors such as presentation karyotype (Hiddemann *et al.*, 1999; Lowenberg, 1996). In addition, whilst prognostic factors are often taken into consideration when treating younger patients, they are seldom utilised in elderly patients, who are frequently selected for curative treatment on clinical grounds rather than objective criteria that may influence treatment outcome.

Over a thirty year period, 420 consecutive adults (age>60 years) with AML were referred to the ICRF Medical Oncology Unit at St Bartholomew's Hospital for management. This represents a cohort of patients treated at a single centre during the era in which the modern chemotherapeutic management of AML began, and

subsequently evolved, in unison with important advances in supportive care. In order to evaluate the impact of supportive care and intensive chemotherapy in a historic setting, a retrospective analysis of the clinical outcome data from elderly patients with AML treated at St Bartholomew's Hospital over a thirty year period will be performed. The analysis will include the analysis of cytogenetic data for a subgroup of elderly patients who were successfully karyotyped. This data will be presented in relation to clinical outcome so that the prognostic importance of presentation karyotype in older patients can be evaluated,

Patients and Methods

Patients

Four hundred and twenty consecutive adults (age>60 years) with newly diagnosed AML were managed at St Bartholomew's Hospital between June 1969 and December 1999 (the notes of 192 patients were reviewed retrospectively in order to obtain additional clinical information not recorded at the time of diagnosis). A total of 288 patients (69%) received combination chemotherapy with curative intent. Patients were selected for treatment based on clinical grounds and individual patient wishes. The clinical details of patients included in the study are shown in Table 2-1.

Cytogenetic analysis

Cytogenetic analysis was introduced for newly diagnosed patients with AML in 1987. Standard cytogenetic analysis was performed in the Medical Oncology Unit using bone marrow or peripheral blood from patients at the
time of diagnosis. Short term cultures were established using complete medium (RPMI 1640 with glutamax, 20% fetal calf serum, 1% Streptomycin and Penicillin). Metaphase preparations were harvested, and GTG banding performed (Czepulkowski *et al.*, 1992). Patient karyotypes were described according to the International System For Human Cytogenetic Nomenclature (ISCN) (Mitelman, 1995).

Table 2-1
Clinical and biological characteristics at presentation of 420 previously untreated patients with AML: Comparison between curative and conservative treatment

Parameters		Curative treatment n=288	Conservative treatment n=132	Total n=420	*p*-value
Age (years)					
Median		66	70	67	<0.01
Range		60-83	60-86	60-86	
Sex M:F		170:118	74:58	244:176	0.72
Blast count ($x10^9$/l)					
Median		4.5	1.0	3.2	0.003
Range		0-329	0-389	0-389	
Hb (g/dl)					
Median		9.0	8.6	8.9	0.64
Range		3.2-16.5	4-17.6	3.2-17.6	
WBC ($x10^9$/l)					
Median		12.2	5.4	10.1	0.01
Range		0.3-350	0.5-475	0.3-475	
Platelets ($x10^9$/l)					
Median		47	50	47	0.55
Range		5-658	7-398	5-658	
Serum LDH (IU/l)					
Median		560	2964	560	0.18
Range		210-5658	451-4750	210-5658	
Albumin (g/l)					
Median		37	35	36.5	<0.001
Range		24-49	22-44	22-49	
FAB	M0	1	2	3	
	M1	99	27	126	
	M2	81	30	111	
	M3	8	3	11	
	M4	58	25	83	0.001
	M5	24	4	28	
	M6	12	5	17	
	M7	1	4	5	
Hypoplastic AML		4	15	19	
Antecedent haematological disorder		47	23	70	0.63
Hepatomegaly alone		27	16	43	
Splenomegaly alone		19	2	21	0.13
Hepatosplenomegaly		31	9	40	
Neither		114	46	160	

Treatment regimens

Between 1969 and 1999 six generations of combination chemotherapy based on an anthracycline/anthracendione in combination with cytarabine were investigated in adult patients with AML 60 years of age or older, 18/420 patients received treatment with alternative regimens; details of all these therapies are included in Table 2-2.

From 1969 to 1972, patients over 60 years of age received identical chemotherapy to younger adults, whilst between July 1972 and 1978, older patients were treated in a separate program (Barts IVb). At the beginning of the Barts IX trial, all adults with AML received the same combination chemotherapy until the introduction of mitoxantrone and cytarabine in July 1987. The first 26 patients received mitoxantrone 12 mg/m^2 intravenously for 3 days and cytarabine at 100 mg/m^2 twice daily intravenously, for 7 days. Due to a high early recurrence rate, the remaining 49 patients received mitoxantrone 10 mg/m^2 intravenously for 5 days, with the same dose and schedule for cytarabine (MacCallum *et al.*, 1995). The aim was to administer a total of 4 cycles of treatment irrespective of the number of cycles required to achieve complete remission.

Supportive care

The antibiotic regimens used to treat neutropenic fever evolved over the three decade period to reflect a greater emphasis on gram negative cover provided by first line antibiotics. From 1969 to 1978, patients with neutropenic fever were initially treated with ampicillin and flucloxacillin, whilst aminoglycoside and broad-spectrum cephalosporin antibiotic combinations were introduced as first line therapy thereafter. Patients treated on the Barts IX trial were prescribed non-absorbable prophylactic antibiotics in the form of 'FRACON' (framycetin, colistin and nystatin) for gastro-intestinal decontamination. Although G-CSF and GM-CSF became commercially available in the early 1990's, these colony-stimulating factors were not routinely used as an adjunct to the management of neutropenic fever in elderly patients. Red cell products were initially available in the form of fresh blood, and later as packed cells. Between 1969 and1971, platelet support was

Table 2-2
Details of the intensive treatment regimes

Trial/Drugs	Daily dose/m^2	Days	Year	Number of patients
Barts I& III			1969-1972	21
Daunorubicin	55mg iv	1		
Cytarabine	70mg iv	1-5		
Barts II			1970-1971	5
Daunorubicin	55mg iv	6		
Cytarabine	140mg iv	1-2		
	70mg iv	6		
Barts IVb			1972-1978	62
Danorubicin	55mg iv	1+3		
Cytarabine	100mg iv	1-5		
Barts IX			1978-1980	15
Adriamycin	50mg iv	1		
Cytarabine	100mg bd iv	1-5		
6-Thioguanine	100mg bd oral	1-5		
Barts X			1978-1980	26
Adriamycin	25mg iv	1-3		
Cytarabine	100mg bd iv	1-7		
6-Thioguanine	100mg bd oral	1-5		
Barts XI			1981-1983	21
m-Amsacrine	100mg iv	1-5		
Cytarabine	100mg bd iv	1-7		
'3+7'			1983-1986	45
Adriamycin	25mg iv	1-3		
Cytarabine	100mg bd iv	1-7		
Mitoxantrone/cytarabine			1987-1999	75
Mitoxantrone	10mg iv	1-3		
	or			
	12mg iv	1-5		
Cytarabine	100mg bd iv	1-7		
Non trial chemotherapy			1969-1999	18

provided in the form of fresh blood, whilst pooled and single donor platelets were used between 1972 and 1974 and from 1974 onwards, respectively. Prophylactic platelet and red cell transfusions were administered to maintain counts above $10x10^9$/l and 10g/dl respectively.

Definitions

The morphological diagnosis of AML was made according to the French American British classification of acute leukaemia (Bennett *et al.*, 1976; Bennett *et al.*, 1985a; Bennett *et al.*, 1985b; Bennett *et al.*, 1991). The presence of coexisting trilineage myelodysplasia with *de novo* AML was determined according to recognised criteria (Brito-Babapaulle *et al.*, 1987). Complete remission (CR) was defined as patients being well, in the presence of a normocellular bone marrow containing less than 5% blast cells, together with peripheral blood counts demonstrating Hb>10g/dl, neutrophils>$1x10^9$/l and platelets>100 $x10^9$/l.

Patients were classified as having 'resistant disease' (RD) if there was either no change or an increase in the percentage of leukaemic blast cell infiltration at the time of evaluation after one or two cycles of treatment. Patients who did not achieve CR because of a hypocellular bone marrow or peripheral blood cytopenia, despite the absence of leukaemic bone marrow infiltration were also included in this group. Deaths attributable to organ failure, infection or bleeding during the first two cycles of chemotherapy, in the absence of resistant disease were defined as treatment-related deaths (TRD)

Statistical methods

Potential prognostic markers for survival were assessed using a logrank test, with multivariate analysis performed using Cox regression. For achievement of CR, the same factors were assessed using logistic regression. The comparatively small number of patients with resistant disease made the derivation of a multivariable prognostic model for this outcome unfeasible. Therefore, univariate analysis was used to analyse potential factors for resistant disease. For categorical variables, this was performed using Fisher's exact test, whilst a Mann-Whitney test was used to investigate the equality of the distributions of continuous measurements between

patients with and without resistant disease. Analyses of all cytogenetic data tables were performed by exact or monte-carlo methods. The natural ordering of all ordered categorical variables (eg age group) was preserved by using an "exact" version of the Kruskal Wallis test (for singly-ordered tables) or the Jonckheere-Terpstra test (for doubly-ordered tables) as appropriate; otherwise, Fisher's test was used.

Results

Patient characteristics

Comparisons between the sex of the patient, haemoglobin, platelet count, serum LDH, antecedent haematological disorder and abdominal organomegaly revealed no significant difference for the two patient groups (Table 2-1). Patients receiving treatment with curative intent had a statistically significant lower median age, and higher median white count, blast count and serum albumin compared to those managed conservatively. The frequency of FAB types was also significantly different between the two groups, although the three most frequent FAB types overall in decreasing order were M1, M2 and M4.

Cytogenetics

From July 1987 to December 1999 mitoxantrone/cytarabine was the regimen used to treat elderly patients with curative intent. This coincided with the introduction of routine cytogenetic analysis for all new cases of acute leukaemia. The cytogenetic data for patients eligible for treatment with mitoxantrone/cytarabine is presented below.

Cytogenetic analysis

Cytogenetic analysis was performed using bone marrow or peripheral blood from 108/124 patients who were eligible for treatment with mitoxantrone/cytarabine. Informative cytogenetic results were obtained in 95% of cases, normal karyotype was noted in 44% of patients with informative results, and was the commonest karyotype noted in patients who received chemotherapy with curative intent (49%), whilst normal karyotype accounted for 34% in patients who were managed conservatively.

Modal Number

Pseudodiploidy was found in 18 patients (17%). Twenty two cases were hypodiploid (21%): of these, 14 had 45 chromosomes, 6 cases had 44 chromosomes, and the remaining two cases had 43 and 42 chromosomes respectively. In most patients with 45 chromosomes hypodiploidy was the result of monosomy 7 (n=7) or monosomy 5 (n=5).

Numerical chromosomal aberrations

The most frequent numerical aberrations included monosomy 5, monosomy 7 and trisomy 8 (Figure 2.1). Monosomy 7 was noted in 9% of patients, and was the sole abnormality in 3%. The remaining cases had a complex karyotype that included monsomy 5 and other numerical aberrations as well as structural rearrangements. Abnormalities resulting in complete or partial deletion of chromosome 5 were associated with complex karyotype, and occurred in 10% and 7% of patients respectively. An extra copy of chromosome 8 was observed in 9% of patients, and was the sole abnormality in 6%. The acquisition of an extra copy of chromosome 21 occurred in 4% cases and in 3/4 of these cases, was associated with other chromosomal aberrations.

Recurrent chromosomal translocations

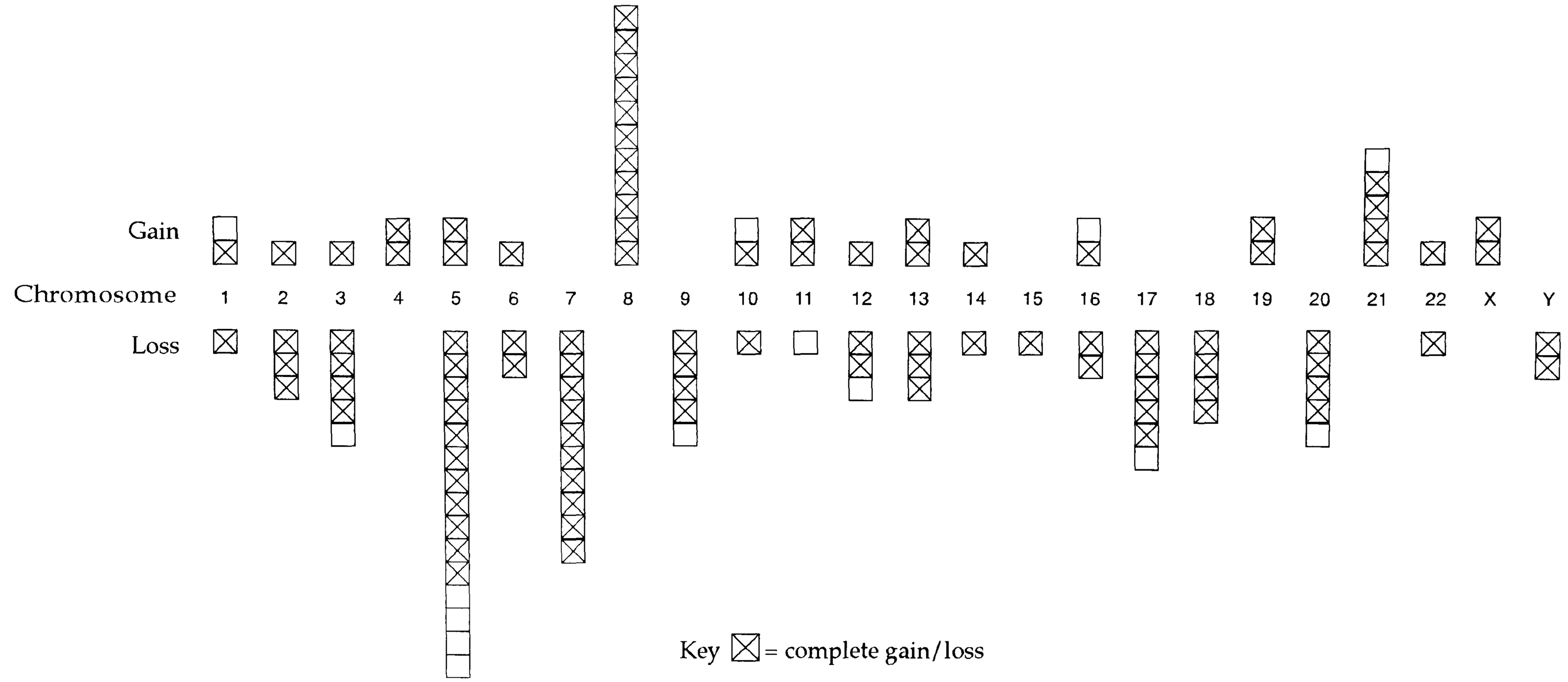
Non-random translocations normally associated with *de novo* AML were less frequent than numerical aberrations; [t(8;21) 3%, t(15;17) 3%, inv(16) 2%]. In the three patients with the t(8;21)(q22;q22) translocation, one case was pseudodiploid, the remaining two cases had 47 and 45 chromosomes as a result of trisomy 8 and loss of the Y chromosome respectively. The t(15;17)(q22;q21) translocation was found in 3 cases, one had an extra copy of chromosome 8. The inv(16)(p13q22) rearrangement was noted in two patients, both of which were pseudodiploid.

Presentation karyotype and prognostic subgroups

For the purposes of clinical outcome patients were divided into 3 cytogenetic subgroups retrospectively based upon previously defined MRC prognostic criteria (Grimwade *et al.*, 1998): [1] 'favourable' [t(8;21), t(15;17), inv(16)], [2] 'unfavourable' [-5/5q-,-7, and complex karyotype (3 or more distinct

Figure 2.1

Frequency of numerical cytogenetic aberrations in patients with AML aged>60years (1987-1999)



chromosomal rearrangements)], [3] 'intermediate' [other chromosomal abnormalities (miscellaneous) and normal karyotype] (Tables 2-3 & 2-4).
Considering only those patients with informative results, presentation karyotypes consistent with an 'intermediate' prognosis were the most frequent prognostic subgroup in patients who received treatment with curative intent, accounting for 75% of informative cytogenetic results. 'Unfavourable' chromosomal aberrations were the second most frequent cytogenetic subgroup in patients managed with curative intent accounting for 14%, whilst 'favourable' karyotype accounted for 11% of informative results in this group of patients. When patients who were managed conservatively were considered, 'unfavourable' aberrations accounted for 42% of informative results. Presentation karyotypes associated with 'intermediate' prognosis accounted for 55% of informative results in this group, with normal and miscellaneous karyotypes at 34% and 21% respectively, whilst 'favourable' karyotype occurred in only 3%. The distribution of prognostic subgroups between the two patient cohorts was statistically different (p=0.003) with patients receiving curative therapy having significantly more favourable karyotypes than patients who were managed conservatively.

Cytogenetic subgroup and patient age

When patients were stratified according to age, chromosomal aberrations associated with 'intermediate' prognosis were the commonest chromosomal aberrations in each age group (Table 2-5). 'Favourable' karyotypes were the least frequent subgroup overall, accounting for 11% in patients 60-65 years old and 66-70 years old. Interestingly, 'favourable' cytogenetic aberrations were not noted in patients over 70 years of age. There was a trend toward an increase in the frequency of 'unfavourable' cytogenetic aberrations with increasing age. For patients aged 60-65 years old, 'unfavourable' cytogenetics aberrations were noted in 19% of patients and 43% of patients over 76 years old. By comparison, the frequency of partial deletion of chromosome 5, and monosomy 7 showed no discernible relationship with patient age (Table 2-6). Although the total numbers were small, when the specific chromosomal aberrations associated with poor outcome were grouped together there was a trend toward an increase in the frequency of these aberrations with increasing age. The total number of

Table 2-3
Abnormal presentation karyotypes for AML patients eligible for treatment with mitoxantrone/cytarabine

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Favourable n=8

46,XY,t(8;21)
47,XY,+8/46,XY,t(15;17)
45,X,-Y,t(8;21)
46,XX,inv(16)
46,XXt(15;17)
47,XY,t(8;21),+8
46,XY,inv(16)
46,XY,t(15;17)

Unfavourable n=25

47,XX,+X,del(1)(p13),-5,der(7),+der(7),
+8,+9,add(10)(q2?),-11,-18,20,add(22)(q13),+mar [cp10]

45,XY,-7

50,XY,+5,+8,16q+,+19,+21/46,XY

44-45,XX,-2,-3,-5,-7,add(11)(p15),-14,-17,-21,-22,+5-9mar [cp]
80-86,chromosomes with evolution of the above clone

45,XY,-5,-18,+mar1,del(3p)/44,XY,-5,
-7,18,+mar1,del(3p),+3q,del12(p12),?+17p/45,XY,-5,-7,-18,+mar1,
?+mar2,del(3p),+3q,del(12)(p12)/46,XY

44,XY,-4,-5,der(7),t(7;?)(q11.2;?),
-8,-8,-16,-20,+mar1,+mar2,+mar3,+mar4/46,XY

44,XY,t(1;17)(p13;p1?),-3,
del(5)(q22;q34),add(6)(p2?),der(7)t(3;7)(q21;q36),?8,
der(9),t(6;9)(p21;p22-23),-12,-13,+2mar

45,XY,-7

44,XX,-4,add(5)(q11.2),t(12;16)(q13;q12),add(15)(q11.2),-17,-18,+mar/46,idem,+add(5)(q11.2),+11

45,XY,-5,add(7)(p22),del(12)(p12)
43,XY,der(3),-5,add(8)(q24),-17,add(17),-18,-20,+mar,+r

45,XY,-7,+mar

45,XY,-7

47,XX,-5,+mar(1),+mar(2)/48,XX,-5,
-16,+20,+C,+mar(3),+mar(4)/46,XX,-14,+mar(5)

45,XY,r(3),-5,-17,+mar2/45,idem,del(12)(p1?)/45,XY,r(3),
-5,-17,+mar3/46,XY,del(3)(p1?;p2?),-5,-7,+mar1,+mar2/46,XY

47,XX,del(20)(q11.2),+del(20)(q11.2)/48,idem,+8/47,idem,
del(5)(q32)

45,XX,-7,add(8)(q24)/48,idem,+4,+12,+22

46,XX,der(5)t(1;5)(q21;q21),add(19)(p13)/46,XX,dup(1)(q21;q4?)/
46,XX

44,XY,t(11;22)(q13;q13),r(3),+10p,+16p,-5,-6,
-7,+r/43,XY,t(11;22)(q13;q13),r(3),+10p,+16p,-5,-6,-7/46,XY

42,X,-Y,del(5)(q13;q35),dic(12;16)(p11.2;q11.?),
-18,dic(19;20)(p13.3;q11.1)/43,idem,+dic(19;20)(p13.3;q11.1)/61,X,
-Y,+1,+1,+2,+3,+5,del(5)(q12;q35),+6,+8,+8,+10,+13,+14,+14,+19,
add(19)(p13.3),+21,+2mar (composite karyotype)

76-81,XXYY,-2,-2,add(2)(p1?),-3,-6,-7,-8,add(8)(q2?),-9,-9,
-10,add(11)(q2?),add(11)(q2?),-13,-14,-15,-16,-17,-22,+4-9mar

46,XY,del(5)(q11.2q33 orq13;q35),dic(7;17)(q11.2;p11.2),+8,
-12,add(22)(q13),+mar/47,idem,-mar1,+mar2,+mar3/46,XY

45,XY,-5,add(9)(p13),del(11)(q23;q25),-13,der(15;17)(q10;q10),
del(20)(q11.2;q13.3),add(21)(q22),+der(?)t(13;?)(q12;?),+mar/45,XY,
-5,add(9)(p13)del(11)(q23;q25),der(15;17)(q10;q10),add(16)(q22),
del(200(q11.2;q13.3),add(21)9q22),+mar/45,XY,-5,add(8)9q24),
add(9)(p13),del(11)(q23;q25),der(15;17)(q10;q10),add(16)(q22),
del(20)9q11.2;q13.3),add(21)(q22),+mar

44,XY,-2,-3,der(4)t(2;4)(q1;p15),-5,add(7)(q11.2),t(7;15)(p22;q1),
del(12)(p11.2p13),-20,der(20)t(3;20)(p21;q13.3),+mar,+r/46,XY

44,XX,add(5)(q1),dic(6;22)(p11;p12),der(7)t(6;7)(p11;q22),
idic(11)(p11),-12,-17,+mar[10]

Miscellaneous n=25

45,XX,-9,i(17)(q10)/46,XX

47,XY,+21

46,XY,del(17)(p12)/46,XY

47,XX,+8

47,XY,+8/46,XY

47,XY,+11

46,XY,t(9;11)(p22;q23)

46,XY,del(9)9q13;q22)/46,XY

47,XY,+8

47,XXX

46,XY,dup(1)(p22;p3?6)/46,XY,dup(1)(p22;p3?6),dup(1)(p22;p3?6)/
46,XY

46,XY,add(10)(q2)/46,XY

47,XY,+21/46,XY,add(14)(q32)

47,XY,+13/46,XY

46,XY,t(12;17)(p12;p13)

47,XY,+8/46,XY

47,XY,+13

46,XY,t(12;13)(q24;q14),del(9)(q12)/46,XY

47,XY,+8/46,XY

45,XY,der(14;15)(q10;q10)/46,XY

46,XX,?del(12)(p12)inc/46,XX

46,XY,i(7)(p10)

47,XX,+8

46,XY,add(21)(q2)

45,X,-Y,-9,+mar/45,X,-Y

Table 2-4
Cytogenetic prognostic subgroup at presentation: Comparison between elderly patients receiving curative therapy and elderly patients treated conservatively.

Prognostic group	Curative chemotherapy n	(%)	Conservative treatment n	(%)	Total n
Favourable[1,2]	7	(11)	1	(3)	8
Unfavourable[1,2]	9	(14)	16	(42)	25
Intermediate[1]	49	(75)	21	(55)	70
(Normal[2])	32	(49)	13	(34)	45
(Miscellaneous[2])	17	(26)	8	(21)	25
Total	65		38		103

Key 'Favourable' group: t(8;21),t(15;17), inv(16)

'Unfavourable' group: -5/5q-,-7, complex karyotype (3 or more distinct chromosomal rearrangements)

'Intermediate' group: other chromosomal abnormalities (miscellaneous) and normal karyotype

[1]Distribution of cytogenetic subgroups (favourable, unfavourable and intermediate) between the two patient groups p=0.003

[2]Distribution of cytogenetic subgroups (favourable, unfavourable miscellaneous and normal) between the two patient groups p=0.01

cytogenetic anomalies associated with each abnormal karyotype varied significantly with regard to patient age (p=0.02).

Younger patients were more likely to have presentation karyotypes with 1 or 2 distinct abnormalities, whilst older patients had a tendency to present with three or more cytogenetic aberrations. Thus there was a significant correlation between complex karyotype and increasing age.

Clinical outcome

Complete remission

An evaluable outcome was noted in 287/288 patients who received chemotherapy with curative intent. Complete remission was achieved in 88 patients, representing a CR rate of 31%, (but a real CR rate of only 21% if the entire patient cohort is considered). When reviewed by era, CR rates increased with each chronological change in remission induction chemotherapy, (with the exception of the period between 1981 and 1983), rising from 13% between 1969 and 1972 to 45% in the era 1987 and 1999 (Table 2-7). Similarly, when patients were analysed on an 'intention-to-treat' basis, CR rates within each era again increased, (with the exception of patients treated with Barts XI) from 10% in the period 1969-1972 to 28% between 1987 and 1999. Overall, CR was achieved with the first cycle of chemotherapy in 55% of patients. Seventy six percent of patients treated with mitoxantrone/cytarabine achieved CR with the first cycle of chemotherapy, compared to 29% and 27% for patients treated between 1983 and1987 and 1978 and 1980 respectively.

Factors associated with achievement of CR on univariate analysis included initial chemotherapy received, serum albumin, and hepatosplenomegaly (p=0.01, p=0.01 and p=0.02 respectively) (Table 2-8). When patients receiving mitoxantrone/cytarabine were considered in isolation, factors associated with achievement of CR on univariate analysis included, presentation karyotype, serum lactate dehydrogenase (LDH), and presence of hepatosplenomegaly.

Patients with 'favourable' and normal karyotype had a significantly higher CR rate compared to patients with 'unfavourable' karyotype (71% v 22%; p=0.05), and (46% v 22%; p=0.04) respectively. Serum LDH was routinely measured at the time of presentation from 1990, and was available on 44 patients who received

mitoxantrone/cytarabine. For these patients, the median serum LDH for patients in whom CR was achieved was 559 IU/l (normal range 240-480 IU/l) compared to 755 IU/l in patients in whom treatment failed. Patients with LDH<twice upper limit of normal (n=32) had a significantly higher CR rate compared to patients with LDH>twice upper limit of normal (n=12) (86% v 14%; p=0.04). Likewise patients without hepatosplenomegaly (n=52) had a significantly better CR rate than patients with hepatosplenomegaly (n=10) (52% v 10%; p=0.01).

On multivariate analysis factors predictive of reduced CR included treatment era (1969-1972, 1972-1978, 1978-1980), and hepatosplenomegaly (Table 2-8), whilst for patients treated with mitoxantrone/cytarabine, age (increment) was the only factor that correlated with a decreased probability of achieving CR (p=0.01). Although patients with serum LDH>twice upper limit of normal illustrated a trend towards reduced CR, it was not a statistically significant association. In addition, 'unfavourable' karyotype was weakly predictive of a reduced probability of achieving CR when serum LDH was excluded from multivariate analysis.

Disease-free survival

The median disease-free survival (DFS) for all remitters (n=88) was 10 months (range 3 weeks-22 years). There was no significant difference between the duration of DFS and the era in which patients were treated, although patients treated in the period between 1969 and 1972, and 1972 to 1978 were noted to have the shortest DFS at 4 and 7.5 months respectively (Figure 2.2). Statistical analysis failed to identify any association between DFS and clinical or laboratory parameters at the time of presentation.

Table 2-5
Patient age in relation to cytogenetic subgroup

	Age (years)								
	60-65		66-70		71-75		>76		
	n	(%)	n	(%)	n	(%)	n	(%)	Total
Prognostic subgroup									
Favourable	4	(11)	4	(11)	0	-	0	-	8
Unfavourable	7	(19)	9	(26)	6	(25)	3	(43)	25
Intermediate	26	(70)	22	(63)	18	(75)	4	(57)	70
Normal	*12*	*(32)*	*16*	*(46)*	*15*	*(63)*	*2*	*(29)*	*45*
Miscellaneous	*14*	*(38)*	*6*	*(17)*	*3*	*(13)*	*2*	*(29)*	*25*
Total	37		35		24		7		103

Table 2-6
Karyotypic patterns and specific chromosomal aberrations in AML in relation to age

	Age (years)								
	60-65 n=37 (36%)		66-70 n=35 (34%)		71-75 n=24 (23%)		>76 n=7 (7%)		
	n	(%)	n	(%)	n	(%)	n	(%)	*p*-value
Normal	12	(32)	16	(46)	15	(62)	2	(29)	
Abnormal	25	(68)	19	(54)	9	(38)	5	(71)	0.10
1 anomaly	15	(40)	10	(28)	4	(17)	1	(14)	
2 anomalies	5	(14)	1	(3)	1	(4)	0	0	}0.02
≥3 anomalies	5	(14)	8	(23)	4	(17)	4	(57)	
t(8;21)	1	(3)	2	(5)	0	0	0	0	0.71
t(15;17)	2	(5)	1	(3)	0	0	0	0	0.30
inv(16)	1	(3)	1	(3)	0	0	0	0	0.67
Total	4	(11)	4	(11)	0	0	0	0	
-5	2	(5)	5	(14)	3	(13)	0	0	0.60
5q-	1	(3)	3	(9)	1	(4)	2	(29)	0.14
-7	4	(11)	1	(3)	3	(13)	1	(14)	0.84
7q-	1	(3)	3	(9)	0	0	1	(14)	0.74
3q	1	(3)	0	0	0	0	0	0	0.43
Total	9	(24)	12	(34)	7	(29)	4	(57)	

Table 2-7 **Results of initial chemotherapy for elderly AML patients treated with curative intent (1969-1999)**

Trial	Era	CR n	(%)	†*p* value	n	RD (%)	n	TRD (%)	Not treated n	Total
Barts I-III	1969-1972	4	(13)	0.004	1	(3)	27	(84)	10	42
Barts IVb	1972-1978	17	(26)	0.01	4	(6)	45	(68)	44	110
Barts IX/X	1978-1980	11	(27)	0.04	11	(27)	19	(46)	4	45
Barts XI	1981-1983	5	(23)	0.05	4	(18)	13	(59)	13	35
'3+7'	1983-1986	14	(31)	0.04	14	(31)	17	(38)	9	54
Mitoxantrone/ cytarabine	1987-1999	37	(45)	-	22	(27)	22	(27)	53	134
Total		88	(31)		56	(19)	143	(50)	133	420

Treatment -related death attributable to infection and haemorrhage in relation to treatment era

	1969-1972		1972-1978		1978-1980		1981-1983		1983-1986		1987-1999		Tota;	
	n	(%)	n	(%)	n	(%)	n	(%)	n	(%)	n	(%)	n	(%)
Infection	11	(41)	15	(33)	17	(89)	9	(69)	14	(82)	15	(68)	79	(55)
Hemorrhage	5	(19)	10	(22)	1	(5)	1	(8)	0	0	1	(5)	18	(13)

†*p* value for comparison of CR rates for trials compared to mitoxantrone/cytarabine

Table 2-8
Factors associated with and predictive of complete remission for elderly patients with AML (1969-1999)

			p-value
Univariate analysis			
Treatment era			0.01
Serum albumin			0.01
Hepatosplenomegaly			0.02
Multivariate analysis	**OR**	**95% confidence interval**	
Treatment era (base line 1987-1999)			
1969-1972$^{\Delta}$	0.17	0.05-0.53	0.002
1972-1978$^{\Delta}$	0.41	0.21-0.83	0.01
1978-1980	0.44	0.19-0.99	0.05
Hepatosplenomegaly	0.26	0.09-0.75	0.01
*Age (5 year increment)	0.14	0.03-0.57	0.01
Cytogenetic sub-group† (base line: Normal)			
'Unfavourable'	0.17	0.02-0.97	0.06
'Favourable'	1.33	0.20-8.61	0.77
Miscellaneous	0.35	0.08-1.46	0.15

Key:

OR= odds ratio (ratio<1reduced probability of CR with presence of the term)

$^{\Delta}$Multivariate analysis on an intention to treat basis [treatment era 1969-1972 OR=0.28, p=0.02; 1972-1978 OR=0.48, p=0.02]

*Patients treated with mitoxantrone/cytarabiine
† multivariate analysis on mitoxantrone/cytarabine patients if LDH excluded

Treatment-related death and resistant disease

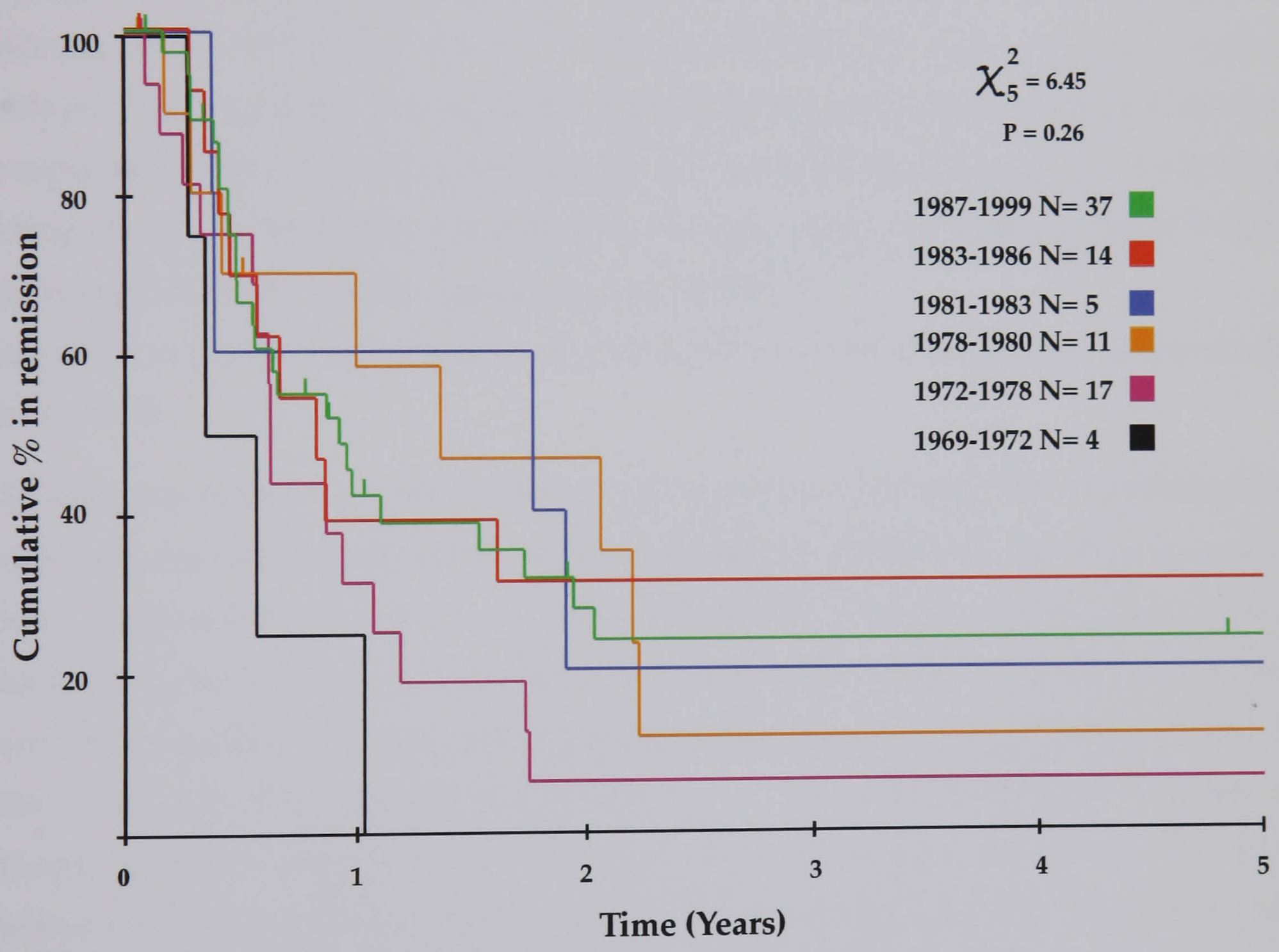
When the complications associated with remission induction chemotherapy were considered, treatment-related deaths accounted for treatment failure in 50% of patients treated overall (Table 2-7). Reviewed by era, TRD declined appreciably from 84% between 1969 and 1972, to 27% for the period between 1987 and 1999, (with the exception of early deaths associated with Barts XI).

Severe infection during the period of bone marrow aplasia remained the major cause of TRD for patients over the thirty year period, accounting for 55% of TRD overall. Between 1969 and 1978, approximately 30-40% of TRD were attributable to infection, this figure increased to approximately 70-90% in trials conducted between 1978 and 1999. The introduction of non-absorbable antibiotics for prophylactic gastro-intestinal tract decontamination in patients treated with Barts IX was not associated with a reduction in TRD due to infection. Deaths attributable to haemorrhage accounted for 20% of TRD between 1969 and 1978, falling to less than 10% thereafter, reflecting better haemostatic support by the provision of prophylactic platelet concentrates.

Resistant disease was noted in 20% of patients who received chemotherapy with curative intent between 1969 and 1999 (Table 2-7). However, treatment failure due to RD increased four-fold from 6% in the period between 1972 and 1978, rising to approximately 26% thereafter, despite an improvement in CR rate. This trend was probably attributable to improvements in supportive care, and the subsequent reduction in treatment-related death rate amongst patients with AML resistant to chemotherapy. For patients who received mitoxantrone/cytarabine, resistant disease occurred more frequently in patients with 'unfavourable' karyotype (44%) than in any other cytogenetic subgroup. Patients with 'favourable' karyotype who received chemotherapy did not demonstrate disease resistance at all, whilst patients with normal karyotype and miscellaneous cytogenetic aberrations had similar rates of resistant disease at 19% and 18%, respectively. These differences were not statistically significant. Furthermore statistical analysis failed to identify any clinical or laboratory factors associated with RD.

Figure 2.2

Duration of remission of elderly AML patients by treatment era



Survival

With a median follow-up of 11 years, actuarial survival for all patients at 1, 3 and 5 years was 20%, 7% and 4% respectively, whilst median survival from time of diagnosis was 2 months (Figure 2.3). For patients who were treated with curative intent, patients in whom CR was achieved had a significantly better median survival than patients in whom treatment failed (14 months v 6 months; $p<0.00001$). Not surprisingly, the overall survival (OS) of patients who received treatment with curative intent was significantly better than that of patients who were managed conservatively (3 months v 1 month; $p=0.0001$).

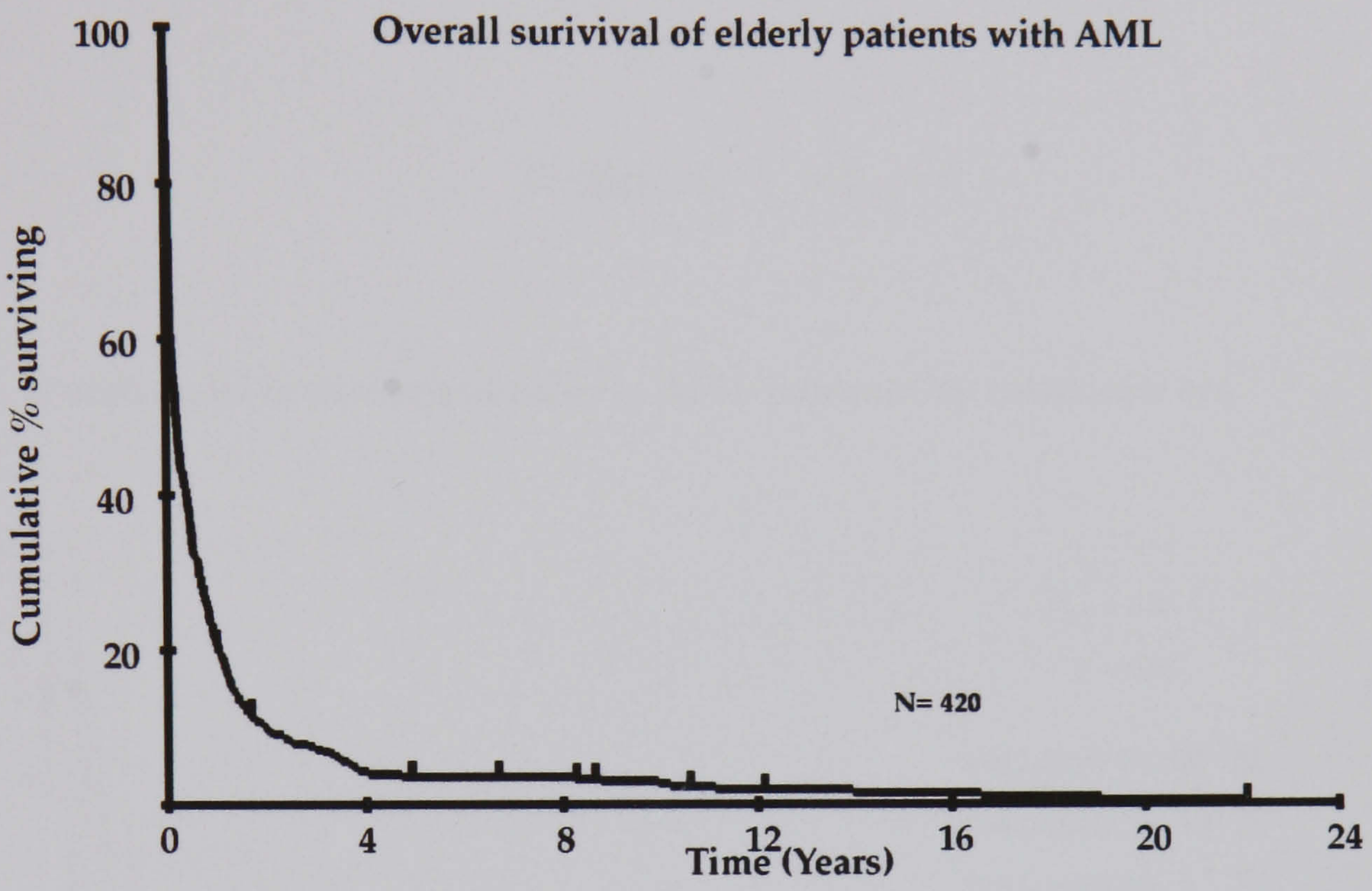
Long term survival (greater than 5 years) was noted in 13 patients. Informative cytogenetics were available in 9/13 at the time of diagnosis; ('favourable' n=4, normal n=4, miscellaneous n=1). Two patients who were in first remission relapsed at 8 months ('favourable' karyotype), and 2 years (normal karyotype) respectively, and subsequently entered second remission having received further chemotherapy. These two patients are currently alive and disease-free at 7 and 12 years respectively. Of the remaining 11 patients, 4
are alive in first CR ('favourable' n=2, normal n=1). The remaining 7 patients died in first CR.

Factors associated with survival by univariate analysis included initial treatment received, age, serum albumin, and hepatomegaly (Table 2-9). For patients treated with mitoxantrone/cytarabine, factors correlating with OS on univariate analysis included presentation karyotype and serum LDH. Patients in the 'favourable' and 'intermediate' cytogenetic subgroups had better OS compared to patients in the 'unfavourable' subgroup (10 months v 1 month, $p=0.04$), (6 months v 1 month, $p=0.03$) respectively. Patients in the 'favourable' subgroup also had a better OS than patients in the 'intermediate' subgroup ($p=0.05$), whilst the OS of patients with a normal karyotype, and miscellaneous chromosomal aberrations were similar. In addition, the median survival of patients with serum LDH<twice upper limit of normal was significantly better than that of patients with an serum LDH>twice upper limit of normal (10 months v 1.25 months; $p<0.002$).

Several clinical and laboratory factors retained predictive value for OS on multivariate analysis (Table 2-9). Curative therapy between 1969 and 1983 predicted for reduced OS, whilst the presence of hepatosplenomegaly also

predicted for reduced OS, whilst the presence of hepatosplenomegaly also

Figure 2.3



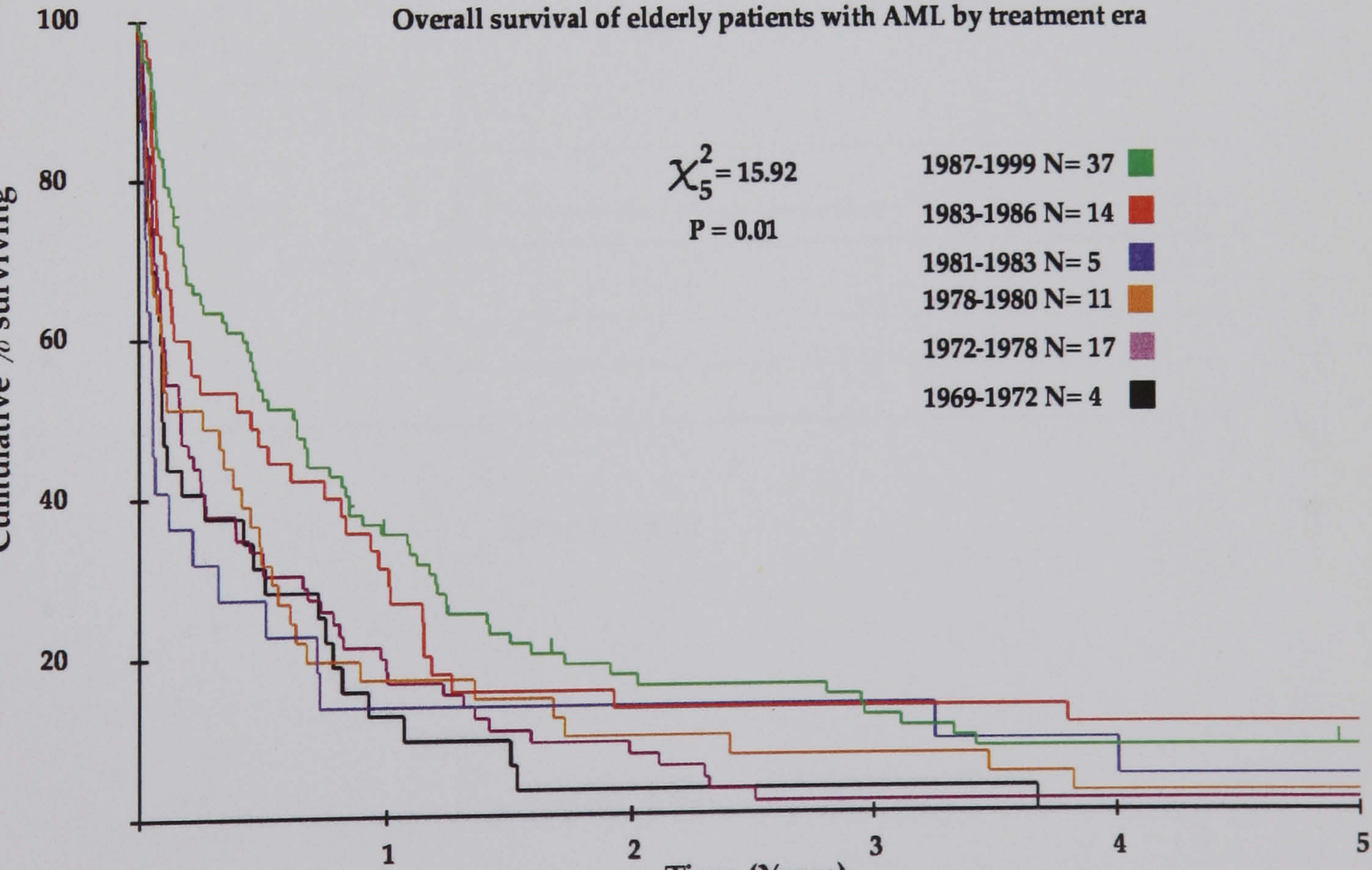


Table 2-9
Factors associated with overall for elderly patients with AML treated with curative intent (1969-1999)

			p-value
Univariate analysis			
Treatment era			0.001
Age			0.01
Serum albumin			<0.001
Hepatosplenomegaly			0.004
Multivariate analysis	**HR**	**95% confidence interval**	
White blood count	1.06	1.03-1.08	<0.001
Platelet count	0.82	0.73-0.92	0.001
FAB type			
M1	1.48	1.18-1.87	0.001
M3	1.93	0.99-3.74	0.05
Hepatosplenomegaly	1.72	1.13-2.61	0.01
Treatment era (base line 1987-1999			
1969-1972	2.07	1.42-3.00	<0.01
1972-1978	1.67	1.26-2.21	<0.01
1981-1983	1.64	1.11-2.43	0.01
*LDH (>twice upper limit of normal†)	3.29	1.38-7.86	0.01
*Age (5 yea increment)	1.84	1.15-2.95	0.01
*Cytogenetic sub-group			
'Unfavourable'	3.58	1.24-10.3	0.02
'Favourable'	0.29	0.06-1.46	0.13
Miscellaneous	1.04	0.48-2.28	0.92

Key: HR= hazard ratio (ratios>1 predict for reduced overall survival).

*Patients treated with mitoxantrone/cytarabine

†Serum LDH normal range: 240-480IU/l

predicted for reduced OS. A number of haematological parameters were found to be predictive of reduced OS, and included raised white blood count, and M1 and M3 FAB types. Interestingly, normal platelet count at presentation was predictive of better OS. For patients treated with mitoxantrone/cytarabine, both raised serum LDH and 'unfavourable' karyotype remained predictive of reduced OS, but in addition to these factors, increasing age was also found to predict for reduced OS (Table 2-9).

Time in hospital (in-patient stay)

For patients managed conservatively, the median days in hospital was 2 weeks, compared to 6 weeks in those managed with curative intent. This reflected the time required for haemopoietic recovery after chemotherapy. It was not possible to accurately record recovery times for neutrophils and platelets in the majority of patients who received chemotherapy. However, for patients who received mitoxantrone/cytarabine, the median time to recovery of neutrophils>$0.5x10^9$/l was 25 days and did not differ significantly in relation to the number of cycles of mitoxantrone/cytarabine received, whilst the median time to recover platelets>$50x10^9$/l was 22 days. Importantly, only 15% of patients who received mitoxantrone/cytarabine were fit enough to receive two or three courses of chemotherapy, whilst only 4% received the desired 4 courses of therapy due to the significant morbidity and mortality associated with the treatment. These data illustrate the difficulty in delivering consecutive courses of chemotherapy to elderly patients and tentatively suggest that this was not due to excessive haematological toxicity.

In order to measure quality of life in the absence of a performance status score, it was assumed that patients receiving therapy in hospital had a worse quality of life relative to patients discharged home. The median days in hospital relative to OS for patients managed conservatively and patients managed with curative therapy was 82% and 51% respectively, this difference was not significantly different ($p=0.74$). Thus there was no difference in this quality life measure for the two patient cohorts.

Discussion

Although AML is predominantly a disease that occurs in adults over 60 years of age, the majority of published clinical data pertain to selected patients below 60 years of age. Thus clinical trial data often reflects a 'best case scenario' (view) which can not readily be extrapolated to elderly adults. Many older patients selected for treatment with curative intent experience significant morbidity and mortality, with reduced remission rates and survival compared to younger patients treated similarly. Consequently it is commonly assumed that all elderly patients have a poor clinical outcome. In this study the cytogenetic and clinical data for elderly AML patients treated at a single centre over thirty year period were presented.

Although the total number of patients in the study at 420 was sizeable it was only possible to analyse cytogenetic data for patients referred between 1987 and 1999, as cytogenetic analysis for patients with acute leukaemia was routinely available from 1987. Analysis of the cytogenetic data confirmed that the majority of elderly patients with AML had an abnormal karyotype, although the number presenting with normal karyotype was appreciable (44%). In the group as whole, informative cytogenetic results were obtained in 95% of cases analysed and abnormal karyotype was documented in 56%. This figure is similar to that reported by the MRC, which noted an abnormal karyotype in 58% of patients with AML below 56 years of age (Grimwade *et al.*, 1998). In two studies in which cytogenetic aberrations were documented specifically in elderly patients, abnormal karyotype was noted in 55% and 42% of patients respectively (Baudard *et al.*, 1994; Leith *et al.*, 1997). Thus although some authors suggest that abnormal karyotype can be detected in up to 80-90% of patients with AML using conventional banding methods (Yunis *et al.*, 1981), for older patients, the results from this study and others would suggest that abnormal karyotype is detected less frequently.

In those patients who presented with an abnormal karyotype the majority were found to have numerical aberrations. Although patients were not selected for treatment with curative intent on the basis of presentation karyotype, the distribution of cytogenetic aberration as defined by prognostic subgroup was found to differ significantly between patients managed conservatively and patients managed with curative intent. The most striking difference was the

higher frequency of 'unfavourable' aberrations in patients managed conservatively at 42%, compared to 14% in patients managed with curative intent. 'Favourable' cytogenetic aberrations were the least frequent chromosomal aberrations in both groups. However, 11% of patients managed with curative intent had a 'favourable' karyotype compared to only 3% in patients managed conservatively.

Numerical aberrations particularly monosomy 5, monosomy 7, and trisomy 8 were the most frequent aberrations noted, whilst the non-random chromosomal rearrangements, t(8;21), t(15;17) and inv(16) occurred infrequently. Numerous studies have documented a relationship between patient age and the incidence of specific aberrations, with 'unfavourable' aberrations occurring more frequently in older patients and 'favourable' aberrations occurring more frequently in younger adults (Baudard *et al.*, 1994; Fenaux *et al.*, 1989; Fourth international Workshop on Chromosomes in Leukaemia, 1984; Taylor et al., 1995). Whilst determining the incidence of chromosomal aberrations in young adults with AML was not an aim in this study, there was a significant trend towards an increase in the incidence of 'unfavourable'-complex karyotypes with increasing age amongst the patients analysed.

Between 1969 and 1999, 420 patients were eligible for treatment with curative intent. One hundred and thirty two patients (31%) were deemed unsuitable for intensive chemotherapy due to severe co-morbid disease, poor general condition or the patient declined treatment. This figure is concordant with most series (Ånstrom *et al.*, 2000; Baudard *et al.*, 1994; Ferrara *et al.*, 1998b). Thus, the above data illustrates the first major clinical problem in the management of AML in older patients ie, a significant number of elderly adults are unsuitable for curative therapy at the time of diagnosis. The clinical outcome for these patients is extremely poor, and in this study those patients managed conservatively were characterised by a median survival of only 4 weeks.

For patients treated with curative intent, the overall CR rate was 31%. However, the correct interpretation of the CR data should take into account those patients excluded from curative treatment. Thus considering all patients, CR was achieved in only 88/420 patients (21%). Therefore, in reality, CR was achieved in a minority of elderly patients. Furthermore, the CR rate in this study was approximately half that quoted in published series in which elderly patients

ineligible for intensive chemotherapy are excluded from analysis (Leith *et al.*, 1997; Liu Yin *et al.*, 1991; Lowenberg *et al.*, 1998; Lowenberg *et al.*, 1989). Despite these disappointing figures a meaningful improvement in CR was documented over a thirty year period, with CR rates increasing significantly from 13% to 45% in treated patients.

Another significant problem encountered when managing AML in elderly patients is the inability to deliver consecutive courses of chemotherapy to those in whom achieve CR is achieved. As a direct consequence of treatment related morbidity and mortality, only 4% of patients who received mitoxantrone/cytarabine went onto receive the desired 4 courses of chemotherapy. Thus the median DFS of 10 months documented in this study probably reflects the clinical consequence of this phenomenon. Furthermore, the similarity in DFS associated with each era was probably a function of reduced intensity of treatment and an ability to deliver more courses of chemotherapy in earlier eras, coupled with an increased intensity and the inability to deliver sequential courses of chemotherapy in later eras. However, this can only be an assumption given the inability to clearly document the number of courses of consolidation chemotherapy administered in each era.

Several clinical/biological factors were found to influence CR on multivariate analysis. The less intensive regimens in use between 1969 and 1980 were associated with a lower probability of achieving CR. However, on an 'intention-to-treat' basis, only regimens used for curative therapy between 1969 and 1978 remained predictive on multivariate analysis (Table 2-8). The presence of hepatosplenomegaly also correlated with a lower probability of achieving CR.

By analysing patients who received mitoxantrone/cytarabine separately, it was possible to assess the impact on CR of a number of biological factors which were not available for analysis in earlier eras, namely presentation karyotype, and serum LDH. For these patients, increasing age was the only factor predictive of a lower probability of achieving CR, whilst 'unfavourable' karyotype was weakly predictive of reduced CR rate when serum LDH was not included in the multivariate analysis. Although 'unfavourable' karyotype was identified as an independent factor predictive of a decreased likelihood of achieving CR for older patients in a recent study (Leith *et al.*, 1997), it is important to note that the authors grouped 'favourable' and 'intermediate' karyotypes together for the

purposes of statistical analysis. The cytogenetic subgroups were considered separately for the purposes of this study, despite the relatively low number of patients in each subgroup of patients.

By comparing clinical outcome according to era, it was not only possible to confirm that intensification of therapy, coupled with improved supportive care led to an increase in the CR rate, and a dramatic reduction in TRD. Of note, patients treated between 1981-83 with Barts XI experienced a relatively high TRD rate with only a modest CR rate of 23% compared to other regimens. Indeed Dhaliwal and co-workers reported excessive toxicity amongst older patients who received this regimen, with a particularly high rate of gastrointestinal and hepatic toxicity (Dhaliwal *et al.*, 1986). Considering all patients who received treatment with curative intent, TRD occurred in 50% overall, although the TRD rate in the era between 1987-1999 was only 27%. Importantly, despite the availability of broad-spectrum antibiotics and antifungal agents TRD due to severe infection remained the most frequent cause of treatment failure amongst elderly patients receiving remission induction therapy.

The relatively high rate of TRD associated with treatment of AML in the elderly has led some authors to advocate dose attenuation as a way of reducing treatment-related mortality (Manoharan, 1998). However, recent trials have clearly shown that older patients receiving intensive therapy have a better CR and OS, without significantly increased mortality (Buchner *et al.*, 1995; Rees *et al.*, 1996). Thus although the delivery of supportive care to patients receiving treatment with curative intent is of paramount importance, the rapid achievement of CR also makes an essential contribution to the reduction in TRD rate (Hiddemann *et al.*, 1999). Therefore, the fact that CR was achieved in 76% of patients with the first cycle of mitoxantrone/cytarabine, may have contributed to the reduction in TRD in this cohort, compared to earlier regimens.

The OS of patients within this study was in keeping with other reported series in that it was disappointingly low. The majority of patients died within 1 year of diagnosis, and only 4% of patients were alive 5 years from diagnosis. Multivariate analysis identified laboratory and clinical factors predictive of OS, including the presence of hepatosplenomegaly, and treatment era (1969-1983), both of which predicted for reduced OS. A number of haematological indices were also similarly predictive of reduced OS, including raised white blood count,

and M1 and M3 FAB types. It is important to emphasise that although M3 FAB type is strongly associated with the t(15;17) translocation, which predicts for favourable outcome, ATRA was not included in the curative regimens for elderly patients who presented with AML M3. It was interesting to note that normal platelet count predicted for better OS despite the provision of platelet concentrates from 1972.

For patients who received mitoxantrone/cytarabine as curative therapy, 'unfavourable' karyotype, serum LDH>twice upper limit of normal and increasing age were all predictive of a significantly poorer OS by multivariate analysis (Table 2-8). These findings are in keeping with similar studies in older patients with AML (Ånstrom *et al.*, 2000; Ferrara & Mirto, 1996; Leith *et al.*, 1997).

The decision to treat elderly patients should take into consideration quality of life issues as well as prognostic factors that independently influence patient outcome. An objective assessment of quality of life was only possible retrospectively, and required the assumption that quality of life was better for those patients who spent time at home rather than in hospital. Patients treated with curative intent had a lower median number of days in hospital as a percentage of OS, although this did not reach statistical significance, suggesting that quality of life was similar for patients in the two treatment groups.

For patients treated with curative intent, the prognostic data obtained in this study clearly demonstrated that patient age and treatment era predict for two measurable outcomes namely CR and OS, whilst serum LDH and cytogenetic subgroup predicted for OS. Prognostic factors identified in other studies include multi-drug resistance phenotype, antecedent haematological disorder and performance status at the time of presentation (Baudard *et al.*, 1994; Johnson *et al.*, 1995; Leith *et al.*, 1997). Although it was not possible to assess the impact of multi-drug resistance phenotype and performance status on clinical outcome, in this study antecedent haematological disorder was not found to be predictive of CR or OS.

Prognostic models based upon the results of multivariate analysis can be used to stratify patients and thus bring objectivity to the therapeutic decision making process. For example, performance status, peripheral blood count, hepatomegaly and blood urea can be used in a model to predict survival in older patients with AML (Johnson *et al.*, 1995). Similarly, other authors have reported the successful

use of serum LDH and presentation karyotype as a way of objectively selecting patients for aggressive chemotherapy (Ferrara *et al.*, 1998b). The development and validation of a prognostic scoring system was not an aim of this study. However, given the potential problems that face the older patient, and their physicians when curative treatment is administered, such scoring systems should be developed and evaluated in future studies.

Summary

The results of this study revealed that although 69% of patients received treatment with curative intent, CR occurred in a minority of patients (21%). Importantly CR rate increased in the three decades studied, whilst ED declined. These findings were attributable to more intensive therapy, and better supportive care. Prognostic factors associated with treatment outcome included patient age, treatment era, serum LDH, and 'unfavourable' karyotype. By utilising these factors, it may be possible to direct curative therapy to older patients with AML more appropriately.

Chapter 3

Background

The cytogenetic study of leukaemia has led to a better understanding of leukaemogenesis and to a 'molecular' appreciation of leukaemia biology. Clinical AML studies incorporating cytogenetic data have also highlighted the prognostic importance of chromosomal aberrations in AML. These two aspects of AML cytogenetics were summarised in the previous chapter.

Since routine cytogenetic analysis of AML became commonplace in the early 1970's banding analysis have yielded the most reliable assessment of patient karyotype. With the introduction of FISH based techniques, it has been possible to achieve chromosomal analysis at the molecular level with a higher detection sensitivity than can be achieved with conventional methods. Thus new molecular cytogenetic FISH techniques complement conventional banding methods and offer the ability to detect cryptic aberrations. Comparative genomic hybridisation (CGH) originally described in 1992 (Kallioniemi *et al.*, 1992) is a relatively new molecular cytogenetic technique which allows the detection of amplification and deletion of genetic material from tumour specimens, without the need for cell culture. Unlike conventional FISH, it is possible to screen the entire genome for DNA copy number change in a single hybridisation step by using CGH. The technique can also be applied to frozen, or fixed tumour tissues, so in theory large tissue banks can be screened retrospectively using this technique.

The primary clonal aberrations associated with AML in older patients tend to result in numerical change, with complete or partial loss of chromosome 5 or 7 being the commonest abnormalities detected using conventional methods. This suggests that CGH may be an appropriate technique that could be exploited in the preliminary screening for oncogenes and tumour suppressor genes in elderly patients with AML.

Comparative Genomic Hybridisation: Studies in Haematological Malignancy

Over the past seven years there have been at least fifteen studies in which CGH has been utilised in the cytogenetic study of leukaemia (Table 3-1). Bentz and co-workers were the first to document concordance between DNA copy number change and conventional banding results in a series of cases (8 AML, 2 MDS), and suggested that AML was a model tumour for the evaluation of CGH methodologies (Bentz *et al.*, 1995). In subsequent studies in which AML was studied, CGH provided important additional cytogenetic information. Comparative genomic hybridisation was used in the cytogenetic analysis of a patient with AML with complex karyotype, and was able to provide information not provided by G-banded analysis (Nacheva *et al.*, 1995). In a study of 25 adult patients with refractory AML, CGH detected interstitial deletions in 6 patients with complex karyotype in which complete monosomies had been identified using banding analysis (El-Rifai *et al.*, 1997). In a another study CGH correctly localised aberrations poorly defined by banding techniques (Wilkens *et al.*, 1998). By utilising CGH with other molecular cytogenetic techniques the karyotypic abnormalities associated with the human AML cell line GF-D8 has been possible including the identification of two regions associated with amplifications namely: 8q2.3-qter and 11q21-qter (Tosi *et al.*, 1999). Comparative genomic hybridisation was successfully applied in a case of AML M3 with double minutes, and made it possible to localise an amplified region to 8q24, implicating the MYC oncogene (Mohamed *et al.*, 1993). In a similar case, CGH was used to localise regions of amplification in a case of AML to 11q23→qter and 9p11→pter, enabling the identification of the amplified genes by using Southern blot (Crosen *et al.*, 1999).

In acute lymphoblastic leukaemia (ALL) conventional banding analysis frequently produces poor quality chromosomes, especially in cases with complex karyotype. This may partly explain the lower rates of success for cytogenetic analysis in ALL compared to AML, (86% v 92%, UK NEQAS 1998-1999). Thus CGH studies in ALL have often yielded complementary data to banding analysis, particularly in cases with hyperdiploid ALL. Consequently, a number of authors have advocated the routine use of CGH in the routine

Table 3-1 Comparative genomic hybridisation studies in haematological malignancy

Study	Disease	Gains	Losses	Comments
Mohammed 1993	AML	8q24(MYC)		
Bentz 1995	AML/MDS			additive information in 1 case
Nacheva 1995	AML			concordance with banded analysis
Avet-Loisea 1997	Myeloma	1q12qter 7,3q22q29 11q13.3qter 8q21qter	13q12.1-q21 14,11.2q31	
El-Rafai 1997	AML	8q	5q14q32 7q31.2q32 12p12	DNA copy number change in 36% of cases
Karhu 1997	CLL	12	11q14-24 13q	
Rodley 1997	CML	13q31 1q,5p,6p,16q 9q31-->9q34	8q, 9q 10q,17q	
Cigudosa 1998	Myeloma	19,19p	13,13p	
Laramendy 1998	ALL	21(25%) 18(22.2%) X(19.4%) 17(19.4%)	9p22pter(12.5%) 12p13-pter(11.1%)	
Wilkens 1998	Haematological malignancy			CGH clarified location of defects
Wong 1998	ALL			Concordance and discrepancies with banded analysis
Crosen 1999	AML	11q23->11q24 9p		genes in amplified regions identified
Huhuta 1999	AML (paediatric)			Concordance with banded analysis
Su 1999	CML	+8,+7,+17 20,17q 1(q12-21qter)	17q	1(q12-21qter) implicated in transformation
Rice 2000	ALL			

cytogenetic analysis of childhood leukaemia (Larramendy *et al.*, 1998b; Paszek-Vigier *et al.*, 1997; Rice *et al.*, 2000).

The cytogenetic aberrations in CML that accompany acceleration or blast crisis frequently result in the acquistion of multiple copies of the Philadelphia chromosome, isochromosome 17q, and trisomy 8 and 19. CGH analysis of CML in accelerated phase or blast crisis has confirmed DNA copy number change in a number of cases, and documented a novel region of amplification 1(q12-21qter) thought to be involved in transformation, in at least 3 patients (Su *et al.*, 1998).

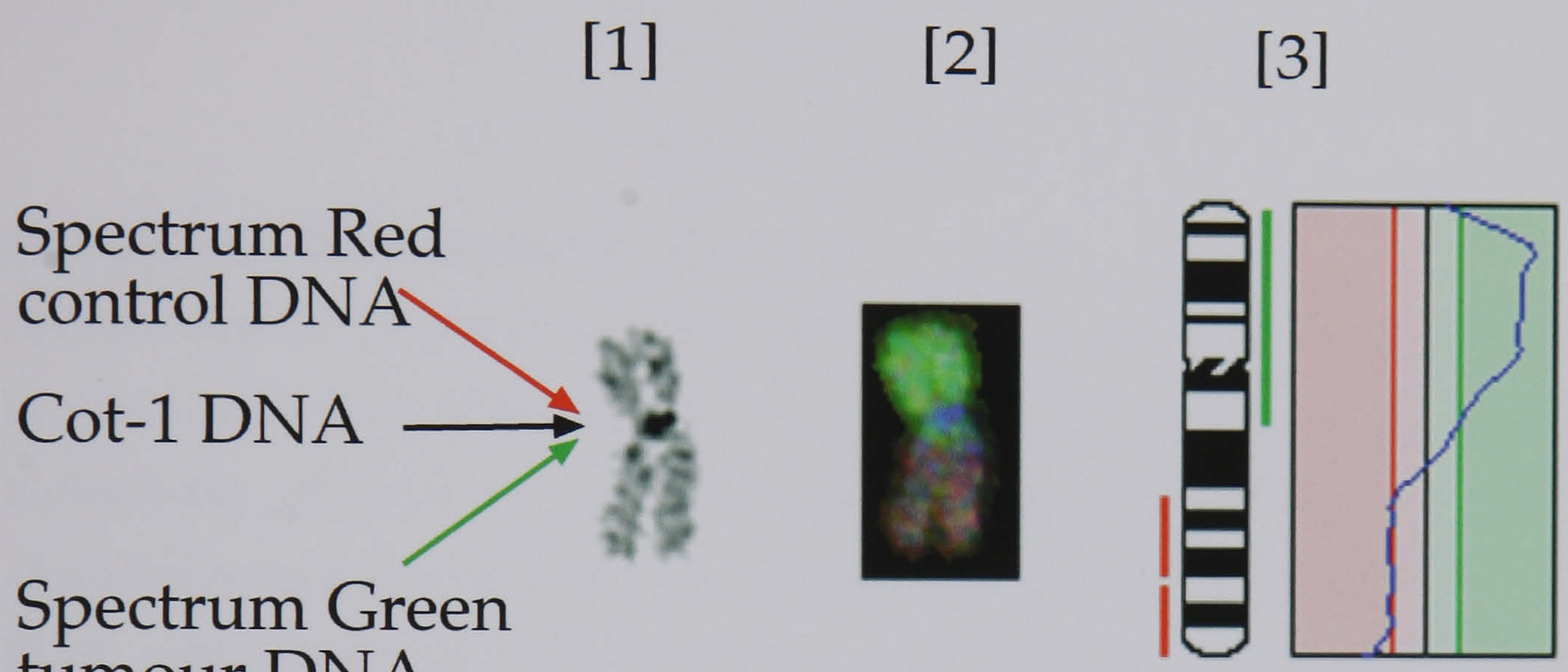
Until recently the routine cytogenetic analysis of multiple myeloma at the time of presentation was carried out infrequently. Recent reports suggest that karyotypic aberrations in multiple myeloma, like in AML, may predict for clinical responsiveness and severity of the disease. For example, the partial deletion of chromosome 13, (13q14) has been shown to correlate with the increased proliferative activity of myeloma, and predicts for a poor prognosis (Zojer *et al.*, 2000). In two studies in which CGH was utilised in the cytogenetic study of myeloma, CGH resulted in a higher rate of detection of karyotypic aberrations compared to conventional banding analysis (Avet-Loiseau *et al.*, 1997; Cigudosa *et al.*, 1998). In addition both studies confirmed that complete and partial deletion of chromosome 13 was the most frequent cytogenetic aberration in the cases of myeloma studied.

Comparative genomic hybridisation has mainly been exploited in the cytogenetic analysis of solid tumours primarily because the technique does not require a source of dividing tumour cells. However, the reports cited in Table 3-1 clearly illustrate that CGH can also provide useful additive cytogenetic data when applied to haematological malignancy.

Principles of Comparative Genomic Hybridisation

Comparative genomic hybridisation requires the simultaneous *in situ* hybridisation of differentially labelled total tumour DNA (Spectrum Green) and normal DNA (Spectrum Red), in equal proportion, to normal human

Figure 3.1



Schematic illustration of comparative genomic hybdridisation. Step[1] Spectrum Green labelled tumour DNA (test DNA) and Spectrum Red labelled normal (control DNA) are hybridised simultaneously with Cot-1 DNA to normal human metaphase chromosomes. Step[2] images of the metaphase preparations are captured using a fluorescence microscope fitted with a CCD camera. Step[3] image analysis using dedicated software. Amplification or polysomy for chromosomes/regions present in the tumour results in more intense green fluorescence at that site on the target chromosome due to excess copies in the tumour compared to control DNA. Likewise deletions or monosomies existing in the tumour will be highlighted by more intense red fluorescence since control DNA will preferentially hybridise.

metaphase chromosome preparations, under conditions of repeat sequence suppression (Figure 3.1). The chromosomal location at which under or over representation of tumour DNA sequence map was identified by an imbalance between green and red fluorescence ratio. By using a DNA counter stain, the metaphase chromosomes are banded, and therefore the sites of DNA copy number change can be assigned to chromosome a band region.

The routine successful application of CGH relies upon several critical steps in the protocol:

i. Metaphase chromosome preparation
ii Isolation of high molecular weight DNA from tumour
iii Image acquisition.

Metaphase Chromosome Preparation

Normal human metaphase chromosomes serve as the hybridisation targets for tumour/reference DNA probes in CGH, and as a result the quality of the metaphase spreads are critical to the successful application of CGH. The chromosomes used for CGH are prepared according to routine procedures from PHA stimulated peripheral blood lymphocytes. Optimal CGH metaphase spreads have a minimal number of overlapping chromosomes, and are of an adequate length for accurate fluorescence ratio measurements. The preparations should also be devoid of cytoplasm, which if present prevent satisfactory denaturation. Chromosomes should also retain their morphology after denaturation in order to allow accurate karyotyping and chromosome band identification (Karhu *et al.*, 1997a).

Tumour DNA isolation and Labelling

In order to obtain an accurate CGH profile, DNA extracted from the tumour in question must be of high molecular weight, and ideally be derived from a high percentage of tumour cells, (60% or more) (Kallioniemi *et al.*, 1994). Clearly this may not be possible in cases of acute leukaemia, since the morphological diagnosis of AML is achieved when the bone marrow is infiltrated by leukaemic blast cells which need only be as little as 30% (Bennett *et al.*, 1976).

Once total genomic DNA is extracted, tumour and reference DNA are labelled directly with different fluorochrome-conjugated nucleotides by nick translation to achieve probe fragment lengths of between 300-3000 bp, which is considered optimal for hybridisation.

Image Acquisition

Digital images from at least five high quality metaphase preparations are routinely captured using a fluorescence microscope fitted with a single bandpass filter set, and a charge coupled device camera (CCD). The CCD is made from silicon, and functions as a 'photon-detector'. When a CCD is exposed to photons generated by an excited fluorochrome, an electron potential is generated and propagated within its structure, proportional to the incident photons. The electron potential is then converted into a digital signal which can then be interpreted (Aikens *et al.*, 1989; Hiraoka *et al.*, 1987).

Aims of this Study

The description of non-random chromosomal aberrations in leukaemia is the initial step that hopefully leads to the identification of oncogenes and or tumour suppressor genes, which drive leukaemogenesis and influence disease biology. Historically this has relied upon conventional banding analysis, which in older patients with AML, demonstrate an increased frequency of numerical aberrations.

CGH is a FISH based method, which can identify DNA copy number change throughout the genome, and can be used to screen tumour DNA for amplifications and deletions. The elderly (age> 60 years) define the largest group of patients with AML. In this group of patients the biology of the disease is distinct from AML in younger patients, partly as a consequence of specific chromosomal aberrations which occur with increasing frequency with age. Thus far, there have been no CGH studies in which DNA from a cohort of elderly patients has been screened for DNA copy number change. Therefore, the initial aim of this study is an appraisal of CGH in elderly patients with AML in order to determine whether or not the technique can provide reliable cytogenetic data, concordant with conventional analysis. Archival leukaemic blast cells from patients aged 60 years or more, will be used as the source of DNA.

Conventional cytogenetic analysis requires *in vitro* cell culture, which may introduce selection bias towards a particular clone of cells. CGH analysis provides an 'average' DNA copy number karyotype, based upon the proportion of clones present in a tumour sample at the time it was taken from the patient.

Thus the second aim of this study is to determine whether CGH can provide additional cytogenetic information compared to G-banded analysis in elderly AML patients with either normal or abnormal karyotype, and to determine the prognostic significance of any additional cytogenetic data found.

Patients and Methods

Patient material

Between 1985 and 1999, 202 patients (aged>60 years) with newly diagnosed acute myeloid leukaemia (AML) had bone marrow or peripheral blood sent for cytogenetic analysis at St Bartholomew's Hospital. Fifteen patients were selected as having presentation archival material available for analysis. Bone marrow or peripheral blood mononuclear cells were collected from patients at the time of diagnosis, and stored at –70°C until they were used. The clinical laboratory data of the patients are listed in Table 3-2.

Conventional Cytogenetic Analysis

Standard cytogenetic analysis was performed by clinical cytogeneticists in the Department of Medical Oncology using bone marrow or peripheral blood from patients at the time of diagnosis. Short term cultures were established using complete medium (RPMI 1640 with glutamax, 20% fetal calf serum, 1% Streptomycin and Penicillin). Metaphase preparations were harvested, and GTG banding performed (Czepulkowski *et al.*, 1992). Patient karyotypes were described according to the ISCN (Mitelman, 1995), and were later compared with CGH DNA copy number change profiles of patients analysed in the CGH study.

Preparation of genomic DNA

Archival bone marrow/peripheral blood mononuclear cells from elderly patients with AML (age>60 years) stored at –70°C were retrieved and thawed quickly, but gently, at 37°C in a water bath. The cells were transferred to a sterile culture vessel and 10ml of culture medium (RPMI 1640/ 10% FCS) added slowly. The cells were washed twice and resuspended in 1ml culture

Table 3-2
Clinical and laboratory characteristics of patients included in the CGH study

Patient	Age	Year of diagnosis	Sex	FAB	BM (%)	Source of DNA	[DNA] µg/µl	Karyotype
01	69	1998	F	M1	54	BM	2.3	A
02	67	1998	M	M4	73	BM	1.8	A
03	60	1998	M	M4	46	BM	1.5	A
*04	64	1995	F	-	70	PB	1.0	A
05	71	1995	M	M1	70	BM	0.5	A
06	60	1998	F	M2	54	BM	0.4	A
07	75	1985	F	M1	95	BM	0.3	N
08	60	1996	M	-	-	BM	0.6	N
09	68	1996	F	M1	97	BM	0.7	N
10	64	1983	F	M1	85	BM	3.2	N
11	62	1983	M	M2	45	BM	1.2	N
12	73	1994	F	M4	90	PB	0.5	N
13	68	1997	F	M4	90	BM	0.9	N
14	69	1985	F	M1	90	BM	0.06	N
15	67	1989	F	M5	90	BM	0.8	N

Key A=Abnormal karyotype, N=Normal karyotype
*Secondary AML (Myelofibrosis→AML)
BM= bone marrow
PB= peripheral blood

medium. Viable cells were counted in a haemocytometer using trypan blue dye exclusion.

The cell suspension was added to 2ml DNA digestion buffer 50µl. Proteinase K (0.1mg/ml) were added and incubated overnight at 55°C for 18 hours. The lysate was then extracted with equal volumes of phenol, phenol/chloroform and chloroform to remove denatured proteins. DNA was precipitated with $1/10^{th}$ (original) volume 3M sodium acetate followed by 2x (original) volume 100% ethanol, recovered by centrifugation in an eppendorf centrifuge for 10min at 14000 rpm. The DNA was washed in 70% ethanol, and left to dissolve overnight in distilled water.

Estimation of DNA concentration

The optical density (OD) of the solutions was measured relative to water at a wavelength of 280nm on a Pharmacia Biotech Gene Quant II spectrophotometer. In order to check the purity of DNA the OD at 280nm was measured. A ratio OD_{260}/OD_{280} of 1.7-1.95 indicated a protein free sample. In addition, DNA quantitation was estimated visually by running DNA on a 1% agarose gel alongside a standard of known concentration.

Agarose gel electrophoresis

Gels were prepared by heating agarose in 1xTris/borate/EDTA (TBE) electrophoresis buffer until completely resuspended to a final concentration of 1% w/v. They were cast at 4°C using 8x6 cm mini gel trays containing appropriate sized combs, and submerged under 1xTBE. DNA samples, and a 1 Kb DNA ladder (298-12,216 bp) were mixed with $1/6^{th}$ volume of 6x loading buffer (40%w/v sucrose, 0.2% w/v bromophenol blue) and loaded into wells. Gels were run at between 0.5-10 V/cm. The gels were stained with ethidium bromide (1µg/ml) for 5min and visualised on an ultraviolet transilluminator (UVP inc). Images were captured using a polaroid camera (Kodak).

Comparative genomic hybridisation

Nick Translation

Nick translation of genomic DNA was performed using nick translation kit reagents (Vysis, UK). One microgram of genomic tumour DNA was mixed with (2.5µl) 0.2mM Spectrum Green, dUTP, (5µl) 0.1 mM dTTP, (10µl) 0.1 mM

dNTP, (5µl) 10x nick translation buffer, and made up to 40µl with nuclease free water. Ten microlitres of nick translation enzyme (DNAse 1/DNA polymerase 1) was added. The mixture was incubated at 15°C for 2-3 hours, and the reaction terminated by denaturing at 70°C for 10 minutes.

One hundred nanograms of probe was mixed with 1µl of loading buffer and run on a 1% agarose gel alongside a 1Kb DNA ladder to determine probe size. The incubation time was adjusted to ensure a final product size of 300-3000 bp.

Preparation of unsynchronised metaphase slides

Whole blood from a healthy volunteer (0.4ml) was cultured in 10ml complete medium, and phytohaemagglutinin 0.1ml (Gibco BRL) for 72h at 37°C. One microgram of Colcemid (Gibco BRL) was added, and after one hour at 37°C cells were pelleted, the medium removed and resuspended in 5ml hypotonic solution (KCl 0.075M). After 15min at 37°C the potassium chloride was removed and cells fixed in 10ml of fixative (methanol:glacial acetic acid, ratio 3:1). The fixative was changed three times before storing at –20°C.

Metaphase spreads were prepared by carefully dropping a diluted cell suspension in fixative onto a clean wet slide. Excess cytoplasm was removed by adding 1 drop of fixative to the slide before allowing to air dry at room temperature. Slides with few chromosome overlaps were selected and stored at –20°C before use.

Probe preparation

Working on ice, 500ng of Spectrum Green labelled tumour DNA was mixed with 250ng of Spectrum Red labelled DNA (Vysis, UK). Twenty five micrograms of human Cot-1 DNA was added to block repetitive DNA sequences. The probe was ethanol precipitatedwith3M sodium acetate and 100% ice cold ethanol, dried and resuspended in 3µl nuclease free water and 7µl CGH hybridisation buffer (Vysis, UK).

Metaphase slides were denatured in 70% formamide/2xSSC at 73°C for 2-5 minutes and dehydrated in ice cold ethanol (70%, 95%, 100%). At the same time the DNA probe was denatured at 73°C for 5 minutes. The probe mix was placed on a dry slide and sealed under a cover slip with rubber cement, and hybridised

at 37°C for 72 hours. The slides were then washed by removing the coverslip and agitating in 0.4xSSC/0.3% NP40 at 74°C for 2 minutes and 2xSSC/0.1% NP40 for 1 minute at room temperature. Following dehydration in an ethanol series (70%, 95%, and 100%), the slides were air dried before mounting in DAPI II counterstain (125ng/ml citifluor) under a large coverslip (24x50mm).

Image analysis

Slides were visualised using a fluorescence microscope (Leica DMRXA) and digital images of metaphases were captured using filters specific for Spectrum Green, Spectrum Red and DAPI linked to a charge coupled device (CCD) camera. Images were processed using digital image software specific for CGH (Perceptive Scientific Instruments Inc, UK). Metaphases were selected on the basis of high intensity green and red signal. Those with uneven hybridisation signal, granular pattern or fluorescence spots were avoided. For complete analysis 5-10 metaphases were fully analysed and the mean ratio profile of Spectrum Green to Spectrum Red calculated. Amplifications and deletions were determined by green/red fluorescence ratios of 1.25 and 0.75 respectively in accordance with imaging software algorithm.

Fluorescence in situ hybridisation (FISH)

Conventional FISH analysis was performed in cases where there was a discrepancy between G-banded analysis and CGH profiles. Briefly, for the evaluation of gain or loss of whole chromosomes, a centromeric probe for chromosome 7 and whole chromosome paint for the X chromosome (Oncor, Gaitersburg USA) were used. For interphase FISH, centromeric probes for chromosome 4 and 12 were used (Oncor, Gaithersburg USA). Hybridisation and post hybridisation washes were carried out according to the recommendations of the probe supplier. Fluorescence detection was carried out using a Leica DMRXA microscope fitted with appropriate filter sets, images were captured using Mac Probe software (Perceptive Scientific Instruments Inc, UK).

Results

The CGH results for the fifteen patients included in the study are summarised in Table 3-3. CGH provided no additive information in the nine cases with normal karyotype by G-banded, which all had normal CGH profiles. Six patients included in the study had an abnormal karyotype by conventional G-banded analysis. CGH analysis revealed DNA copy number change in 5 of these cases (01, 02, 04, 05, 06). The remaining case, (03) had a balanced translocation, and had a normal CGH profile. Of the 5 cases with DNA copy number change, CGH was in complete concordance with karyotype data in 3 cases (01, 02, and 05) (Figure 3.2-3.4). In the remaining two cases (04, 06) CGH analysis was discordant with G-banded karyotype. In case 06 CGH demonstrated monosomy 7 and trisomy 4 in 4/8 metaphases captured, but the averaged profile for the 8 metaphases captured was not statistically significant as determined by the image analysis software. In the same case both trisomy 12 and 22 (noted in 60% of dividing cells by G-banded analysis), was not apparent by CGH analysis (Figure 3.5). CGH analysis of case 04 revealed a complete loss of chromosome 7, whilst G-banded analysis described a partial deletion, del(7)(q22q36). In order to elucidate the nature of the discrepancy, metaphase FISH was performed using a centromeric probe for chromosome 7. This confirmed monosomy 7 in six out of seven metaphase cells analysed, thus correlating with the CGH profile in this case (Figure 3.6).

Table 3-3
Summary of the cytogenetic results from 15 elderly patients analysed by CGH

Patient	G-banded karyotype	CGH-karyotype
01	47,X,idic(X)(q12-13),+idic(X)(q12-13)	rev ish enh(Xp),dim(Xq)
02	47,XY,+1,der(1;7)(q10;p10), inv(3)(q21q26),+21	rev ish enh(1q),dim(7q),enh(21q)
03	46,XY,t(12;17)(p12;p13)	rev ish XY
04	48,XX,?+X,del(7)(q22q36), +21/46,X,idic(X)(q11)/45,X,-X/46,XX	rev ish dim(7),enh(21), enh(Xp),dim(Xq)
05	45,XY,-7	rev ish dim(7),
06*	45,XX,-7,add(8)(q24)/ 48,idem,+4,+12,+12	rev ish enh(4),dim(7)
07	46,XX	rev ish XX
08	46,XY	rev ish XY
09	46,XX	rev ish XX
10	46,XX	rev ish XX
11	46,XY	rev ish XY
12	46,XX	rev ish XX
13	46,XX	rev ish XX
14	46,XX	rev ish XX
15	46,XX	rev ish XX

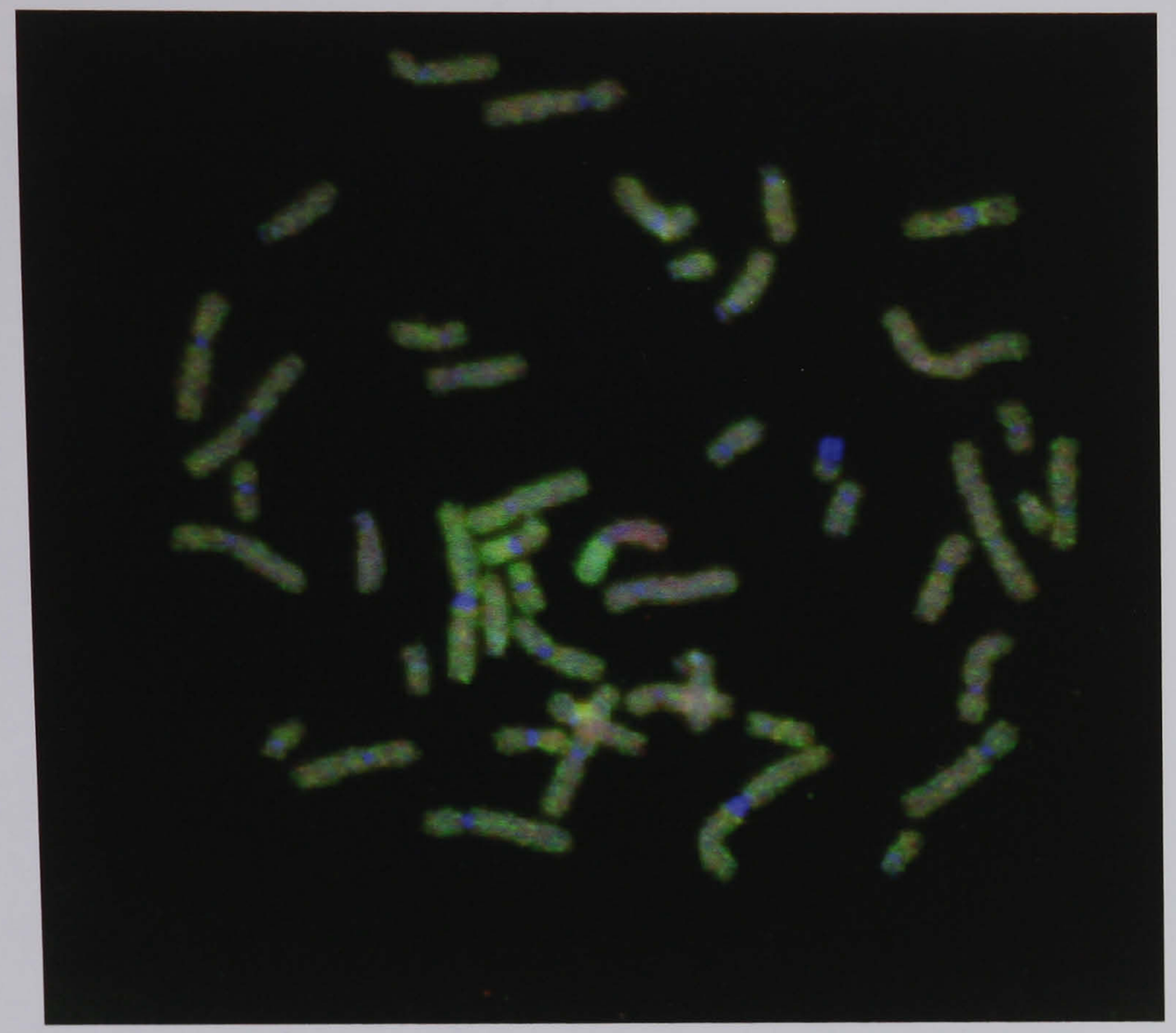
Key enh=enhanced (i.e. gain of that region)
dim=diminished (i.e. loss of that region)
*averaged CGH profile not statistically significant

TEXT BOUND INTO

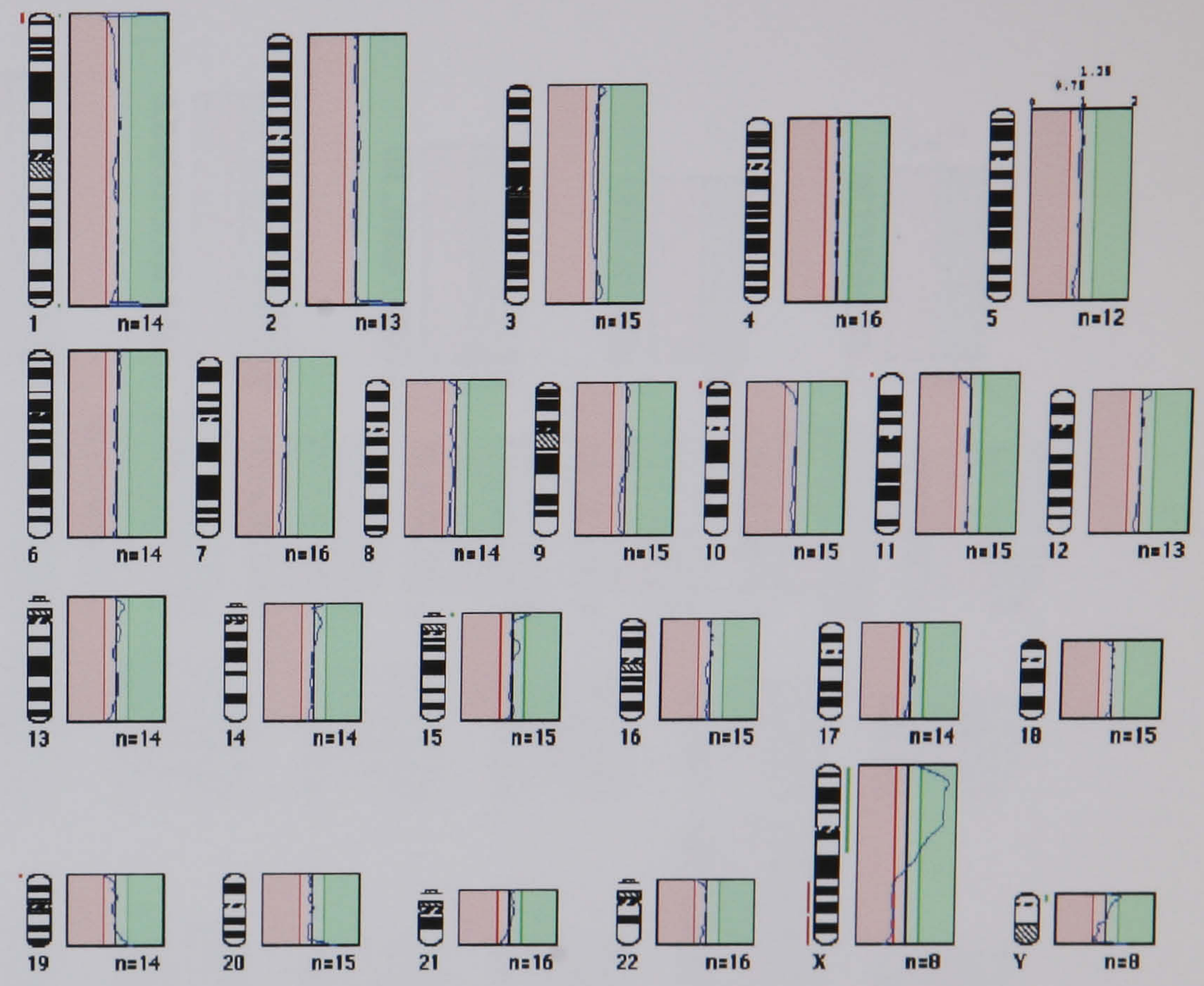
THE SPINE

Figure 3.2

[a]



[b]

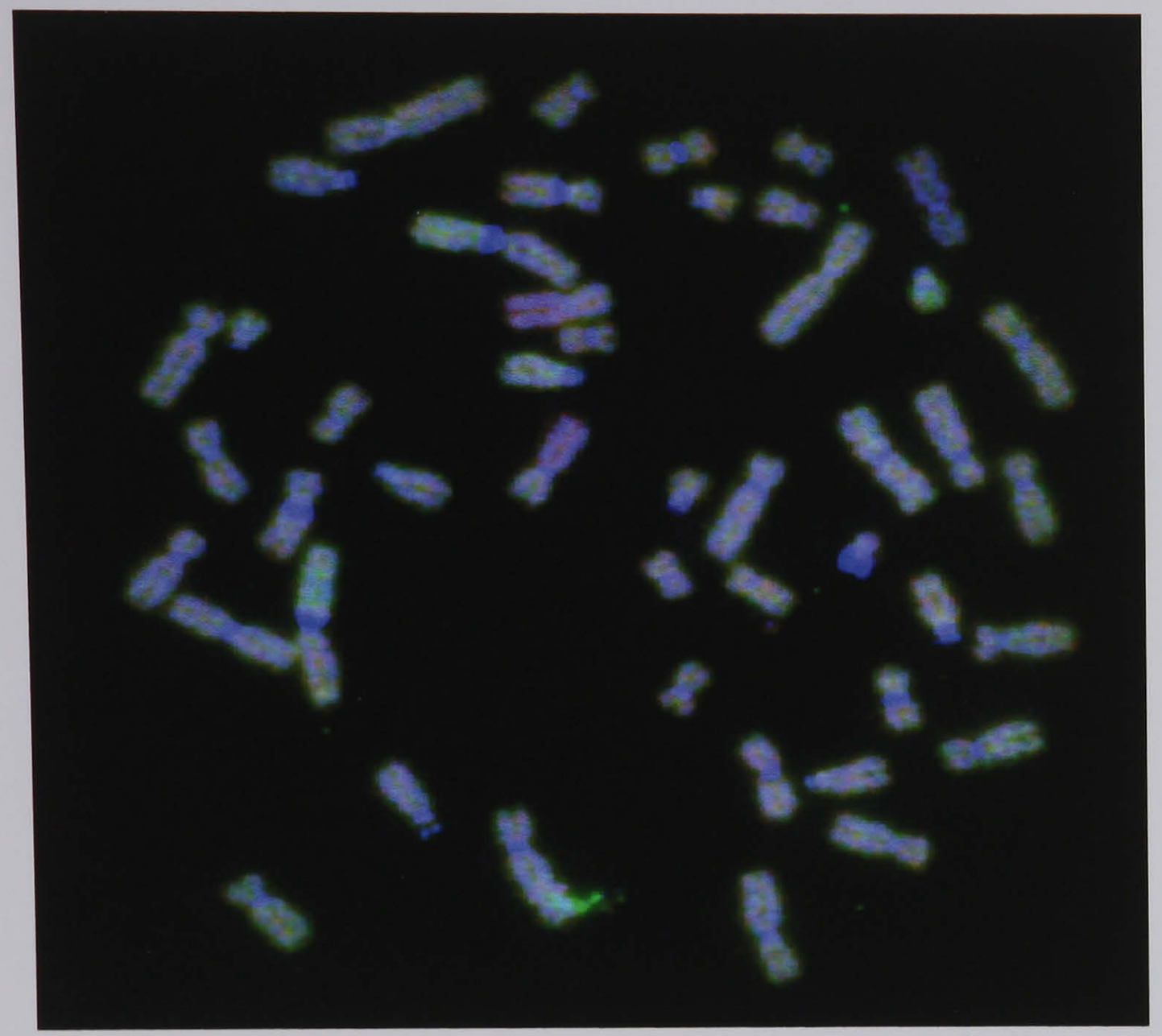


Case 01: 47,X,idic(x)(q12-13),+idic(X)(q12-13)

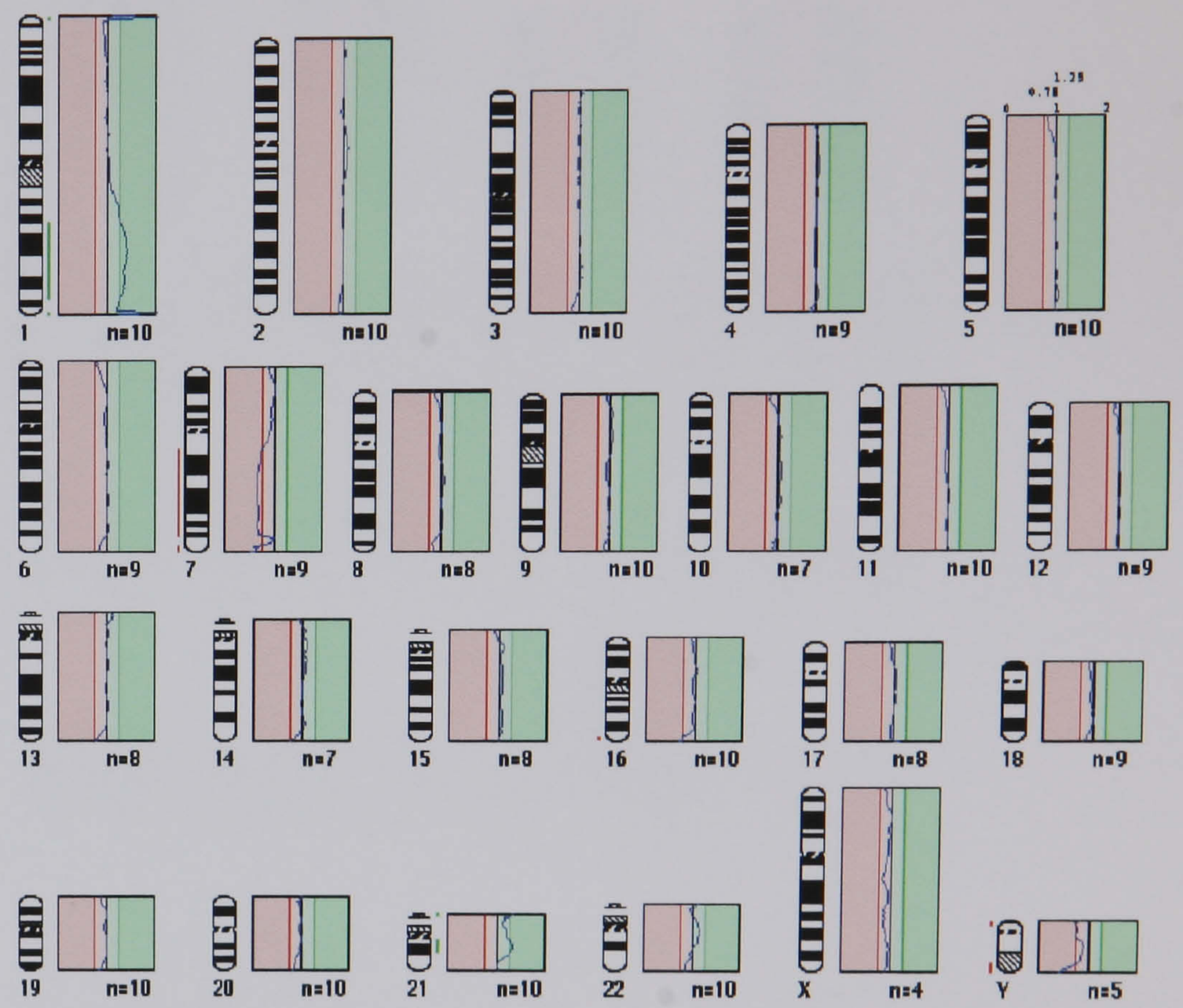
The isodicentric X chromosome with green p arm and red q arm is clearly visible in the captured metaphase (Panel a). The CGH profile (Panel b) demonstrates amplification of p arm DNA and loss of q arm DNA within the tumour, consistent with the G-banded karyotype.

Figure 3.3

[a]



[b]

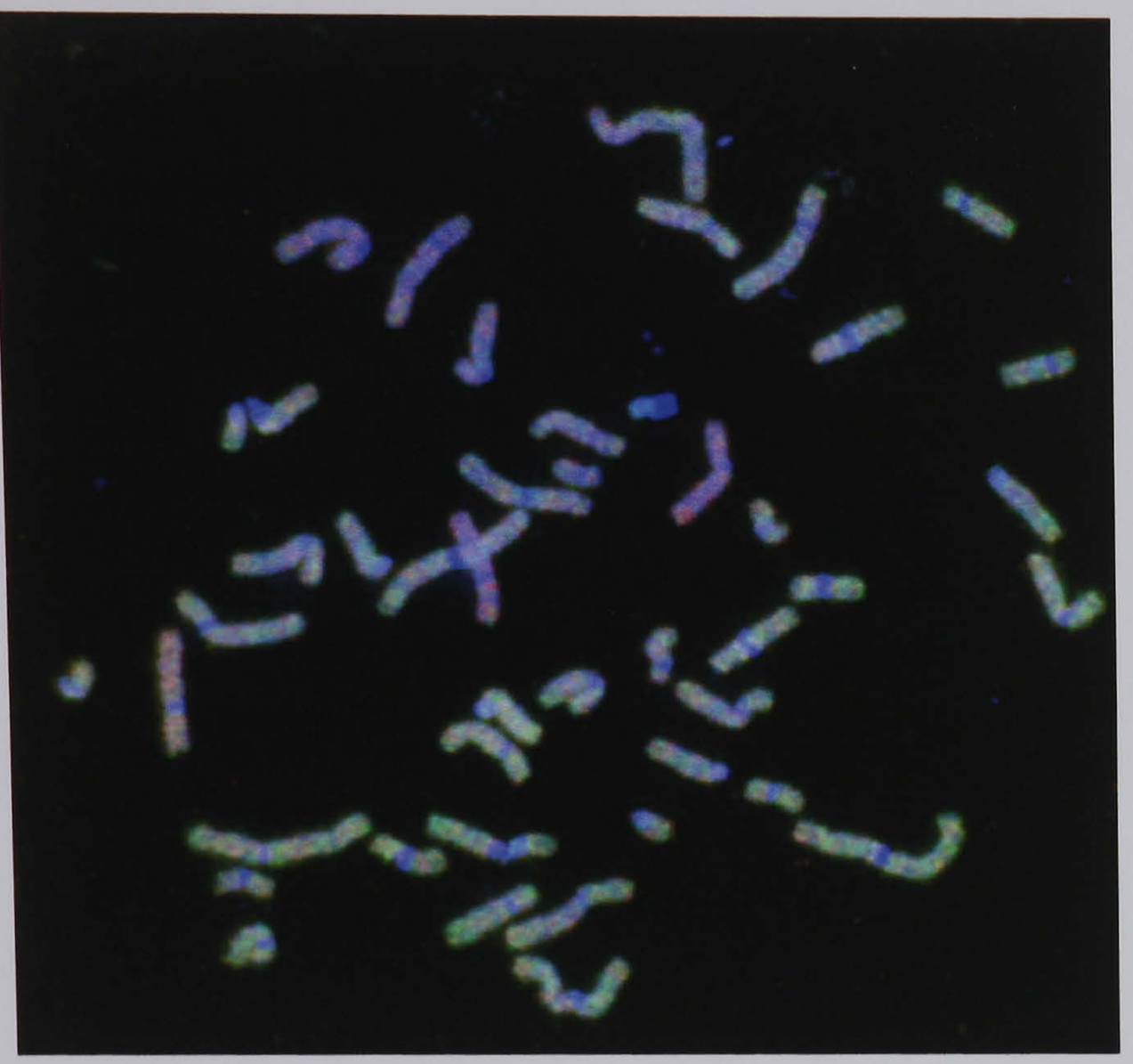


Case 02: 47,XY,+1,der(1;7)(q10;p10),inv(3)(q21q26),+21

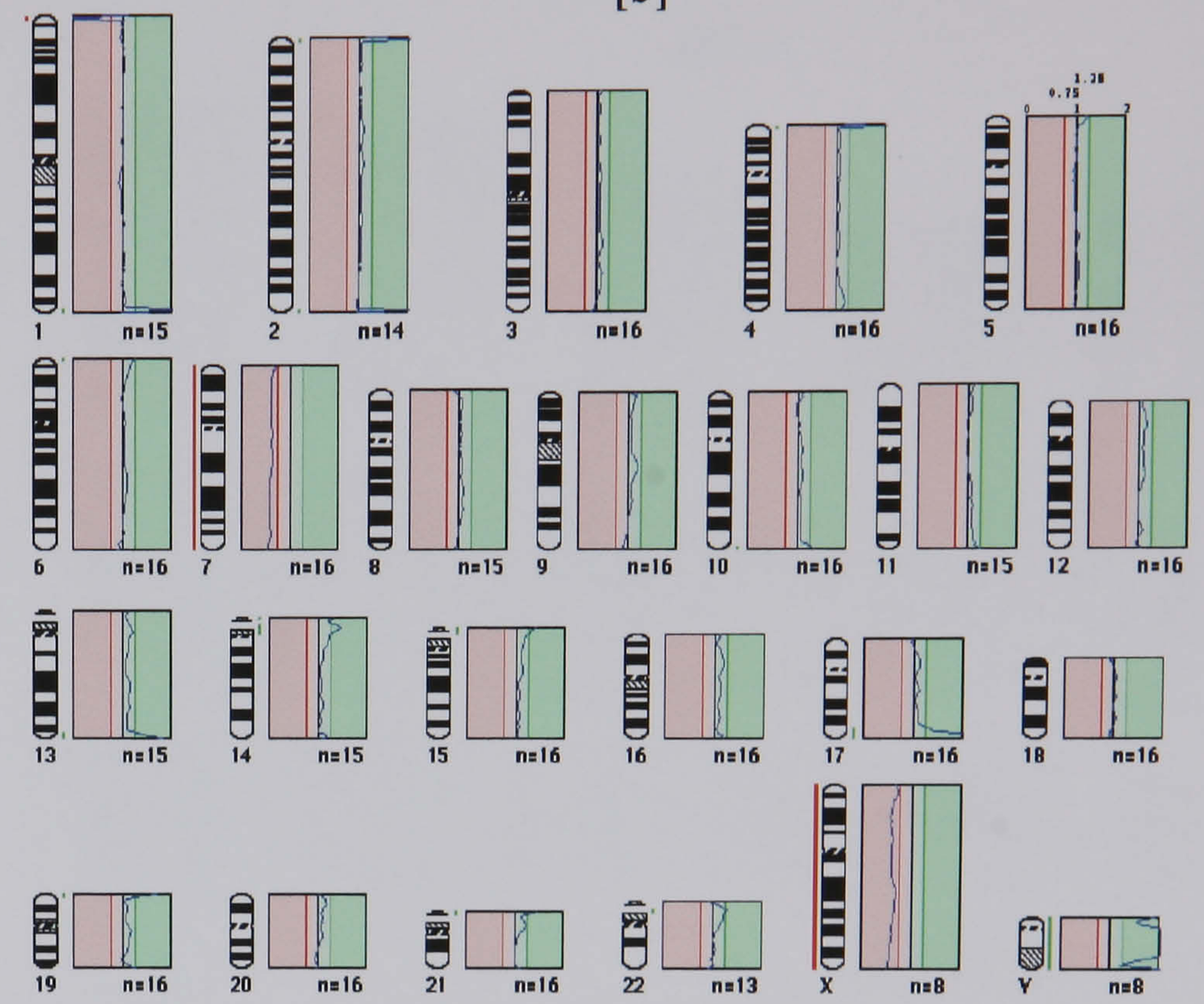
Panel a illustrates the amplified 1q region (green) and deleted 7q region (red) in a captured metaphase. The averaged CGH profile (Panel b) is consistent with the cytogenetic aberrations detected by CGH.

Figure 3.4

[a]



[b]

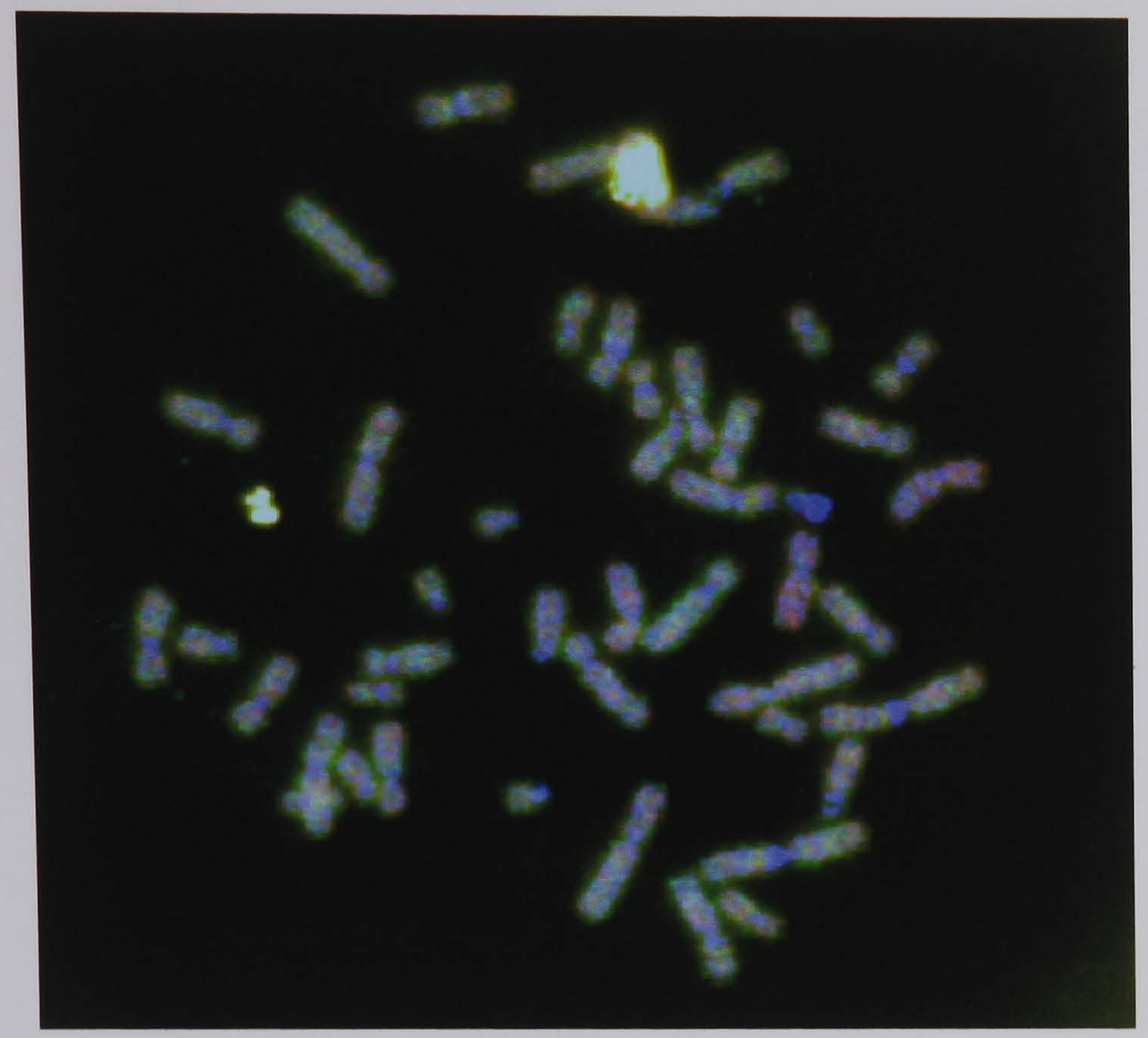


Case 05: 45,XY,-7

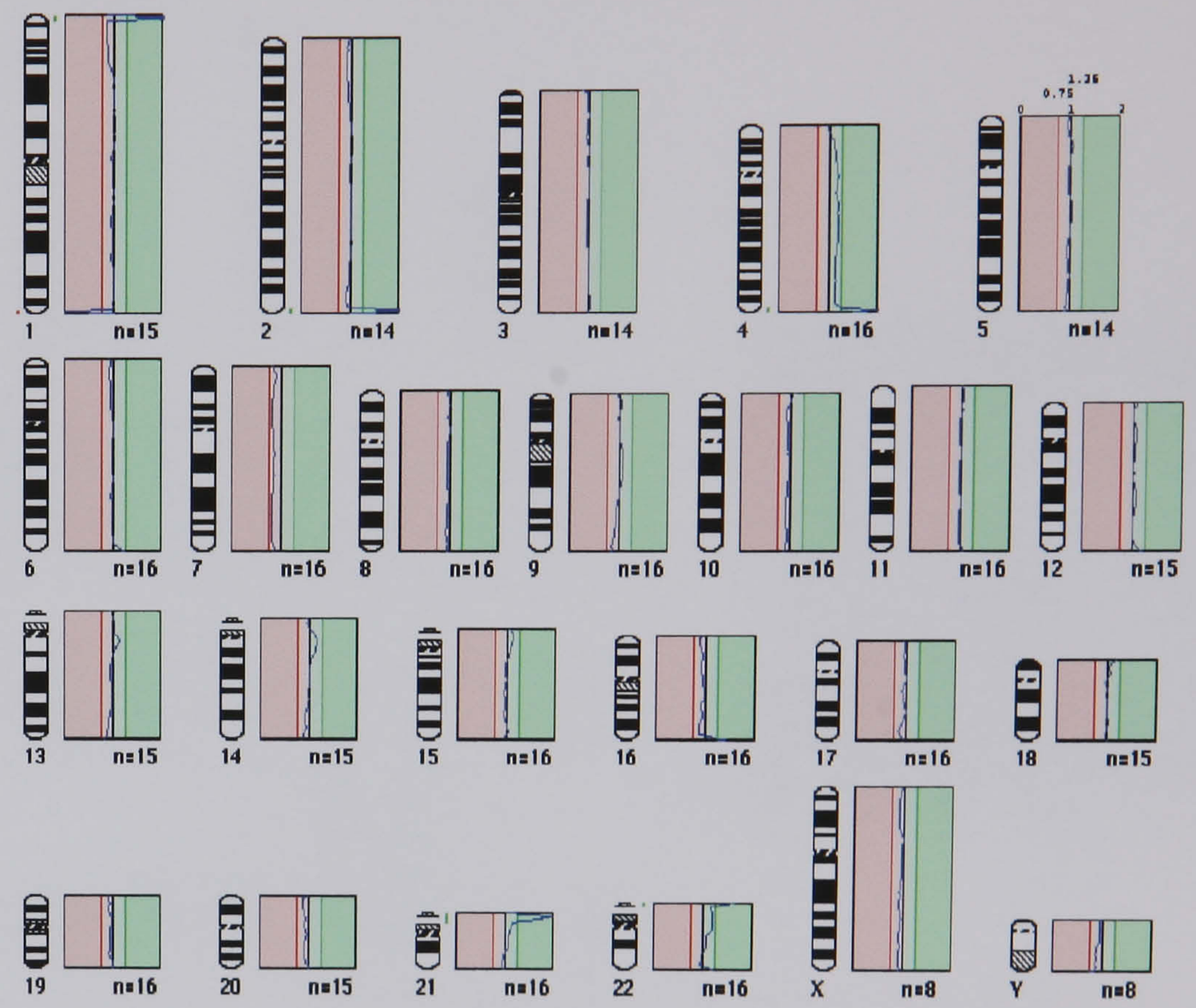
CGH was concordant with G-banded analysis in this case:45,XY,-7. The control DNA used in this experiment was female, whilst the tumour DNA was from a male patient. Thus in panel a, the chromosome 7 homologues and the X chromosome appear red. The averaged CGH ratio confirms monosomy 7, in addition the X chromosome appears deleted whilst the Y chromosome is amplified, both concordant with male and female DNA, from patient and normal control receptively.

Figure 3.5

[a]



[b]

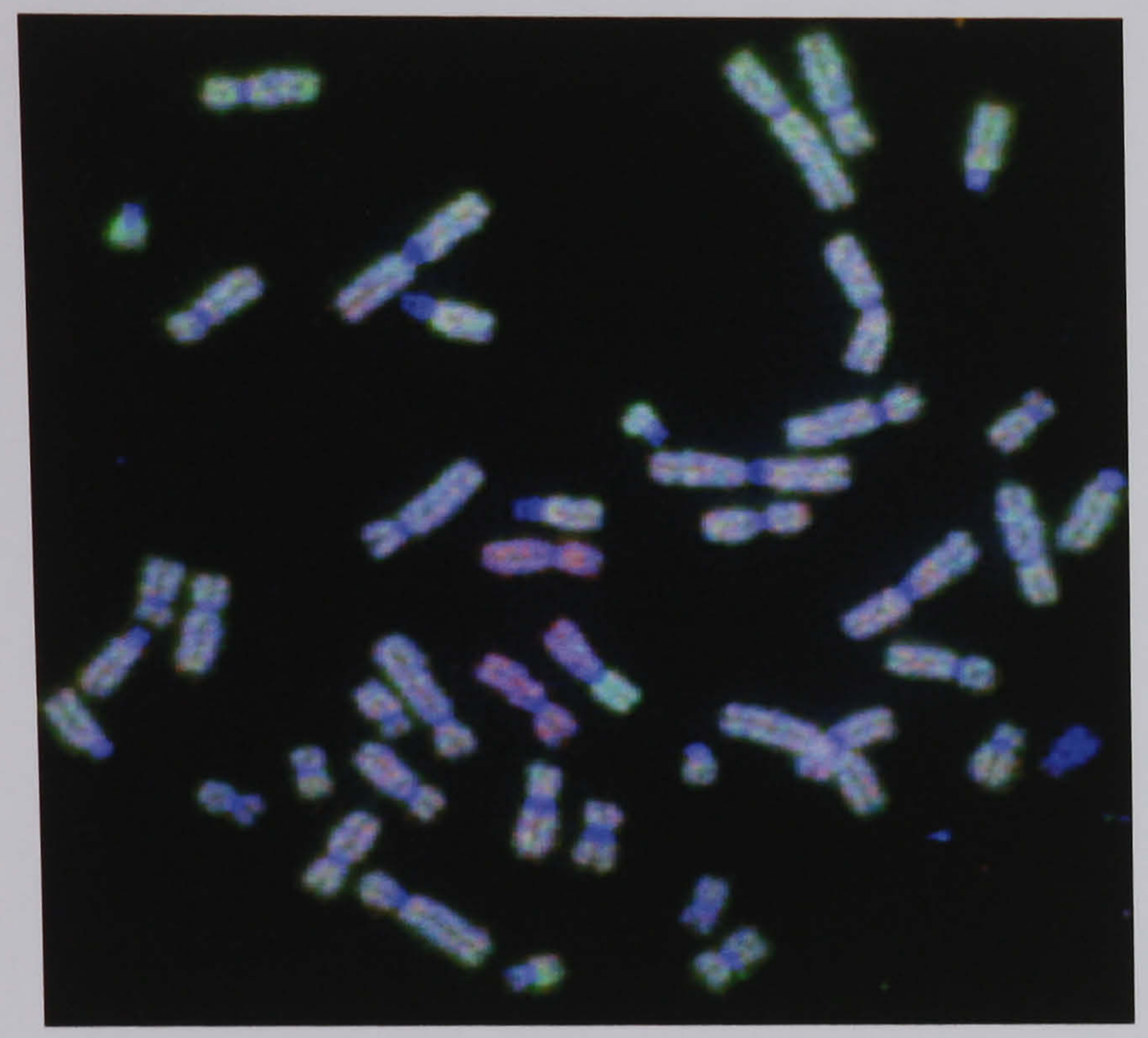


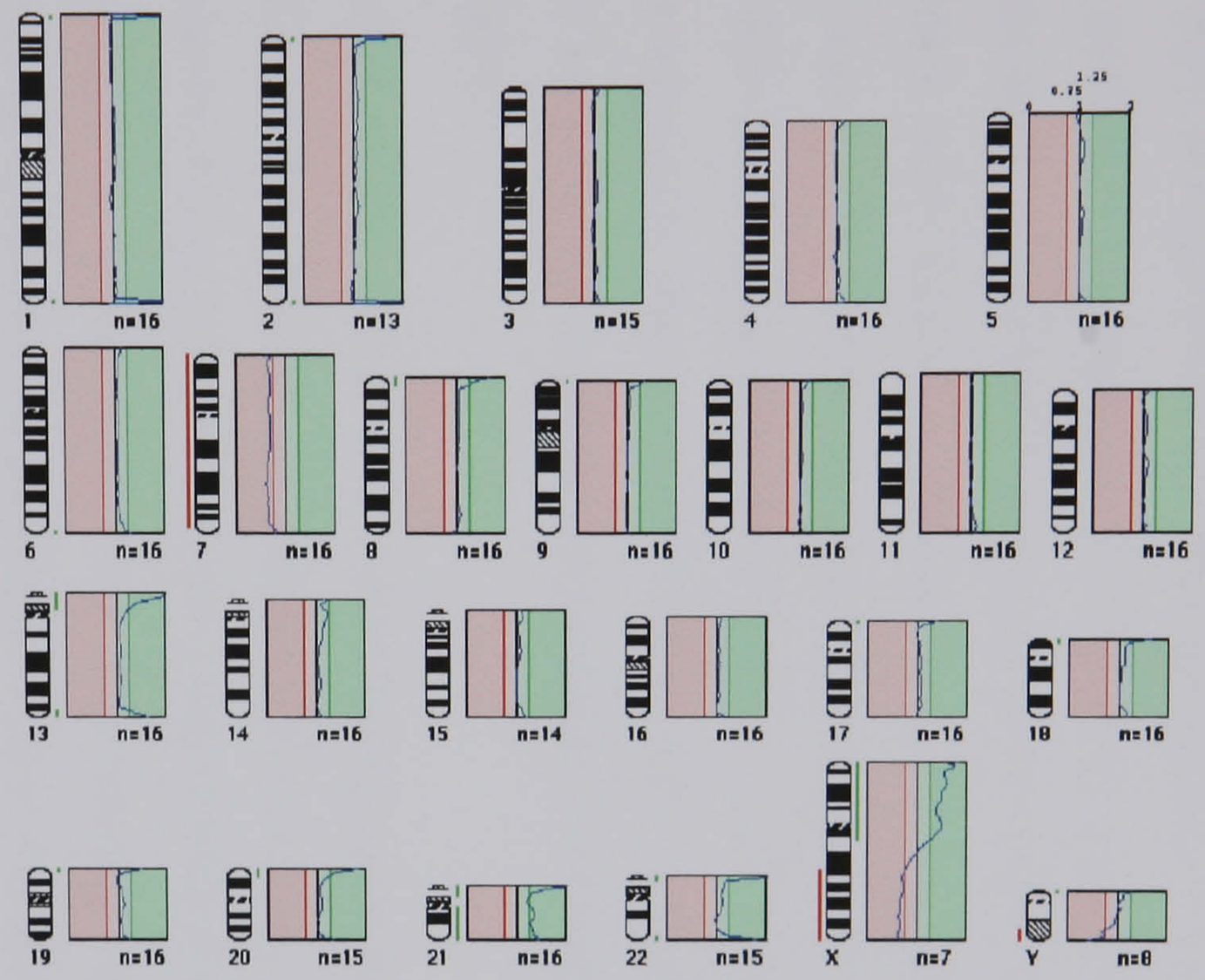
Case 06: 45,XX,-7,add(8)(q24)/48,idem,+4,+12,+22

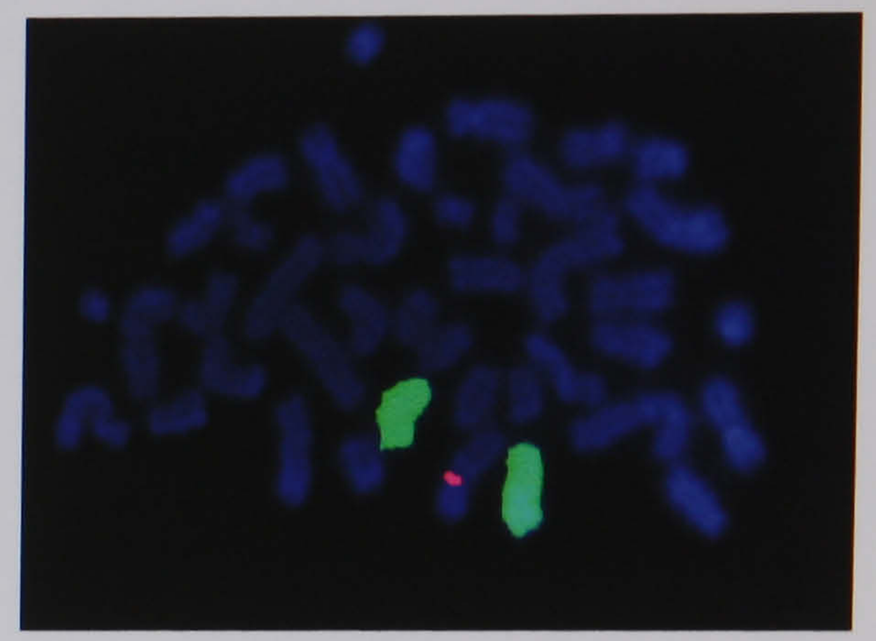
The CGH image profile of this case suggested loss of chromosome 7 and gain of chromosome 4. However, the averaged ratios were not statistically signficant for these DNA copy number changes. Ratio profiles for chromosomes 12, and 22 were also normal.

Figure 3.6

[a] [b] [c]







Case 04: 48,XX,?+X,del(7)(q22q36),+21/46,X,idic(X)(q11)/46,XX

CGH analysis was discordant with the G-banded karyotype in this case. Monosomy of chromosome 7 and an isodicentric X chromosome by CGH is shown in the captured image (Panel a). Image profile analysis (Panel b) demonstrates loss of chromosome 7, loss of Xq and gain of Xp(consistent with idic(X)). FISH analysis (Panel c) confirmed monosomy 7 [chromosome 7 centromere (red), X chromosome (green)].

Discussion

This study demonstrated that archival bone marrow mononuclear cells can be successfully used as a source of DNA for CGH analysis in AML. Furthermore, the results of this study revealed a high level of concordance between CGH profiles and conventional banding analysis in the majority of patients studied. This second finding agrees with previous reported studies in which AML was analysed by CGH (Bentz *et al.*, 1995a; Wilkens *et al.*, 1998). Bone marrow blast counts were available in 8/9 patients with normal karyotype analysed by CGH, and averaged 85% (range 45-97%), indicating that DNA extraction was derived from a highly enriched population of leukaemic blasts in most cases. Thus it can be inferred that in some elderly patients with AML, leukaemic blasts have no evidence of DNA copy number change and have a normal karyotype. Normal CGH profiles have also been reported by others in cases of haematological malignancy with normal karyotype (Huhta *et al.*, 1999; Wilkens *et al.*, 1998). Therefore this suggests that cryptic DNA copy number change may not be a frequent event in patients with haematological malignancy characterised by a normal karyotype.

The results of this study reveal other interesting aspects of CGH. Although CGH and G-banded analysis produced comparable results, the two techniques have fundamental methodological differences. Conventional cytogenetics results in the analysis of metaphase cells. However, the lineage of the haemopoietic cells undergoing mitosis may alter depending on the duration of culture (Keinanen *et al.*, 1986). By comparison, CGH results in the analysis results of metaphase and interphase nuclei, which may include non-malignant cells like fibroblasts, or normal haemopoietic bone marrow mononuclear cells. This represents an advantage of CGH, but also a limitation. In case 06, CGH was unable to detect two numerical aberrations (trisomy 12 and 22), trisomy 12 was in found in a low percentage of interphase cells by interphase FISH, (29%). However, both aberrations were detected using conventional cytogenetic analysis. Thus G-banded analysis can detect small sub-clones with a high mitotic index, whilst chromosomal aberrations present in small subclones may be missed by CGH. Initial studies in which the resolving power of CGH was determined suggested that at least 50% of the studied population must carry the imbalance, and that the chromosomal region must be at least 2Mbp if a gain of genomic material is to be

detected, and at least 10Mbp for deleted material (Kallioniemi *et al.*, 1994; Piper *et al.*, 1995). In case 06 trisomy 12 was found in 29% of interphase nuclei, which is below the level of resolution of CGH reported by Kalioniemi. However, in a recent study of thirty eight cases of haematological malignancy with cytogenetic mosaicism, genomic imbalances could still be detected if the aberration was present when at least 23% of the cells analysed by CGH (Gebhart *et al.*, 2000). Clearly the critical resolving power of CGH remains unknown but may be higher than originally thought.

Several studies have shown CGH to be superior to conventional banding cytogenetics in the detection of numerical aberrations in leukaemia (Bentz *et al.*, 1995b; Karhu *et al.*, 1997b; Larramendy *et al.*, 1998a; Rice *et al.*, 2000). In this study, the CGH profile of case 04 revealed a complete deletion of chromosome 7, whilst G-banded analysis suggested a partial deletion of 7q in 3 metaphases. FISH analysis of case 04 subsequently confirmed monosomy 7. Therefore it is likely that the del(7) was actually an idic(X) hence the revised karyotype of this clone would be: 46,X,idic(X)(q11)/45,idem,-7/46,idem,-7,+21. Thus CGH may detect certain numerical aberrations more effectively compared with conventional banding methods. However, in the cases included in this study, additive cytogenetic data was provided in only one case (04). Using MRC criteria (Grimwade *et al.*, 1998), monosomy 7 is associated with poor prognosis whilst 7q- deletions predict for standard risk. Therefore the additive information provided by CGH in case 04 would have altered the prognostic significance of the presentation karyotype in this patient.

Although CGH has the unique ability to screen the entire genome in a single hybridisation for DNA copy number change, the procedure is technically demanding. A significant number of experiments resulted in either poor hybridisation or no hybridisation at all. The factors most critical to the assay were found to be quality of genomic DNA and metaphase preparations. It was not possible to obtain CGH results from cases in which the concentration of high molecular weight DNA was low. Furthermore, although metaphase preparations selected for CGH appeared to have suitable morphology under phase contrast microscopy, in many cases the DAPI image revealed a C-banded chromosome image consistent with excessive denaturation, and resulted in poor hybridisation. Whilst in some experiments the DAPI image derived from the chromosomes was

poor resulting in incomplete karyotyping unsuccessful CGH analysis. The best results were achieved when metaphase preparations were allowed to 'age' at –20°C for 2 to 4 weeks prior to use. By doing so it was possible to denature chromosomes without incurring deterioration in chromosome architecture.

Summary

Evidence from this study confirms that CGH can be used in the successful cytogenetic study of AML, and in the elderly, produces a high level of concordance with conventional cytogenetic analysis. However, chromosomal aberrations involving small side clones may be missed. Importantly, the technique can be used to analyse cases for DNA copy number change using archival leukaemic blast cells as a source of genomic DNA.

Comparative genomic hybridisation is technically demanding, and requires expensive hardware and software investment. The results obtained in this study indicate that the routine use of CGH in the cytogenetic analysis would add little to conventional banding analysis. However, CGH may prove useful in cases of AML where banding analysis is incomplete, due to poor chromosome morphology, complex karyotype, or in circumstances where conventional analysis fails completely.

Chapter 4

Background

Conventional cytogenetic techniques detect chromosomal aberrations in the majority of patients with AML. The clinical significance of this data in elderly patients with AML was illustrated in chapter 2.

Conventional banding analysis is technically demanding, time consuming, and may be incomplete in cases with complex chromosomal rearrangements and or marker chromosomes. In the late 1980's new FISH protocols were developed which allowed the delineation of whole chromosomes and chromosomal subregions (Kearney, 1999). The increasing number of spectrally distinct fluorochromes that can be utilised for nucleic acid labelling has led to an increase in the number of DNA targets that can be visualised simultaneously. By utilising two different strategies namely combinatorial labelling and ratio labelling it is possible to identify or discriminate more DNA targets than the number of spectrally distinct fluorochromes available (Dauwerse *et al.*, 1992; Nederlof *et al.*, 1990).

Multiplex-FISH (M-FISH) is a new molecular cytogenetic technique which allows the simultaneous visualisation of each human chromosome in a specific colour in a single hybridisation (Speicher *et al.*, 1996). Structural rearrangements are denoted by a change in colour along the length of the derivative chromosome. Multiplex-FISH, and a similar technique spectral karyotyping (SKY) (Schrock *et al.*, 1996), can be used to detect simple or complex chromosomal translocations, as well as interstitial insertions. In addition, unlike conventional banding methods, M-FISH and SKY offer a high degree of automation in the analysis of chromosomal rearrangements (Eils *et al.*, 1998).

Principles of 24 Colour FISH

The M-FISH protocol relies upon the simultaneous hybridisation of specific whole chromosome DNA probes, labelled with a combination of fluorochromes, to the metaphase preparations of a sample to be analysed. Separate digital images of the metaphase preparation are captured for each fluorochrome using a CCD camera. The images are then analysed using dedicated software, which assigns a pseudo-colour to each chromosome, dictated by the fluorochrome composition of the DNA probes (Figure 4.1).

In order to produce a probe mixture capable of identifying the 22 autosomes and 2 sex chromosomes within the human genome, specific DNA probes for each chromosome (generated by micro-dissection or flow cytometry, and PCR amplification) are labelled by nick translation, in a combinatorial manner. The minimum number of fluorochromes required to achieve this is defined by the equation $C=2^N-1$, where C is the number of useful combinations, and N is the number of fluorochromes, thus when N=5, C=31.

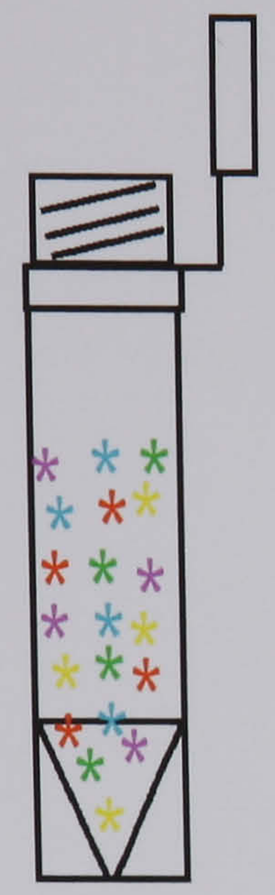
Spectral karyotyping like M-FISH utilises a mixture of whole chromosome DNA probes, labelled in a combinatorial manner, with fluorochromes to 'paint' metaphase preparations. The image is then captured and an interferometer is used to determine the spectrum of fluorescence wavelength for each pixel of the CCD chip. Identification of the components of this spectrum is achieved by dedicated software which applies an algorithm (Fourier transformation) in order to produce a pseudo-colour for each chromosome, resulting in a spectral karyotype (Schrock *et al.*, 1996).

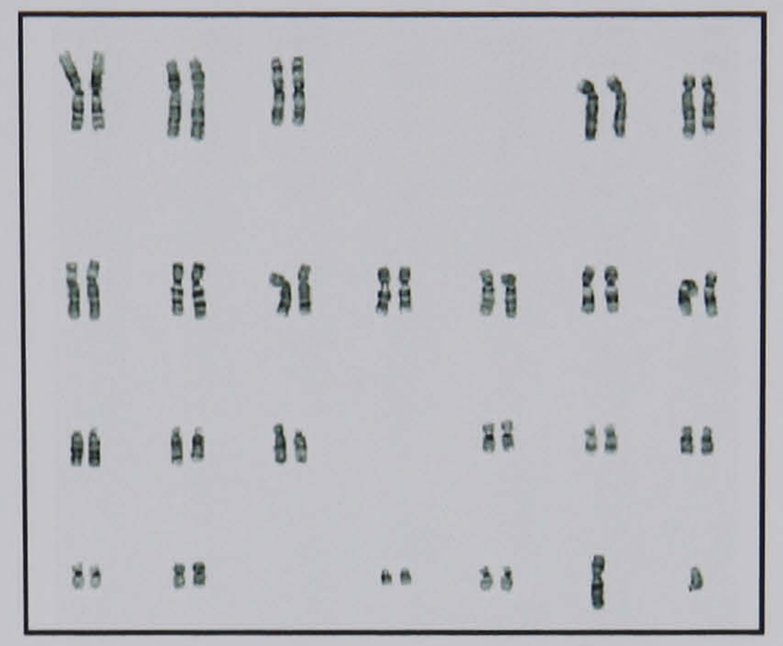
The two methodologies have there own distinct advantages and disadvantages. Multiplex-FISH requires a series of image acquisitions for each fluorochrome. Thus although exposure times can be altered to compensate for weak fluorochrome signal, the final pseudo-colour image may be poor if any one fluorochrome signal is below threshold. Although SKY requires a single image acquisition, the exposure times are longer than in M-FISH, which may result in fluorochrome bleaching and an impaired pseudo-colour image (Lichter *et al.*, 1996).

[a]

[b]

[c]





M-FISH probe

Probe applied to metaphases

Pseudo-coloured image

Figure 4.1

M-FISH probe (Panel a) consists of a 52 probe mixture of whole chromosome paint DNA probes that cover the 24 human chromosome homologues. The probes are directly labeled with 5 different fluorophores in a combinatorial manner. The M-FISH probe is allowed to hybridise with human metaphase preparations (Panel b). After hybridisation chromosome homologues are identified by a distinct pseudo-colour using imaging analysis software (Panel c).

Multiplex-FISH and SKY studies in Haematological Malignancy

Since the introduction of M-FISH and SKY in 1996 there have been a limited number of studies in which these techniques have been employed in the cytogenetic analysis of haematological malignancy (Table 4-1). The largest series have been reported separately in patients with acute leukaemia and multiple myeloma. M-FISH was used to analyse 15 cases of haematological malignancy including 3 cases of AML and was able to identify marker chromosomes, and detect a number of cryptic translocations missed by G-banded analysis (Veldman *et al.*, 1997). When SKY was used to analyse 32 cases of AML additive information was obtained in several cases with abnormal karyotype, but all patients with normal karyotype by G-banded analysis had normal karyotypes using SKY (Mohr *et al.*, 2000). However, in a similar series 2/28 cases of AML with normal karyotype analysed using SKY were found to have cryptic aberrations (Zhang *et al.*, 2000).

In order to determine whether cryptic translocations were present in T-cell ALL analogous to the t(12;21) in childhood B-ALL, SKY was utilised to analyse 15 cases of T-ALL (Rowley *et al.*, 1999). In 3 cases with complex karyotype, SKY was able to clarify the karyotypic aberrations by identifying unknown material present on a series of chromosomes. Concordance with conventional karyotype was noted in 11 other cases, whilst in the remaining case SKY was unable to detect a translocation present in a small clone of cells. When 24-colour FISH was used to analyse metaphase preparations from patients with multiple myeloma marker chromosomes and cryptic translocations were identified (Rao *et al.*, 1998).

The studies cited above clearly illustrate that M-FISH and SKY technologies assays can provide additive cytogenetic data in cases of haematological malignancy, including cases with normal karyotype.

Table 4-1 Multicolour-FISH studies in haematological malignancy

Study	Disease	Assay	Comments
Veldman 1997	AML, CML, MDS ALL, NHL	SKY	marker chromosomes identified cryptic translocations identified
Rao 1998	Myeloma, Myeloma cell lines	SKY	marker chromosomes identified cryptic translocations identified
Sawyer 1998	Myeloma	SKY	156 aberrations resolved by
Rowley 1999	T-ALL	SKY	karyotypes clarified in 3 cases missed translocation in 1 case
Tosi 1999	AML GF-D8 cell line	M-FISH	M-FISH detected der(7)t(7;15)
Gribble 2000	CML K562 cell line	M-FISH / CGH	marker chromosome identified, cryptic t(12;21) identified, del(20q) identified
Harrison 2000	CML(blast crisis)	M-FISH	concordant with G-banded analysis
Mohr 2000	AML/MDS	SKY	SKY detected cryptic aberrations in 2 cases
Wan 2000	T-lymphoblastic NHL	SKY	cryptic translocation identified in a case with complex karyotype
Zhang 2000	AML	SKY	SKY detectedcryptic aberrations in 2/28 cases

Aims of this Study

Acquired cytogenetic aberrations are detected in the majority of patients with AML using conventional banding techniques. Analysis of the presentation karyotype data from elderly patients with AML in chapter 2 revealed that 56% had abnormal karyotype. The majority of these patients, together with patients with normal karyotype were characterised by a clinical outcome intermediate between patients with 'favourable' and 'unfavourable' karyotype. Important cryptic chromosomal rearrangements may go undetected when using conventional banding techniques due to sub-optimal morphology, reduced mitotic index or due to the exchange of chromosomal material of similar contrast. In addition, whilst CGH can detect DNA copy number change, translocations go undetected.

It is clear that cryptic aberrations of prognostic importance in cases of acute leukaemia with normal karyotype, as illustrated by the 12;21 translocation in childhood leukaemia (Shurtleff *et al.*, 1995). The aim of this study is to determine whether M-FISH can detect cryptic chromosomal rearrangements in elderly patients with AML with wither normal or abnormal karyotype, and to compare the cytogenetic data provided by M-FISH with the G-banded karyotype at the time of presentation, and determine the prognostic significance of any additive data provided by the technique.

Patients and Methods

Patients material

Eighteen patients with AML (aged>60 years old) referred to the ICRF Department of Medical Oncology, St Bartholomew's Hospital and successfully karyotyped, with archival material suitable for analysis were selected for the study. Of these cases, five were also included in the previous CGH study (01, 03, 05, 06, 13). The clinical and laboratory data of the patients are listed in Table 4-2. Conventional cytogenetic analysis was performed as previously described at the time of diagnosis.

Table 4-2
Clinical and laboratory details of AML patients included in the M-FISH study

Case	Age	Year of diagnosis	Sex	FAB	BM blast (%)	Karyotype
01	71	1998	F	M1	54	A
03	60	1998	M	M4	46	A
05	71	1995	M	M1	70	A
06	60	1998	F	M2	54	A
13	68	1997	F	M4	90	N
16	74	1999	M	M2	89	A
17	79	1999	F	M1	71	A
18	74	1999	F	M2	63	A
19	60	1999	M	M2	41	A
20	77	1999	M	M5	80	A
21	74	1999	F	M4	90	N
22	68	1999	M	M2	30	N
23	78	2000	F	M4	65	N
24	71	1996	M	M6	35	N
25	64	1999	F	M5	60	N
26	60	1999	M	M2	62	N
27	76	1997	M	M2	68	N
28	73	1999	M	M1	89	A

Key A= abnormal karyotype
N= normal karyotype

Multiplex Fluorescence *in-situ* Hybridisation

Metaphase slide preparation

Metaphase preparations from patients, stored at –20°C in fixative at the time of diagnosis were retrieved and resuspended in fresh fixative. Metaphase slides were then prepared as previously described. Slides with minimal cytoplasm were selected and air dried at 70°C for 2 hours, and then left overnight at room temperature before use.

Probe preparation

Metaphase slides were denatured at 73°C for 2 minutes in 70% formamide/2xSSC dehydrated with ethanol (70%, 95%, and 100%) for 1 minute each and left to dry in air at room temperature. At the same time, 5µl of Spectra Vysion probe (Vysis, UK) was denatured for 5 minutes then applied to the denatured metaphase slide preparation. Hybridisation was performed at 37°C for 18 hours in a humidified light proof box. Slides were then washed by removing the coverslip and agitating in 0.4xSSC/0.3% NP40 (pH 7.0) at 74°C for 2 minutes and 2xSSC/0.1% NP40 (pH 7.0) for 30 seconds at room temperature. After drying, slides were mounted in 10µl DAPI III (42ng/ml) (Vysis UK) under (22x33mm) coverslip.

Image Acquisition

Metaphase images were captured using an Olympus AX70 fluorescence microscope (Olympus, UK) fitted with an M-FISH filter configuration (Vysis, UK), CCD camera. Image analysis was performed using Perceptive Scientific Instrument M-FISH^{TM} software on an Apple Macintosh Power Mac computer. Five to ten metaphase cells were analysed for each patient, and aberrations were classified as clonal when detected in two or more metaphase cells (Mitelman, 1995).

Fluorescence in situ hybridisation (FISH)

Conventional FISH analysis was performed in cases where there was a discrepancy between banding analysis and M-FISH karyotype. For the evaluation of cryptic translocations whole chromosome paints for chromosomes 3, 5, 9, 11, 12, 13, 20, 21 and centromeric probes for chromosome 8 and 17 were used (Oncor, Gaitersburg USA). Hybridisation and post hybridisation washes were carried out according to the recommendations of the supplier. Fluorescence

detection was carried out using a Leica DMXRA microscope fitted with appropriate filter sets, images were captured using digital image analysis software (Perceptive Scientific Instruments Inc, UK).

Results

Metaphase preparations from 18 patients (9 with abnormal karyotypes) were analysed by M-FISH, five of these patients were also analysed by CGH (01, 03, 05, 06, and 13). A summary of the M-FISH results together with conventional cytogenetic data is shown in Table 4-3. The G-banded analysis of case 06 showed additional material on chromosome 8. M-FISH analysis identified the origin of the extra material as being derived from chromosome 3. In addition, M-FISH suggested the presence of a cryptic t(3;11) translocation in 3/7 cells analysed. FISH analysis with whole chromosome paints for chromosomes 3, 8, and 11, confirmed the presence of the t(3;8) translocation, but the t(3;11) translocation could not be demonstrated (Figure 4.2).

G-banded analysis demonstrated a balanced t(12;17)(p12;p13) translocation in case 03. However, M-FISH failed to demonstrate this telomeric rearrangement in the metaphase preparations captured. FISH analysis (on metaphase chromosomes from the same sample) using probes for chromosomes 12 and 17 clearly demonstrated the presence of this translocation (Figure 4.3).

A complex pattern of chromosomal abnormalities was noted in case 028 by G-banded analysis, which included a marker chromosome, and additional material of unknown origin on chromosomes 9, 16 and 21. M-FISH revealed a rearrangement between chromosome 5, 9 and 21. There was also an abnormal chromosome 13 containing a homogenously staining region (hsr) derived from chromosome 11. This hsr was translocated to chromosome 16 in a sideline clone. FISH analysis using whole chromosome paints for chromosome 5, 9, 13, 17 and 21 confirmed these rearrangements (Figure 4.4).

M-FISH karyotypes were concordant with the G-banded analysis in the remaining 16 patients, M-FISH revealed a shortened chromosome 7 in case 16

Table 4-3 Summary of tthe cytogenetic results from elderly patients with AML analysed by M-FISH

Case	Conventional Karyotype	M-FISH karyotype	Comments
01	47,X,idic(X)(q12-3), +idic(X)(q12-3)/46,XX	47,X,idic(X)(q12-3), +idic(X)(q12-3)/46,XX	M-FISH confirmed karyotype
03	46,XY,t(12;17)(p12;p13)	46,XY	t(12;17) not seen with M-FISH t(12;17) confirmed by WCP^{+}
05	45,XY,-7	45,XY,-7	M-FISH confirmed karyotype
06	45,XX,-7,add(8)(q24)/ 48,idem,+4,+12,+22	45,XX,-7der(8)t(3;8)/ 48,idem,+4,+12,+22	M-FISH clarified karyotype, additional material on chromosome 8 derived from chromosome 3 confirmed by WCP^{+}
13	46,XX	46,XX	M-FISH confirmed karyotype
16	46,XY,i(7)(p10)/46,XY	46,XY,i(7)/46,XY	M-FISH confirmed karyotype shortened chromosome 7 seen
17	46,XX,-7,+8	46,XX,-7,+8/46,XX	M-FISH confirmed karyotype
18	47,XX,+8/46,XX	47,XX,+8/46,XX	M-FISH confirmed karyotype
19	46,XY,der(5)del(5)(q13q31) t(5;9)(q31;q34)/49,idem,+3,+8,+18	46,XY,der(5)del(5)(q13q31) t(5;9)(q31;q34)/49,idem,+3,+8,+18	M-FISH confirmed karyotype
20	47,XY,+8	47,XY,+8	M-FISH confirmed karyotype

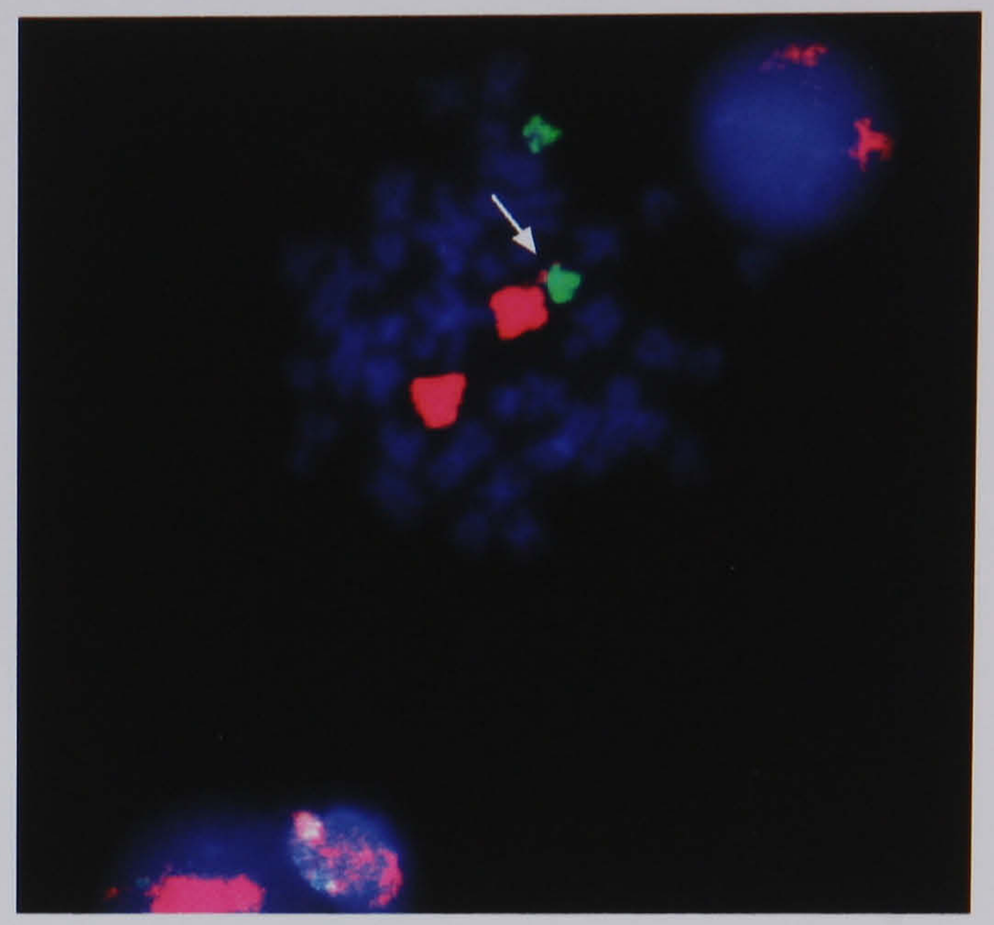
21	46,XX	46,XX	M-FISH confirmed karyotype
22	46,XY	46,XY	M-FISH confirmed karyotype
23	46,XX	46,XX	M-FISH confirmed karyotype
24	46,XY	46,XY	M-FISH confirmed karyotype
25	46,XX	46,XX	M-FISH confirmed karyotype
26	46,XY	46,XY	M-FISH confirmed karyotype
27	46,XY	46,XY	M-FISH confirmed karyotype
28	45,XY,-5,add(9)(p13),del(11)(q23q25), der(15;17)(q10;q10),add(16)(q22), del(20)(q11.2q13.3),add(21)(q22),+mar/ 45,idem,add(8)(q24)/45,XY,-5,add(9)(p13), del(11)(q23q25),-13,der(15;17)(q10;q10), del(20)(q11.2q13.3),add(21)(q22), +der(?)t(13;?)(q12;?),+mar	See text	M-FISH clarified karyotype

Key WCP†= Whole chromosome paints

Figure 4.2

[a]

[b]

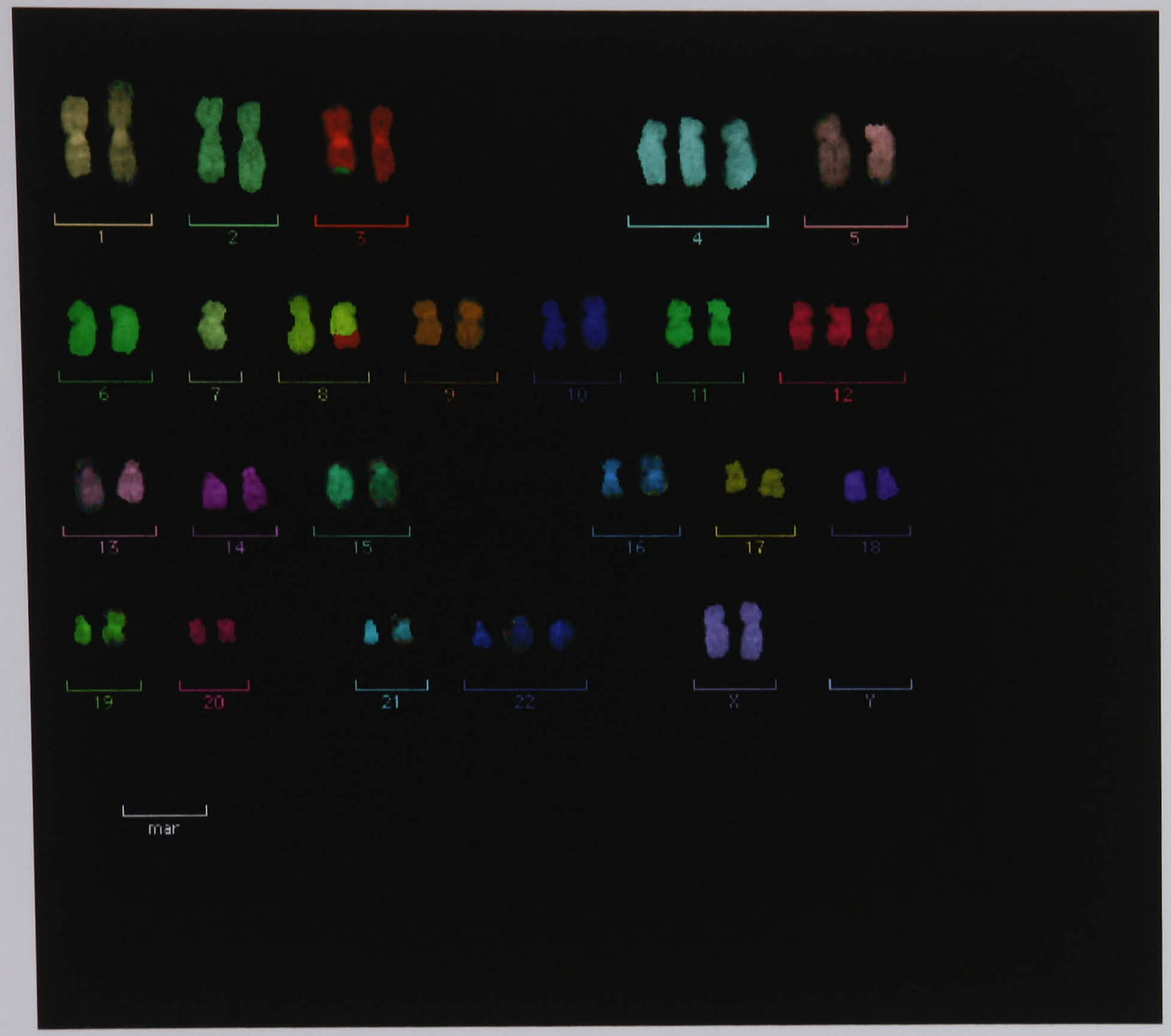


Case 03: 46,XY,t(12;17)(p12;p13)

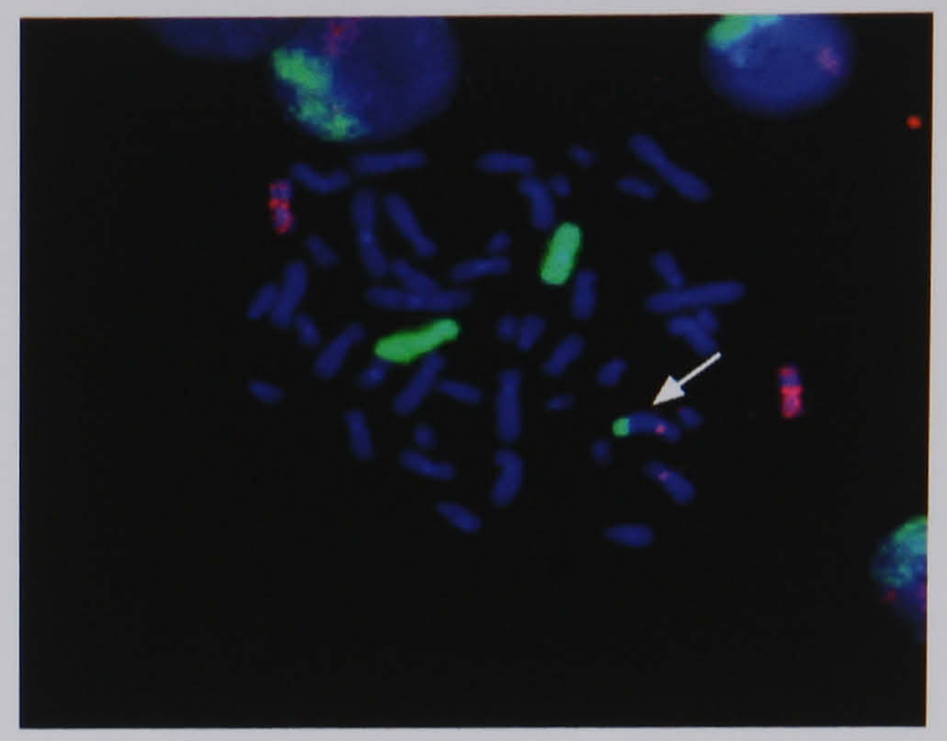
M-FISH analysis of this case revealed a normal karyotype (Panel a). FISH analysis using whole chromosome paints for chromosomes 12 (red) and chromosome 17 (green) confirmed the presence of a telomeric t(12;17) translocation (white arrow) detected by G-banding analysis at the time of diagnosis.

Figure 4.3

[a]



[b]

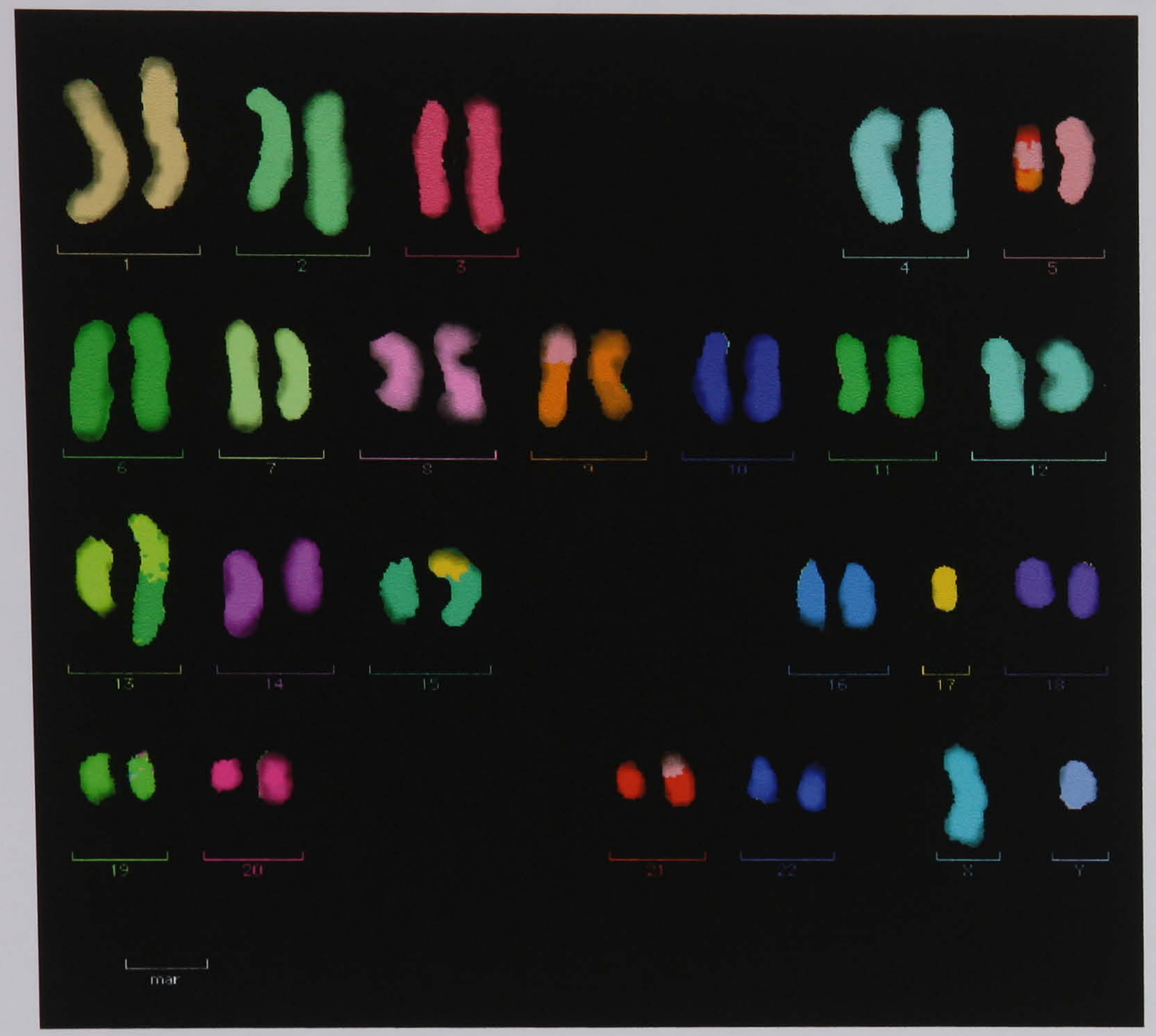


Case 06:45,XX,-7,add(8)(q24)/ 48,idem,+4,+12,+22

Additional material on chromosome 8 was derived from chromosome 3 (Panel a). M-FISH analysis also suggested the presence of a cryptic t(3;11) translocation. FISH analysis was performed (Panel b), using centromeric probe for chromosome 8 [red] and whole chromosome paints for chromosomes 3 [green] and 11 [red]). The t(3;8) translocationcan be clearly seen (white arrow). The t(3;11) tranlocation was not confirmed by FISH.

Figure 4.4

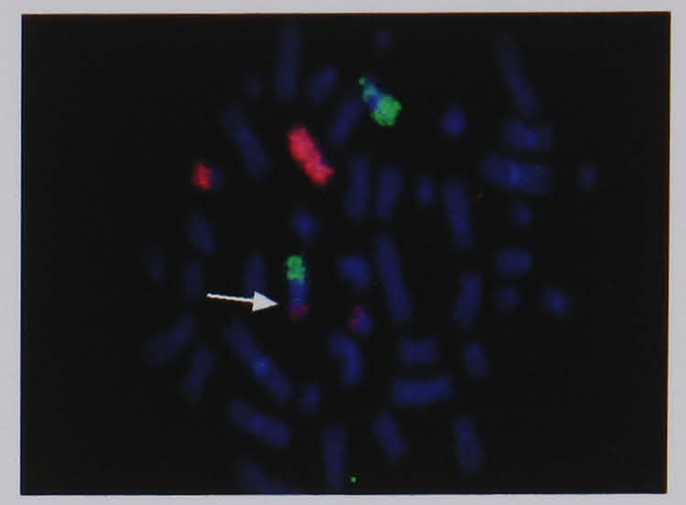
[a]



Case 028: 45,XY,-5,add(9)(p13),del(11)(q23q25),-13,der(15;17)(q10;q10), del(20)(q11.2q13.3),add(21)(q22),+der(?)t(13;?)(q12;?),+mar/45,XY,-5, add(9)(p13),del(11)(q23q25),der(15;17)(q10;q10),add(16)(q22), del(20)(q11.2q13.3),add(21)(q22),+mar/45,XY,-5,add(8)(q24),add(9)(p13), del(11)(q23q25),der(15;17)(q10;q10),add(16)(q22),del(20)(q11.2q13.3), add(21)(q22),+mar

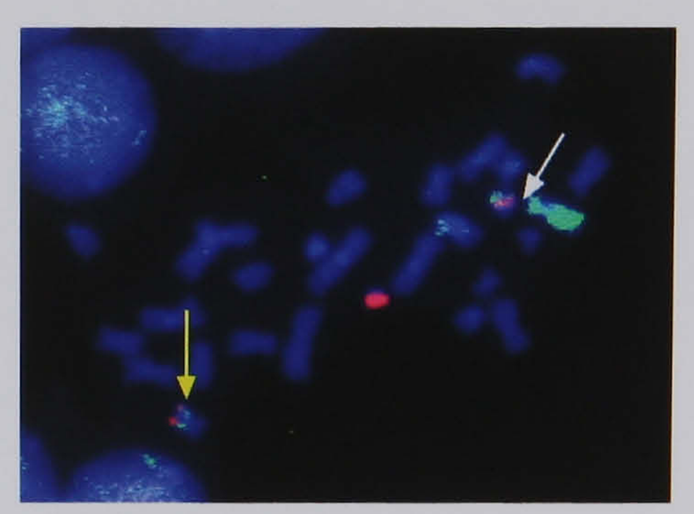
M-FISH analysis of this case revealed a der(9)t(5;9) rearrangement and a second translocationaffecting thesame chromosome 5 and chromosome 21. In addition a t(11;13)translocation was uncovered. FISH analysis confirmed the rearrangements.

[b]



FISH analysis (chromosome 5-red, chromosome 9 green). The white arrow indicates the der(9)t(5;9) rearrangement.

[c]



FISH analysis (chromosome 5 green, chromosome 21 red).The white arrow indicates the der(21) t(5;21) translocation.The yellow arrow indicates the presence of the der(5)t(5;9)t(5;21) rearrangement.

consistent with i(7)(p10), and demonstrated an abnormal X chromosome consistent with idic(X) in case 01. Metaphase cells from nine patients with a normal karyotype had a normal karyotype when analysed using M-FISH. An average of 6 cells per patient was analysed in these cases. Despite this relatively small number, the bone marrow samples from the majority of these patients contained more than 60% blasts. Thus the leukaemic blasts of some patients may have a normal karyotype.

Discussion

M-FISH is an important FISH based molecular cytogenetic technique which makes the identification of marker chromosomes, and the complete analysis of complex karyotypes in haematological malignancy possible (Tosi *et al.*, 1999; Veldman *et al.*, 1997). In this study M-FISH clarified the chromosomal abnormalities of two patients with complex karyotype. In both cases, standard G-banded analysis identified chromosomes that had additional unidentified material. M-FISH was able to identify the origin of this material as well as the origin of a homogenously staining region in one case. In one other case a balanced telomeric translocation identified by G-banded was not detected by M-FISH, but was detected with FISH using centromeric probes. M-FISH was concordant with G-banded analysis in 7 cases that had numerical aberrations, and or balanced translocation. Importantly, no recurring cryptic aberrations were found in patients with normal or abnormal karyotype.

The ability of M-FISH to detect chromosomal translocations is dependent upon the location of the chromosomal aberration, quality of the metaphase preparations, and the size of the chromosomal rearrangement. Multiplex-FISH analysis of case 03 clearly illustrated that M-FISH was unable to detect the presence of a telomeric rearrangement. Similarly, subtelomeric translocations in multiple myeloma involving segment 14q32 were too small to be resolved completely by SKY (Sawyer *et al.*, 1998). The inability to consistently detect subtelomeric rearrangements using M-FISH or SKY may be improved by increasing the relative proportion of telomeric/subtelomeric DNA within the probe mixture (Kearney, 1999; Zhang *et al.*, 2000). Likewise, by spiking the probe

mixture with band probes spanning known translocation breakpoints, may allow M-FISH to target specific chromosomal breakpoints associated with AML or other haematological malignancies (Speicher *et al.*, 1996).

High quality metaphase preparations are essential for accurate M-FISH analysis. Metaphase preparations in this study were prepared from archival material stored in fixative at the time of diagnosis. Although successful M-FISH analysis was possible using this material as a source, it also resulted in some technical difficulties. For example, the chromosomes of a number of metaphase preparations from samples more than 1 year old were frequently of poor quality, or the metaphase preparations themselves were scanty or had abundant cytoplasm. This resulted in suboptimal analysis in a number of cases, and made it impossible to analyse the metaphase preparations from several patients included in the CGH study. In the cases in which M-FISH and CGH were both utilised, M-FISH identified the numerical aberrations detected by CGH, as well the origin of chromosomal material involved in a translocation (case 06). Thus although M-FISH and CGH have their own distinct advantages pertaining to cytogenetic analysis, it can be argued that M-FISH provided a more comprehensive assessment of presentation karyotype than CGH.

As outlined in the previous chapters, cytogenetic data provided at the time of diagnosis may influence the management of young and middle aged AML patients by virtue of its prognostic power (Bloomfield *et al.*, 1984; Grimwade *et al.*, 1998). Likewise, karyotype at the time of diagnosis in patients 60 years or older also carries prognostic significance, predicting for complete remission and overall survival (Dalley *et al.*, 1999; Leith *et al.*, 1997). Published data indicate that M-FISH has the ability to detect chromosomal rearrangements with a resolution between 500kb to 1.0Mb whereas banding techniques have a resolution equivalent to the size of a single band (5-10Mb). Despite this apparent superiority, M-FISH analysis failed to provide additive information in the majority of patients included in this study. Similar findings were noted when SKY was used to analyse patients with T-ALL and AML, suggesting that some blast cells in acute leukaemia may have a normal karyotype (Mohr *et al.*, 2000; Rowley *et al.*, 1999). However, in a recent study in which SKY was used to analyse 28 cases of AML with normal karyotype, 2 cases with normal karyotype were found to have cryptic aberrations (Zhang *et al.*, 2000). The disparity between

these results may reflect sample size, as only 8 patients with normal karyotype were included in this study and an average of 5-10 cells per patient were analysed, whilst Zhang et al analysed 20 cells per patient. In addition the possibility remains that M-FISH may have missed abnormal clones in the patients analysed due to a low mitotic index. Multiplex-FISH was able to provide additive cytogenetic data in 2 cases where G-banded analysis was incomplete. However, in both these cases the banded karyotypes were complex and therefore associated with unfavourable outcome (Grimwade *et al.*, 1998). Thus the additive cytogenetic data provided by M-FISH for these cases would not have changed the prognostic significance of the presentation karyotype of these patients had M-FISH been used alongside banding analysis at the time of diagnosis. In contrast, the study in which SKY detected cryptic aberrations in 2 cases of AML with normal karyotype resulted, in a change in the prognostic subgroup (Zhang *et al.*, 2000).

Multiplex-FISH requires significant financial investment in product hardware, software and consumables. Although there was good overall concordance between M-FISH and G-banded analysis in this study, it is already clear that in cases where G-banded and M-FISH analysis are discordant, conventional FISH analysis should be used to confirm the results. Furthermore, given the technical limitations of M-FISH as demonstrated in this study, the need for further validation studies in which the clinical utility and diagnostic accuracy of M-FISH can be determined are required. Such studies will be mandatory if M-FISH is to move from being a research tool, to a vital diagnostic technique in clinical cytogenetics.

Summary

Multiplex-FISH is a new molecular cytogenetic technique that can identify marker chromosomes, and fully characterise complex karyotypes in patients with AML. This study illustrated that the technique could be successfully applied to archival metaphase preparations, and illustrated its potential diagnostic and clinical utility in elderly patients with AML, particularly those with complex chromosomal rearrangements. Based upon the findings in this study, there is little to currently recommend the routine use of M-FISH in the cytogenetic

analysis of AML, as the additive cytogenetic data provided by the technique is marginal and unlikely to influence patient prognosis. However, other studies in which 24-colour FISH has been used to analyse AML cases have resulted in important prognostic cytogenetic data been uncovered in cases with normal karyotype. Modifications to M-FISH probe composition and analysis software may improve the sensitivity and resolving power of the assay.

It is clear that M-FISH and SKY have the potential to augment conventional cytogenetic analysis and FISH for resolving karyotypes in AML. However, further validation studies are warranted before the true diagnostic and clinical potential of M-FISH can be realised in AML.

Chapter 5

Concluding Remarks

It is clear from the results presented in chapter 2, and from published studies elsewhere that curative treatment strategies often result in poor clinical outcome for the majority of older patients with AML. With current intensive induction therapy and supportive care it is possible to achieve complete remission of the order of 40-50% in selected elderly patients, and if patients not selected for treatment are considered then in reality less than one third of elderly patients referred for treatment will achieve remission. Although these results are disappointing it is clear that improvements in the delivery of supportive care, and the introduction of more intensive curative regimens have considerably improved clinical outcome for older patients with AML. The dramatic reduction in treatment-related deaths documented in elderly patients with AML over three decades was undoubtedly as a result of improved supportive care, which also indirectly accounted for an increase in resistant disease as more patient with resistant disease were able to survive induction therapy. Similarly, the improvement in complete remission rates from 13% to 45% was probably a consequence of more intensive therapy and a greater ability to manage the complications associated with such treatment.

Despite the poor outcome associated with elderly patients with AML it is clear that by using prognostic data, older patients with a better than average clinical outcome can be identified. For example, patients with 'favourable' karyotype had a significantly better complete remission rate compared to patients with 'unfavourable' karyotypes whilst younger age and the absence of hepatosplenomegaly were also associated with improved complete remission. When elderly patients with AML present with these clinical and biological characteristics, the decision to offer treatment with curative intent is relatively straightforward. However, cytogenetic aberrations predictive of 'favourable' outcome occur infrequently in older patients, who commonly present with chromosomal aberrations predictive of intermediate or 'unfavourable' outcome. Furthermore the results from this study demonstrated that 'unfavourable' karyotypes were independent predictor of

reduced overall survival. Indeed when treated with curative intent, elderly patients with 'unfavourable' karyotype had a median survival of 4 weeks, which was identical to that of patients managed conservatively. Thus in the absence of experimental therapies the argument for managing patients with 'unfavourable' karyotype conservatively is compelling. It therefore seems clear that those co-variates which impact on clinical outcome in older patients with AML should be actively utilised in the therapeutic decision making process. Indeed several authors have advocated the use of prognostic scoring systems or prognostic data in order to facilitate the therapeutic decision making when managing elderly patients with AML (Estey, 2000b; Ferrara *et al.*, 1998a; Johnson *et al.*, 1995). One can argue that in older patients curative therapy should await the results of conventional cytogenetic analysis, which normally takes at least 48 hours, this delay would not be improved by implementing M-FISH or CGH technologies which currently require a minimum of 24 and 72 hours, excluding time for analysis. With this in mind the prognostic significance of serum LDH in predicting complete remission and survival is clear given the short turn around time associated with the test. Any future clinical studies in elderly AML patients should hopefully include the development and validation of a prognostic scoring system incorporating the prognostic co-variates identified in this study.

It was hoped that additional cytogenetic data would be uncovered by the use of CGH and M-FISH in elderly patients with AML. Although it was possible to perform CGH using appropriately stored archival material the results obtained in this study suggest that there would be no advantage in the routine use of CGH in the analysis of AML. The technique was technically demanding, time consuming and would require stringent quality control with regard to metaphase preparations, tumour and reference DNA, as well as the routine use of appropriate controls. Although DNA copy number change was consistent with banding analysis in the majority of cases, there was a paucity of additive cytogenetic data. However, new developments namely cDNA microarray based CGH, offer the detection of genome wide DNA copy number change at a higher resolution than is currently possible with conventional CGH (Pinkel *et al.*, 1998). In addition, it is also possible to profile gene expression with this new technology (Pollack *et al.*, 1999). Although in its infancy microarray technology may allow the detection of dysregulated genes
in AML, particularly in patients with normal karyotype. Preliminary results

suggest that it is already possible to distinguish between AML and ALL on the basis of gene expression profiling alone (Golub, 1999). It therefore follows that gene expression profiling may offer another way in which genetic data can be interpreted for prognostic purposes.

It was possible to achieve a more comprehensive assessment of cytogenetic aberrations in elderly patients with AML through the use of M-FISH than with CGH. Importantly, the technique was relatively time efficient, requiring 18 hours for hybridisation. Furthermore it was also clear that M-FISH can provide additive cytogenetic data in cases with complex karyotype, provided that metaphase preparations of reasonable quality are available. However although M-FISH is a powerful technique it also has technical limitations, in that telomeric rearrangements may be missed as current commercially available probes contain relatively little PCR product from these regions. In addition it is not possible to detect inversion rearrangements with M-FISH. Based upon the results presented it would seem that the routine use of M-FISH in AML is unlikely to provide results that will alter the prognostic impact of cytogenetic data obtained with conventional banding analysis. Thus the acquisition and use of this technique, with regard to AML, is limited to the more accurate description of complex rearrangements by the cytogeneticist. Furthermore, given the limitations of M-FISH its use as a research tool in screening for cryptic aberrations in AML, particularly in those patients with normal karyotype, can not yet be recommended.

Although the objective selection of older patients with AML for curative therapy is essential, it is important to emphasise that current curative therapies only benefit a minority of patients. Furthermore, if patients ineligible for curative therapy are considered then the necessity for experimental curative regimens, and innovative supportive care strategies is even more immediate. Severe infection, particularly during the period of bone marrow aplasia, was identified as the major cause of early death amongst patients receiving curative therapy. Thus interventions aimed at reducing infection in these patients would be advantageous. One author has advocated the use of parenteral antifungal therapy, and or granulocyte transfusions in
febrile, in order to reduce the incidence of fungal pneumonia, although there is no obvious data to support these interventions (Estey, 2000b). The introduction of

novel therapeutic agents with less myelosuppressive activity than current curative regimens would seem to offer another approach to reducing early deaths. Calicheamicin is a cytotoxic antibiotic that induces double stranded DNA breaks when introduced into cells. When conjugated to humanised anti-CD33 monoclonal antibody, and administered to patients with refractory AML, a CR rate of 13% and overall response rate of 43% has been documented (Sievers *et al.*, 1998). Importantly, patients who received this therapy in phase II trials experienced less toxicity than would be expected through the use of conventional therapy. Common side effects reported included fever, asymptomatic hypotension, nausea, vomiting and leucopenia. And in contrast to conventional curative therapy, there was an absence of mucositis and alopecia in those patients who received the monoclonal antibody. Thus by using this agent first line, it may be possible to offer curative therapy to elderly patients in whom conventional regimens would be contraindicated. Furthermore, it may be possible to avoid the significant morbidity associated with the delivery of consolidation chemotherapy by using this agent in those patients who achieve CR and are too frail to receive further conventional therapy. Thus the results of phase III trials in which anti-CD33/Calicheamicin conjugate is evaluated, should be eagerly awaited.

The chimeric proteins that result from non-random chromosomal translocations may repress gene expression by recruiting histone-deacetylase (HDAC) to DNA/chromatin structures. The enzyme reduces the accessibility of transcription factors to DNA by removing acetyl groups from lysine residues on histones, leading to a closer association between DNA and histone complexes. The butyrates are a group of drugs that inhibit HDAC, and have been shown to induce remissions in patients with refractory AML (Zwiebel, 2000). The development of these drugs may lead to novel therapies for AML devoid of significant myelotoxicity, which would clearly be an advantage to older patients. Other drugs with novel activities include decitabine which promotes DNA demethylation when used with HDAC inhibitors. Decitabine acts synergisticaly to promote the re-expression of repressed genes silenced by chromosomal rearrangements (Estey, 2000b; Singal & Ginder, 1999). Therapeutic interventions aimed at modulating multi-drug resistance in older patients with AML have been evaluated in clinical trials. Unfortunately the promising results obtained with the cyclosporin analogue PSC833 in early phase I and II trials have not been repeated in phase III trials involving elderly patients (Stone, 1999).

In summary, an improvement in supportive care and the introduction of intensive chemotherapy has led to a significant improvement in clinical outcome for elderly patients treated with curative intent. Prognostic factors such as serum LDH, presentation karyotype and age can be used to objectively select elderly patients most likely to benefit from intensive chemotherapy. Comparative genomic hybridisation and M-FISH can be utilised in the cytogenetic analysis of AML. However, the additive cytogenetic data provided by these two techniques is limited, and can not justify their routine use in elderly AML.

References

Aikens, R.S., Agard, D.A. & Sedat, J.W. (1989). Solid-state imagers for microscopy. *Methods in Cell Biology*, **29,** 291-313.

Andrieu, V., Radford-Weiss, I., Troussard, X., Chane, C., Valensi, F., Guesnu, M., Haddad, E., Viguier, F., Dreyfus, F., Varet, B., Flandrin, G. & Macintyre, E. (1996). Molecular detection of t(8;21)/AML-ETO in AML M1/M2: Correlation with cytogenetics, morphology and immunophenotype. *British Journal of Haematology*, **92,** 855-865.

Ånstrom, M., Bodin, L., Nilsson, I. & Tidefelt, U. (2000). Treatment, long-term outcome and prognostic variables in 214 unselected AML patients in Sweden. *British Journal of Cancer*, **82,** 1387-1392.

Appelbaum, F.R., Gilliland, D.G. & Tallman, M.S. (1998). The biology and treatment of acute myeloid leukemia. *Hematology: American Society of Hematology Education Program Book*, 15-43.

Arthur, D.C., Berger, R., Golomb, H.M., Swansbury, G.J., Reeves, B.R., Alimena, G., Van Den Berghe, H., Bloomfield, C.D., de la Chapelle, A., Dewald, G.W. & et al. (1989). The clinical significance of karyotype in acute myelogenous leukemia. *Cancer Genetics and Cytogenetics*, **40,** 203-16.

Avet-Loiseau, H., Andree-Ashley, L.E., Moore, D., 2nd, Mellerin, M.P., Feusner, J., Bataille, R. & Pallavicini, M.G. (1997). Molecular cytogenetic abnormalities in multiple myeloma and plasma cell leukemia measured using comparative genomic hybridization. *Genes Chromosomes and Cancer*, **19,** 124-33.

Balducci, L., Phillips, D.M., Davis, K.M., Files, J.C., Khansur, T. & Hardy, C.L. (1988). Systemic treatment of cancer in the elderly. *Archives of Gerontology and Geriatrics*, **7,** 119-50.

Barbui, T., Finazzi, G. & Falanga, A. (1998). The impact of all-trans retinoic acid on the coagulopathy of acute promyelocytic leukemia. *Blood*, **91,** 3093-3102.

Baudard, M., Marie, J.P., Cadiou, M., Viguie, F. & Zittoun, R. (1994). Acute myelogenous leukaemia in the elderly: retrospective study of 235 consecutive patients. *British Journal of Haematology*, **86,** 82-91

Bennett, J.M., Catovsky, D., Daniel, M.-T., Flandrin, G., Galton, D.A.G., Gralnick, H.R. & Sultan, C. (1976). Proposals for the classification of the acute leukaemias. *British Journal of Haematology*, **33,** 451-458.

Bennett, J.M., Catovsky, D., Daniel, M.-T., Flandrin, G., Galton, D.A.G., Gralnick, H.R. & Sultan, C. (1985a). Criteria for the diagnosis of acute leukaemia of megakaryocytic lineage (M7). *Annals of Internal Medicine*, **103,** 440-462.

Bennett, J.M., Catovsky, D., Daniel, M.-T., Flandrin, G., Galton, D.A.G., Gralnick, H.R. & Sultan, C. (1985b). Proposed revised criteria for the classification of acute myeloid leukaemia. *Annals of Internal Medicine*, **103,** 626-629.

Bennett, J.M., Catovsky, D., Daniel, M.-T., Flandrin, G., Galton, D.A.G., Gralnick, H.R. & Sultan, C. (1991). Proposal for the recognition of minimally differentiated acute myeloid leukaemia (AML-MO). *British Journal of Haematology*, **78,** 325-329.

Bennett, J.M., Young, M.L., Andersen, J.W., Cassileth, P.A., Tallman, M.S., Paietta, E., Wiernik, P.H. & Rowe, J.M. (1997). Long-term survival in acute myeloid leukemia: the Eastern Cooperative Oncology Group experience. *Cancer*, **80,** 2205-9.

Bentz, M., Dohner, H., Huck, K., Schutz, B., Ganser, A., Joos, S., du Manoir, S. & Lichter, P. (1995a). Comparative genomic hybridization in the investigation of myeloid leukemias. *Genes Chromosomes Cancer*, **12,** 193-200.

Bentz, M., Huck, K., du Manoir, S., Joos, S., Werner, C.A., Fischer, K., Dohner, H. & Lichter, P. (1995b). Comparative genomic hybridization in chronic B-cell leukemias shows a high incidence of chromosomal gains and losses. *Blood*, **85,** 3610-8.

Bishop, J.F. (1999). Adult acute myeloid leukaemia: Update on treatment. *Medical Journal of Australia*, **170,** 39-43.

Bloomfield, C.D., Goldman, A., Hassfeld, D. & de la Chapelle, A. (1984). Fourth International Workshop on Chromosomes in Leukemia 1982: Clinical significance of chromosomal abnormalities in acute non-lymphoblastic leukemia. *Cancer Genetics and Cytogenetics*, **11,** 332-50.

Bloomfield, C.D., Lawrence, D., Byrd, J.C., Carroll, A., Pettenati, M.J., Tantravahi, R., Patil, S.R., Davey, F.R., Berg, D.T., Schiffer, C.A., Arthur, D.C. & Mayer, R.J. (1998). Frequency of prolonged remission duration after high-dose cytarabine intensification in acute myeloid leukemia varies by cytogenetic subtype. *Cancer Research*, **58,** 4173-9.

Bodey, G.P., Bolivar, R. & Fainstein, V. (1982). Infectious complications in leukemic patients. *Seminars in Hematology*, **19,** 193-226.

Bodley Scott, R. (1957). Leukaemia. *The Lancet*, 1053-1057.

Brinker, H. (1982). Population-based age and sex-specific incidence rates in the four main types of leukaemia. *Scandinavian Journal of Haematology*, **29,** 241-249.

Brito-Babapaulle, F., Catovsky, D. & Galton, D.A.G. (1987). Clinical and laboratory features of de novo acute myeloid leukaemia. *British Journal of Haematology*, **66,** 44-45.

Broome, J.D. (1961). Evidence that the l-asparaginase activity of guinea pig serum is responsible for its antilymphoma effects. *Nature*, **191,** 1114-1115.

Buchner, T. (1993). Acute leukemia. *Current Opinion in Hematology*, 172-182.

Buchner, T., Hiddemann, W., Fonatsch, C., Gassmann, W., Maschmeyer, G., Ludwig, W.G., Hossfeld, G., Sauerland, M.C. & Heinecke, A. (1995). Treatment of

AML in the elderly: Full-dose versus reduced dose induction treatment. *Blood*, **supplement 1,** 434a.

Burnett, A.K., Grimwade, D., Solomon, E., Wheatley, K. & Goldstone, A.H. (1999). Presenting white blood cell count and kinetics of molecular remission predict prognosis in acute promyelocytic leukemia treated with all-trans retinoic acid: result of the randomized MRC trial. *Blood*, **93,** 4131-43.

Byrd, J.C., Edenfield, W.J., Shields, D.J. & Dawson, N.A. (1995). Extramedullary myeloid cell tumours in acute non-lymphoblastic leukemia: A clinical review. *Journal of Clinical Oncology*, **13,** 1800-1816.

Byrd, J.C., Lawrence, D., Arthur, D.C., Pettenati, M.J., Tantravahi, R., Qumsiyeh, M., Stamberg, J., Davey, F.R., Schiffer, C.A. & Bloomfield, C.D. (1998). Patients with isolated trisomy 8 in acute myeloid leukemia are not cured with cytarabine-based chemotherapy: Results from Cancer and Leukemia Group B 8461. *Clinical Cancer Research*, **4,** 1235-41.

Campana, D. & Pui, C. (1995). Detection of minimal residual disease in acute leukaemia: Methodological advances and clinical significance. *Blood*, **85,** 1416-1434.

Caldwell, G., Kelly, D., Zack, M., Falk, H. & Heath, C.J. (1983). Mortality and cancer frequency among military nuclear test (Smoky) participants, 1957 through 1979. *Journal of American Medical Association*, **250,** 620-624.

Caligiuri, M.A., Schichman, S.A., Strout, M.P., Mrozek, K., Baer, M.R., Frankel, S.R., Barcos, M., Herzig, G.P., Croce, C.M. & Bloomfield, C.D. (1994). Molecular rearrangement of the ALL-1 gene in acute myeloid leukemia without cytogenetic evidence of 11q23 chromosomal translocations. *Cancer Research*, **54,** 370-3.

Caron, P.C., Dumont, L. & Scheinberg, D.A. (1998). Supersaturating infusional humanized anti-CD33 monoclonal antibody HuM195 in myelogenous leukemia. *Clinical Cancer Research*, **4,** 1421.

Cartwright, R.A., McNally, R.J.Q., Rowland, D.J. & Thomas, J. (1997). *The Descriptive Epidemiology of Leukaemia and Related conditions in Parts of the United Kingdom 1984-1993*. Leukaemia Research Fund.

Caspersson, T., Zech, L., Johansson, C. & Modest, E.J. (1970). Identification of human chromsomes by DNA-banding fluorescent agents. *Chromosoma*, **30,** 215-217.

Cheson, B.D., Jasperse, D.M., Simon, R. & Friedman, M.A. (1986). A critical appraisal of low-dose cytosine arabinoside in patients with acute non-lymphocytic leukemia and myelodysplastic syndromes. *Journal of Clinical Oncology*, **4,** 1857-64.

Cigudosa, J.C., Pulivarthi, H.R., Calasanz, M.J., Odero, M.D., Michaeli, J., Jhanwar, S.C. & Changanti, R.S.K. (1998). Characterization of nonrandom chromosomal gains and losses in multiple myeloma by comparative genomic hybridization. *Blood*, **91,** 3007-3008.

Cohen, H.J. (1999). Geriatric Hematology: Biology and physiology of aging. *Hematology: American Society of Hematologyu Education Program Book*, 500-504.

Copplestone, J. & Prentice, A. (1988). Acute myeloblastic leukaemia in the elderly. *Leukaemia Research*, **12,** 617-625.

Craigie, D. (1845). *Edinburgh Medicine Surgery Journal*, **64,** 400.

Crosen, P.E., Morrison, M.J., Rodley, P., Cochrane, J. & Morris, C.M. (1999). Identifcation of amplified genes in a patient with acute myeloid leukemia and double minute chromsomes. *Cancer Genetics and Cytogenetics*, **113,** 126-133.

Crowther, D., Powles, R.L., Bateman, C.J., Beard, M.E., Gauci, C.L., Wrigley, P.F., Malpas, J.S., Fairley, G.H. & Scott, R.B. (1973). Management of adult acute myelogenous leukaemia. *British Medical Journal*, **1,** 131-7.

Czepulkowski, B.H., Bhatt, B. & Rooney, D.E. (1992). Basic techniques for the analysis of chromosomes from bone marrow and leukaemic blood. In *Human cytogenetics: A practical approach*, Rickwood, D. & Hames, B.D. (eds), Vol. II Malignancy and acquired abnormalties. pp. 1-25. Oxford University Press: Oxford.

Dalley, C.D., Lillington, D.M., Neat, M., Foot, N., Burridge, M., Carter, M., Slater, S., Young, B.D., Amess, J.A., Lister, T.A. & Rohatiner, A.Z. (1999). Prognostic importance of cytogenetics and the application of molecular cytogentics in elderly patients with acute myeloid leukemia treated at a single center. *Blood*, **94 (10)**, 495a.

Dastugue, N., Payen, C., Lafage, P. & et.al. (1995). Prognostic significance of karyotype in de novo acute myeloid leukaemia. *Leukemia*, **9,** 1491-1498.

Dauwerse, J.G., Wiegant, J., Raap, A.K., Breuning, M.H. & van Ommen, G.J.B. (1992). Multiple colors by fluorescence *in situ* hybridization using ratio labelled DNA probes create a molecualr karyotype. *Human Molecular Genetics*, **1,** 593-598.

Desoize, B. & Robert, J. (1994). Individual dose adaptation of anticancer drugs. *European Journal of Cancer*, **30A,** 844-851.

Devereux, S. (1991). Therapy associated leukaemia. *Blood Reviews*, **5,** 138-45.

Dhaliwal, H.S., Shannon, M.S., Barnett, M.J., Prentice, H.G., Bragman, K., Malpas, J.S. & Lister, T.A. (1986). Treatment of acute leukaemia with m-AMSA in combination with cytosine arabinoside. *Cancer Chemotherapy and Pharmacology*, **18,** 59-62.

Dohner, K., Hehmann, U., Stewart, J., Lowther, G., Scholl, C., Fohhling, S., Cuneo, A., Tsui, L., Lichter, P., Scherer, S. & Dohner, H. (1998). Molecular cytogenetic characterization of a critical region in bands 7q35-q36 commonly deleted in malignant myeloid disorders. *Blood*, **92,** 4031-4035.

Doll, R. (1995). Hazards of ionising radiation: 100 years of observations on man. *British Journal of Cancer*, **72,** 1339-1349.

Dombret, H., Chastang, C., Fenaux, P., Reiffers, J., Bordessoule, D., Bouabdallah, R., Mandelli, F., Ferrant, A., Auzanneau, G., Tilly, H. & et al. (1995). A controlled study of recombinant human granulocyte colony-stimulating factor in elderly patients after treatment for acute myelogenous leukemia. AML Cooperative Study Group. *New England Journal of Medicine*, **332,** 1678-83.

Ebstein, W. (1889). Ueber die acute leukamie und pseudoleukaimie. *Dtsch Arch Klin Med.* **44,** 343.

Eils, R., Uhrig, S., Saracoglu, K., Satzler, K., Bolzer, A., Petersen, I., Chassery, J.-M., Ganser, M. & Speicher, M.R. (1998). An optimized, fully automated system for fast and accurate idenification of chromosomal rearrangements by multiplex-FISH (M-FISH). *Cytogenetics and Cell Genetics*, **82,** 160-171.

Ellison, R., Holland, J.F., Weil, M., Jacquilt, C., Boiron, M., Bernard, J. (1968). Arabinosyl cytosine: A useful agent in the treatment of acute leukemia in adults. *Blood*, ***32,*** 507-523.

El-Rifai, W., Elonen, E., Larramendy, M., Ruutu, T. & Knuutila, S. (1997). Chromosomal breakpoints and changes in DNA copy number in refractory acute myeloid leukemia. *Leukemia*, **11,** 958-63.

Estey, E. (2000a). Treatment of relapsed and refractory acute myelogenous leukemia. *Leukemia*, **14,** 476-479.

Estey, E.H. (2000b). How I treat older patients with AML. *Blood*, **96,** 1670-1673.

Evans, W., Relling, M.V., Rodman, J.H., Crom, W.R., Boyett, J.M. & Pui, C.H. (1998). Conventional compared with individualized chemotherapy in childhood acute lymphoblastic leukaemia. *New England Journal of Medicine*, **338,** 499-505.

Faderl, S., Kantarajian, H.M., Talpaz, M. & Estrov, Z. (1998). Clinical significance of cytogenetic abnormalities in adult acute lymphoblastic leukaemia. *Blood*, **91,** 3995-4019.

Fenaux, P., Preudhomme, C., Lai, J.L., Morel, P., Beuscart, R. & Bauters, F. (1989). Cytogenetics and their prognostic value in de novo acute myeloid leukaemia: A report on 283 cases. *British Journal of Haematology*, **73,** 61-7.

Ferrara, F., Annunziata, C., Copia, C., Margin, S. & Mirito, S. (1998a). Acute Myeloid Leukaemia in Patients over 75 Years: Clinico-hematological characteristics and treatment results. In *Acute Leukaemas VII: Experimental Approaches and Novel Therapies*, al., H.e. (ed) pp. 855-861. Springer-Verlag Berlin Heidelberg 1998.

Farber, S., Diamond, L.K., Mercer, R.D. (1948). Temporary remissions in leukemia in children produced by folic acid antagonist 4-aminopter-oyl-glutamic acid (aminopterin). *New England Journal of Medicine*, **238,** 787-793.

Ferrara, F. & Mirto, S. (1996). Serum LDH value as a predictor of clinical outcome in acute myelogenous leukaemia of the elderly. *British Journal of Haematology*, **92,** 627-31.

Ferrara, F., Mirto, S., Zagonel, V. & Pinto, A. (1998b). Acute myeloid leukaemia in th elderly: A critical review of therapeutic approaches and appraisal of results of therapy. *Leukaemia and Lymphoma*, **29,** 375-382.

Fialkow, P.J., Singer, J.W., Raskind, W.H., Adamson, J.W., Jacobson, R.J., Bernstein, I.D., Dow, L.W., Najfeld, V. & Veith, R. (1987). Clonal development, stem-cell differentiation, and clinical remissions in acute nonlymphocytic leukemia. *New England Journal of Medicine*, **317,** 468-73.

Fourth International Workshop on Chromosomes in Leukemia: Overview of association between chromosome pattern and cell morphology, age, sex, and race. (1984). *Cancer Genetics and Cytogenetics,* **11,** 265-274.

Freireich, E.J. (1987). Arabinosyl cytosine: A 20-year update. *Journal of Clinical Oncology,* **5,** 523-524.

Friedrick, N. (1857). Ein neuer Fall von Leukamie. *Arch Pathol Anat,* **12:37**.

Gajewski, J.L., Ho, W.G., Nimer, S.D., Hirji, K.F., Gekelman, L., Jacobs, A.D. & Champlin, R.E. (1989). Efficacy of intensive chemotherapy for acute myelogenous leukemia associated with a preleukemic syndrome. *Journal of Clinical Oncology,* **7,** 1637-45.

Gebhart, E., Verdorfer, I., Saul, W., Trautmann, U. & Brecevic, L. (2000). Delimiting the use of camparative genomic hybridization in human myeloid neoplastic disorders. *International Journal of Oncology,* **16,** 1099-1105.

Gloor, W. (1930). Ein fall von geheiltes myeloblastenleukamie. *Munchin Med Wochenschr,* **77,** 1096-1098.

Golub, T.R. (1999). Acute myeloid leukemia:The genetics of AML: An update. In *Hematology:American Society of Hematology Education program Book,* Schecter, G. (ed) pp. 102-111. American Society of Hematology.

Goodman, L.S., Wintrobe, M.M., Damshek, W., Gilman, A., Mclennan, M.T. (1946). Nitrogen mustard therapy: Use of methyl-bis (beta-chloroethyl)amine hydrocholride and tris (beta-chlororethyl)amine hydrochloride for Hodgkin's disease, lymphosarcoma, leukemia, and certain allied miscellaneous disorders. *Journal of the American Medical Association,* **132,** 126-132.

Grimwade, D., Walker, H., Oliver, F., Wheatley, K., Harrison, C., Harrison, G., Rees, J., Stevens, R., Burnett, A. & Goldstone, A. (1998). The importance of diagnostic cytogenetics on outcome in AML: Analysis of 1612 patients entered into the MRC AML 10 trial. *Blood,* **92,** 2322-2333.

Handwashing liason goup (2000). Lavate vestras manus. *Annals of the Royal College of Surgeons*, **82,** 133-336.

Haferlach, T. (1996). More individual markers are necessary for patients with acute myeloid leukaemia (AML). Does cytomorphology or cytogenetics define the biological entity? *Leukaemia*, S5-S9.

Harris, N.L., Jaffe, E.S., Diebold, J., Flandrin, G., Muller-Hermelink, H.K., Varidiman, J., Lister, T.A. & Bloomfield, C.D. (1999). World health organization classification of neoplastic disease of the hematopoietic and lymphoid tissues: Report of the clincal advisory commitee meeting-Airlie House, Virginia, November 1997. *Journal of Clincal Oncology*, **17,** 3835-3849.

Heim, S. & Mitelman, F. (1995). Acute myeloid leukaemia. In *Cancer Cytogenetics (Chromosomal and Molecular Genetic Aberrations of Tumor Cells)*, Heim, S. & Mitelman, F. (eds) pp. 69-140. Wiley-Liss,Inc: New York.

Hiddemann, W., Kern, W., Schoch, C., Fonatsch, C., Heinecke, A., Wormann, B. & Buchner, T. (1999). Management of acute myeloid leukaemia in elderly patients. *Journal of Clinical Oncology*, **17,** 3569-3576.

Hiraoka, Y., Sedat, J.W. & Agard, D.A. (1987). The use of a charge-coupled device for quantitative optical microscopy of biological structures. *Science*, **238,** 36-41.

Horrigan, S.K., Arbieva, Z.H., Xie, H.Y., Kravarusic, J., Fulton, N.C., Naik, H., Le, T.T. & Westbrook, C.A. (2000). Delineation of a minimal interval and identification of 9 candidates for a tumor suppressor gene in malignant myeloid disorders on 5q31. *Blood*, **95,** 2372-7.

Hoyle, C.F., de Bastos, M., Wheatley, K. & et.al. (1989). AML associated with previous cytotoxic therapy, MDS or myeloproliferative disorders: Results from the MRC's 9th AML trial. *British Journal of Haematology*, **72,** 45-53.

Hughes, W.T., Armstrong, D., Bodey, G.P., Feld, R., Mandell, G.L., Meyers, J.D., Pizzo, P.A., Schimpff, S.C., Shenep, J.L., Wade, J.C., Young, L.S. & Yow, M.D. (1990). Guidelines for the use of antimicrobial agents in neutropenic patients with unexplained fever. *Journal of Infectious Diseases*, **161,** 381-396.

Huhta, T., Vettenranta, K., Heinonen, K., Kanerva, J., Larramendy, M.L., Mahlamaki, E., Saarinen-Pihkala, U.M. & Knuutila, S. (1999). Comparative genomic hybridization and conventional cytogenetic analyses in childhood acute myeloid leukemia. *Leukemia and Lymphoma*, **35,** 311-5.

Hutchins, L.F., Unger, J.M., Crowley, J.J., Coltman, C.A. & Albian, K.S. (1999). Underrepresentation of patients 65 years of age or older in cancer-treatment trials. *New England Journal of Medicine*, **341,** 2061-7.

Infante, P.F., R.A, R., Wagoner, J.K. & Young, R.J. (1977). Leukaemia in benzene workers. *Lancet*, 76-78.

Johansson, B., Heim, F., Kristoffersson, U. & Mitelman, F. (1991). Cytogenetics of secondary myelodysplasia (sMDS) and acute non-lymphocytic leukaemia (sANLL). *European Journal of Haematology*, **47,** 17-27.

Johansson, B., Mertens, F. & Mitelman, F. (1993). Cytogenetic deletion maps of hematologic neoplasms: Circumstantial evidence for tumour supressor loci. *Genes Chromosomes and Cancer*, **8,** 205-218.

Johnson, I.S., Armstrong, J.G., Gorman, M. & Burnett, J.P. (1963). The vinca alkaloids: A new class of oncolytic agents. *Cancer Research*, **23,** 1390-1427.

Johnson, P.R., Ryder, W.D. & Yin, J.A. (1995). Validation of a model to predict survival in elderly patients with acute myeloid leukaemia. *British Journal of Haematology*, **90,** 954-6.

Johnson, P.R.E. & Liu Yin, J.A. (1993). Acute myeloid leukaemia in the elderly: biology and treatment. *British Journal of Haematology*, **83,** 1-6.

Kallioniemi, A., Kallioniemi, O.-P., Sudar, D., Rutovitz, D., Gray, J.W., Waldman, F. & Pinkel, D. (1992). Comparative genomic hybridization for molecular cytogenetic analysis of solid tumours. *Science*, **258,** 818-821.

Kallioniemi, O.P., Kallioniemi, A., Piper, J., Isola, J., Waldman, F.M., Gray, J.W. & Pinkel, D. (1994). Optimizing comparative genomic hybridization for analysis of DNA sequence copy number changes in solid tumors. *Genes Chromosomes and Cancer*, **10,** 231-43.

Kantarjian, H.M., O'Brien, S., Anderlini, P. & Talpaz, M. (1996). Treatment of chronic myelogenous leukaemia: Current status and investigational options. *Blood*, **87,** 3069-3081.

Karhu, R., Kahkonen, M., Kuukasjarvi, T., Pennanen, S., Tirkkonoen, M. & Kallioniemi, O. (1997a). Quality control of CGH: Impact of metaphase chromosomes and dynamic range of hybridization. *Cytometry*, **28,** 198-205.

Karhu, R., Siitonen, S., Tanner, M., Keinanen, M., Makipernaa, A., Lehtinen, M., Vilpo, J.A. & Isola, J. (1997b). Genetic aberrations in pediatric acute lymphoblastic leukemia by comparative genomic hybridization. *Cancer Genetics and Cytogenetics*, **95,** 123-9.

Kearney, L. (1999). The impact of the new FISH technologies on the cytogenetics of haematological malignancies. *British Journal of Haematology*, **104,** 648-658.

Keinanen, M., Knuutila, S., Bloomfield, C.D., Elonen, E. & de la Chapelle, A. (1986). The proportion of mitoses in different cell lineages changes during short-term culture of normal human bone marrow. *Blood*, **67,** 1240-3.

Kimmick, G.G., Fleming, R., Muss, H.B. & Balducci, L. (1997). Cancer chemotherapy in older adults. A tolerability perspective. *Drugs and Aging*, **10,** 34-49.

Larramendy, M.L., Huhta, T., Heinonen, K., Vettenranta, K., Mahlamaki, E., Riikonen, P., Saarinen-Pihkala, U.M. & Knuutila, S. (1998a). DNA copy number changes in childhood acute lymphoblastic leukemia. *Haematologica*, **83,** 890-5.

Larramendy, M.L., Huhta, T., Vettenranta, K., El-Rifai, W., Lundin, J., Pakkala, S., Saarinen-Pihkala, U.M. & Knuutila, S. (1998b). Comparative genomic hybridization in childhood acute lymphoblastic leukemia. *Leukemia*, **12,** 1638-44.

Leith, C.P., Kopecky, K.J., Chen, I.M., Eijdems, L., Slovak, M.L., McConnell, T.S., Head, D.R., Weick, J., Grever, M.R., Appelbaum, F.R. & Willman, C.L. (1999). Frequency and clinical significance of the expression of the multidrug resistance proteins MDR1/P-glycoprotein, MRP1, and LRP in acute myeloid leukemia. A southwest oncology group study [In Process Citation]. *Blood*, **94,** 1086-99.

Leith, C.P., Kopecky, K.J., Godwin, J., McConnell, T., Slovak, M.L., Chen, I., Head, D.R., Appelbaum, F.R. & Willman, C.L. (1997). Acute myeloid leukemia in the elderly: Assessment of multidrug Resistance (MDR1) and cytogenetics distinguishes biologic subgroups with remarkably distinct responses to standard chemotherapy. A Southwest Oncology Group Study. *Blood*, **89,** 3323-3329.

Liang, H., Fairman, J., Claxton, D., Nowell, Green, E. & Nagarajan, L. (1998). Molecular anatomy of chromosome 7q deletions in myeloid neoplasms: evidence for multiple critical loci. *Proceedings of the National Academy of Science*, **95,** 3781-3785.

Licht, J.D., Chomienne, C., Goy, A., Chen, A. & et.al. (1995). Clinical and molecular characterization of a rare syndrome of acute promyelocytic leukemia associcated with translocation (11;17). *Blood*, **85,** 1083-1094.

Lister, T.A., Johnson, A.N., Bell, R., Henry, G. & Malpas, J.S. (1981). Progress in acute myelogenous leukemia. In *Haematology and Blood Transfusion: Modern trend in Human Leukemia IV*, Vol. 26. pp. 38-44. Springer-Verlag: Berlin Heidelberg.

Liu Yin, J.A., Johnson, P.R., Davies, J.M., Flanagan, N.G., Gorst, D.W. & Lewis, M.J. (1991). Mitozantrone and cytosine arabinoside as first-line therapy in elderly patients with acute myeloid leukaemia. *British Journal of Haematology*, **79,** 415-20.

Lowenberg, B. (1996). Treatment of the elderly patient with acute myeloid leukaemia, Vol. 9(1). pp. 147-159. Bailliere Tindall: Baillierre's Clinical Haematology.

Lowenberg, B. & Sonneveld, P. (1998). Resistance to chemotherapy in acute leukemia. *Current Opinion in Oncology*, **10,** 31-5.

Lowenberg, B., Suciu, S., Archimbaud, E., Haak, H., Stryckmans, P., de Cataldo, R., Dekker, A.W., Berneman, Z.N., Thyss, A., van der Lelie, J., Sonneveld, P., Visani, G., Fillet, G., Hayat, M., Hagemeijer, A., Solbu, G. & Zittoun, R. (1998). Mitoxantrone versus daunorubicin in induction-consolidation chemotherapy: The value of low-dose cytarabine for maintenance of remission, and an assessment of prognostic factors in acute myeloid leukemia in the elderly: final report. European Organization for the Research and Treatment of Cancer and the Dutch-Belgian Hemato-Oncology Cooperative Hovon Group. *Journal of Clinical Oncology*, **16,** 872-81.

Lowenberg, B., Suciu, S., Archimbaud, E., Ossenkoppele, G., Verhoef, G.E., Vellenga, E., Wijermans, P., Berneman, Z., Dekker, A.W., Stryckmans, P., Schouten, H., Jehn, U., Muus, P., Sonneveld, P., Dardenne, M. & Zittoun, R. (1997). Use of recombinant GM-CSF during and after remission induction chemotherapy in patients aged 61 years and older with acute myeloid leukemia: final report of AML-11, a phase III randomized study of the Leukemia Cooperative Group of European Organisation for the Research and Treatment of Cancer and the Dutch Belgian Hemato-Oncology Cooperative Group. *Blood*, **90,** 2952-61.

Lowenberg, B., Zittoun, R., Kerkhofs, H., Jehn, U., Debusscher, L., Cauchie, C., Peetermans, M., Solbu, G., Suciu, S. & Stryckmans, P. (1989). On the value of intensive remission induction chemotherapy in elderly patients of 65+years with acute myeloid leukemia:A randomised phae III study of the Euroopean Organization for Reasearch and Treatment of Cancer Leukemia Group. *Journal of Clinical Oncology,* 7, 1268-1274.

MacCallum, P.K., Rohatiner, A.Z., Davis, C.L., Whelan, J.S., Oza, A.M., Lim, J., Love, S., Amess, J.A., Leahy, M. & Gupta, R.K. (1995). Mitoxantrone and cytosine arabinoside as treatment for acute myeloid leukaemia in older patients. *Annals of Hematology,* **71,** 35-39.

Manoharan, A. (1998). Acute myeloblastic leukaemia in the elderly: biology, prognostic factors and treatment. *International Journal of Haematology,* **68,** 235-243.

Martinez-Climent, J.A., Lane, N.J., Rubin, C.M., Johnstone, H.S., Mick, R., Murphy, S.B., Vardiman, J.W., Larson, R.A., Le Beau, M.M. & et.al. (1995). Clinical and prognostic significance of chromosomal abnormalities in childhood acute myeloid leukemia de novo. *Leukemia,* **9,** 95-101.

Mauritzson, M., Johansson, B., Bilstrom, R. & et.al. (1998). A single-center population-based consecutive series of 1500 cytogenetically investigated adult haematological malignancies: Karyotypic features in relation to morphology, age and gender. *European Journal of Haematology,* **62,** 95-102.

Mayer, R.J., Davis, R.B., Schifer, C.A., Berg, D.T., Powell, B.L., Schulman, P., Omura, G.A., Moore, J.O., McIntyre, R.O. & Frei, E. (1994). Intensive Postremmision chemotherapy in adults with acute myeloid leukaemia. *New England Journal Of Medicine,* **331,** 896-903.

Medical Research Council of the working party on the evaluation of different methods of therapy in leukaemia(1963). Treatment of acute leukaemia in adults:Comparison of steroid therapy at high and low dosage in conjunction with 6-mercaptopurine. *British Medical Journal,* 7-13.

Mitelman, F. (1995). ISCN(1995):An International System for Human Cytogenetic Nomenclature., Mitelman, F. (ed). S.Karger: Basel, Switzerland.

Mohamed, A.N., Macoska, J.A., Kallioniemi, A., Kallioniemi, O.-P., Waldman, F., Ratanatharathorn, V. & Wolman, S.R. (1993). Extrachromosmomal gene amplification in acute myeloid leukemia; characterization by metaphase analysis, comparative genomic hybridization, and semi-quantitative PCR. *Genes, Chromosomes and Cancer*, **8,** 185-189.

Mohammed, A.N., Maccoska, J.A., Kallionemi, A., Kallionemi, O-P., Walman, F., Ratatharathorn, V., Wolman, S.R. (1993). Extrachromosomal gene amplification in acute myeloid leukaemia, characterisation by metaphase analysis, comparative genomic hybridization, and semi-quantitative PCR. *Genes Chromosomes and Cancer*, **8,** 185-189.

Mohr, B., Bornhauser, M., Thiede, C., Schakel, U., Schaich, M., Illmer, T., Pascheberg, U. & Ehninger, G. (2000). Comparison of spectral karyotyping and conventional cytogenetics in 39 patients with acute myeloid leukemia and myelodysplastic syndrome. *Leukemia*, **14,** 1031-8.

Moloney, W. (1987). Radiogenic leukaemia revisited. *Blood*, **70,** 905-908.

Morschhauser, F., Cayuela, J.M., Martini, S., Baruchel, A., Rousselot, P., Socie, G., Berthou, P., Jouet, J.P., Straetmans, N., Sigaux, F., Fenaux, P. & Predhomme, C. (2000). Evaluation of minimal ressidual disease using reverse-transcription polymerase chain reaction in t(8;21) acute myeloid leukaemia: A multicenter study of 51 patients. *Journal of Clinical Oncology*, **18,** 788-794.

Mrozek, K., Heinonen, K., de la Chapelle, A. & Bloomfield, C. (1997). Clinical significance of cytogenetics in acute myeloid leukaemia. *Seminars in Oncology*, **24,** 17-31.

Nacheva, E., Grace, C., Holloway, T.L., Green, A.R., (1995). Comparative genomic hybridisation in acute myeloid leukaemia. A comparison between G-banding and chromosome painting. *Cancer Genetics and Cytogenetics*, **82,** 9-16.

Nederlof, P.M., van der Flier, S., Wiegant, J., Raap, A.K., Tanke, H.J., Ploem, J.S. & van der Ploeg, M. (1990). Multiple fluorescence in situ hybridization. *Cytometry*, **11,** 126-131.

Nowell, P. & Hungerford, D.A. (1960). A chromosome in human chronic granulocytic leukaemia. *Science*, **132,** 1497.

O'Donnell, M., Appelbaum, F., Bishop, M., EH, E., Grever, M. & Maslak, P. (1996). NCCN Acute leukemia practice guidelines. The national comprehensive cancer network. *Oncology*, **10,** 205-221.

Okuda, T., Zhongling, C., Shouli, Y., Noel, L., Chuhl-joo, L., Jan, M., Hironori, H. & Downin, J. (1998). Expression of a knocked-in *AMLI1-ETO* leukemia gene inhibits the establishement of normal definitive haematopoiesis and directly generates dysplastic haematopoietic proghenitors. *Blood*, **91,** 3134-3143.

Oscier, D.G. (1987). Myelodysplastic syndromes:. In *Haematological problems in the elderly*, Hambling, T.J. (ed), Vol. 1. pp. 108-113. Bailliere's Clinical Haematology.

Paszek-Vigier, M., Talmant, P., Mechinaud, F., Garand, R., Harousseau, J.L., Bataille, R. & Avet-Loiseau, H. (1997). Comparative genomic hybridization is a powerful tool, complementary to cytogenetics, to identify chromosomal abnormalities in childhood acute lymphoblastic leukaemia. *British Journal of Haematology*, **99,** 589-96.

Pedersen-Bjergaard, J., Philip, P., Larsen, S.O., Jensen, G. & Byrsting, K. (1990). Chromosome aberrations and prognostic factors in therapy-related myelodysplasia and acute nonlymphocytic leukemia. *Blood*, **76,** 1083-91.

Peterson, B.A. & Bloomfield, C.D. (1977). Treatment of acute non-lymphocytic leukemia in elderly patients. *Cancer*, **40,** 647-652.

Pinkel, D., Seagraves, R., Sudar, D., Clark, S., Poole, I., kowbel, D., Collins, C., Kuo, W., Chen, C., Dairkee, S.H., Ljung, B., Gray, J.W. & Albertson, D.G. (1998). High resolution analysis of DNA copy number variation using comparative genomic hybridization to microarrays. *Nature Genetics*, **20,** 207-211.

Piper, J., Rutovitz, D., Sudar, D., Kallioniemi, A., Kallioniemi, O.P., Waldman, F.M., Gray, J.W. & Pinkel, D. (1995). Computer image analysis of comparative gemomic hybridization. *Cytometry*, **19,** 10-26.

Pollack, J.R., Perou, C.M., Alizadeh, A.A., Eisen, M.B., Pergamenschikov, A., Williams, C.F., Jeffrey, S.S., Botstein, D. & Brown, P.O. (1999). Genome-wide analysis of DNA copy-number changes using cDNA microarrays. *Nature Genetics*, **23,** 41-6.

Preisler, H., Davis, R.B., Kirshner, J., Dupre, E., Richards, F.d., Hoagland, H.C., Kopel, S., Levy, R.N., Carey, R., Schulman, P. & et al. (1987). Comparison of three remission induction regimens and two post-induction strategies for the treatment of acute non-lymphocytic leukemia: A Cancer and Leukemia Group B study. *Blood*, **69,** 1441-9.

Rabbitts, T.H. (1994). Chromosomal translocations in human cancer. *Nature*, **372,** 143-149.

Rai, K.R., Holland, J.F., Glidewell, O.J. & et.al. (1981). Treatment of acute myelocytic leukemia: A study by Cancer and Leukemia Group B. *Blood*, **58,** 1203-1212.

Rao, P.H., Cigudosa, J.C., Ning, Y., Calasanz, M.J., Iida, S., Tagawa, S., Michaeli, J., Klein, B., Dalla-Favera, R., Jhanwar, S.C., Ried, T. & Chaganti, R.S. (1998). Multicolor spectral karyotyping identifies new recurring breakpoints and translocations in multiple myeloma. *Blood*, **92,** 1743-8.

Rees, J.K., Gray, R.G., Swirsky, D. & Hayhoe, F.G. (1986). Principal results of the Medical Research Council's 8th acute myeloid leukaemia trial. *Lancet*, **2,** 1236-41.

Rees, J.K., Gray, R.G. & Wheatley, K. (1996). Dose intensification in acute myeloid leukaemia: greater effectiveness at lower cost. Principal report of the Medical Research Council's AML9 study. MRC Leukaemia in Adults Working Party. *British Journal of Haematology*, **94,** 89-98.

Rice, M., Breen, C.J., O'Meara, A., Breatnach, F., O'Marcaigh, A.S. & Stallings, R.L. (2000). Comparative genomic hybridization in pediatric acute lymphoblastic leukemia. *Pediatric Hematology Oncology*, **17,** 141-147.

Rinsky, A., Rinsky, M.S., Smith, A.B., Hornung, R., Filloon, T.G., Young, R.J., Okun, A.H. & Landrigan, P.J. (1987). Benzene and leukaemia. An epidemiologic risk assessment. *New England Journal of Medicine.*, **316,** 1044-1050.

Rohatiner, A. & Lister, T.A.L. (1996). Acute myelogenous leukemia in adults. In *Leukemia,* Henderson, E.S., Lister, T.A. & Greaves, M.F. (eds), Vol. 6. pp. 479-507. W.B.Saunders Company: Philidelphia.

Rowe, J.M. (2000). Treatment of acute myelogenous leukemia in older patients. *Leukemia*, **14,** 480-487.

Rowe, J.M., Andersen, J.W., Mazza, J.J., Bennett, J.M., Paietta, E., Hayes, F.A., Oette, D., Cassileth, P.A., Stadtmauer, E.A. & Wiernik, P.H. (1995). A randomized placebo-controlled phase III study of granulocyte- macrophage colony-stimulating factor in adult patients (> 55 to 70 years of age) with acute myelogenous leukemia: a study of the Eastern Cooperative Oncology Group (E1490). *Blood*, **86,** 457-62.

Rowe, J.M., Neuberg, D., Friedenberg, F., Bennett, J.M., Paietta, E., Makary, A., Tallman, M.S. & Wiernik, P.H. (1998). A Phase III study of daunorubicin vs idarubicin vs mitoxantrone for older adult patients (>55 yrs) with acute myelogenous leukemia (AML): A study of the Eastern Cooperative Oncology Group (E3993). *Blood*, **92,** 1284a.

Rowley, J. (1973). A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and giemsa staining. *Nature*, **243,** 290-293.

Rowley, J.D., Reshmi, S., Carlson, K. & Roulston, D. (1999). Spectral karyotype analysis of T-cell acute leukemia. *Blood*, **93,** 2038-42.

Rubin, E.H., Andersen, J.W., Berg, D.T., Schiffer, C.A., Mayer, R.J. & Stone, R.M. (1992). Risk factors for high-dose cytarabine neurotoxicity: an analysis of a Cancer and Leukemia Group B trial in patients with acute myeloid leukemia. *Journal of Clinical Oncology*, **10,** 948-53.

Rubnitz, J.E. & Look, A.T. (1998). Molecular genetics of childhood leukemias. *Journal of Pediatric Hematology and Oncology*, **20,** 1-11.

Sawyer, J.R., Lukacs, J.L., Munshi, N., Desikan, K.R., Singhal, S., Mehta, J., Siegel, D., Shaughnessy, J. & Barlogie, B. (1998). Identification of new nonrandom translocations in multiple myeloma with multicolor spectral karyotyping. *Blood*, **92,** 4269-78.

Schichman, S., Canaani, E. & Croce, C. (1995). Self-fusion of the *ALL1* gene: A new genetic mechanism for acute leukemia. *Journal of the American Medical Association*, **273,** 571-576.

Schiffer, C.A., Lee, E.J., Tomiyasu, T., Wiernik, P.H. & Testa, J.R. (1989). Prognostic impact of cytogenetic abnormalities in patients with de novo acute non-lymphocytic leukemia. *Blood*, **73,** 263-70.

Schoch, C., Haase, D., Fonatsch, C., Haferlach, T., Loffler, H., Schlegelberger, B., Hossfeld, D.K., Becher, R., Sauerland, M.C., Heinecke, A., Wormann, B., Buchner, T. & Hiddemann, W. (1997). The significance of trisomy 8 in de novo acute myeloid leukaemia: the accompanying chromosome aberrations determine the prognosis. German AML Cooperative Study Group. *British Journal of Haematology*, **99,** 605-11.

Schrock, E., du Manoir, S., Veldman, T., Schoell, B., Wienberg, J., Ferguson-Smith, M.A., Ning, Y., Ledbetter, D.H., Bar-Am, I., Soenkesen, D., Garini, Y. & Ried, T. (1996). Multicolor spectral karyotyping of human chromosomes. *Science*, **273**, 494-497.

Shurtleff, S.A., Buijs, A., Behm, F.G., Rubnitz, J.E., Raimondi, S.C., Hancock, M.L., Chan, G.C., Pui, C.H., Grosveld, G. & Downing, J.R. (1995). TEL/AML1 fusion resulting from a cryptic t(12;21) is the most common genetic lesion in pediatric ALL and defines a subgroup of patients with an excellent prognosis. *Leukemia*, **9**, 1985-9.

Sievers, E.L., Appelbaum, F.A., Spielberger, R.T. & et.al. (1997). Selective ablation of acute myeloid leukemia using anti-CD33 calciheamicin immunoconjugate. *Blood*, **90**, 504a.

Sievers, E.L., Larson, E., Estey, E. & et.al. (1998). Interim analysis of the efficacy and safety of CMA-676 in patients with AML in first relapse. *Blood*, **92**, 613a.

Singal, R. & Ginder, G.D. (1999). DNA methylation. *Blood*, **93**, 4059-4070.

Sorensen, J.T., Gerald, K., Bodensteiner, D. & Holmes, F.F. (1993). Effect of age on survival in acute leukemia. 1950-1990. *Cancer*, **72**, 1602-6.

Southam, L., Craver, L., Daregeon, H. & Burchenal, J. (1951). Study of the natural history of acute leukemia with special reference to the duration of the disease and the occurence of remissions. *Cancer*, 39-59.

Speicher, M.R., Gwyn Ballard, S. & Ward, D.C. (1996). Karyotyping human chromosomes by combinatorial multi-fluor FISH. *Nature Genetics*, **12**, 368-75.

Stark, B., Resnitzky, P., Jeison, M., Luria, D., Blau, O., Avigad, S., Kodman, Y., R, G., S, A. & etal. (1995). A distinct subtype of M4/M5 acute myeloblastic leukemia (AML) associated with t(8;16)(p11;p13), in a patient with the variant t(8;19)(p11;13): A case report and review of the literature. *Leukemia Research*, **19,** 367-379.

Stasi, R., Venditti, A., Del Poeta, G., Aronica, G., Dentamaro, T., Cecconi, M., Stipa, E., Scimo, M., Masi, M. & Amadori, S. (1996). Intensive treatment of patients age 60 years and older with de novo acute myeloid leukaemia analysis of prognostic factors. *Cancer*, **77,** 2476-2488.

Stone, R., Berg, D., George, S. & etal. (1995). Granulocyte-macrophage colony-stimulating factor after initial chemotherapy for elderly patients with primary acute myelogenous leukemia. *New England Journal of Medicine*, **332,** 1671.

Stone, R.M. (1999). Leukemia in the elderly. In *Hematology:The American Society of Hematolgy Education Program book* pp. 510-516. American Society of Hematology.

Strefford, J.C., Lillington, D.M., Young, B.D. & Oliver, R.T.D. The use of multi-colour fluorescence technologies in the characterisation of prostate carcinoma cell lines: A comparison of multiplex-FISH and spectral karyotyping data. *in press*.

Swansbury, G.J., Lawler, S.D., Alimena, G., Arthur, D., Berger, R., Van den Berghe, H., Bloomfield, C.D., de la Chappelle, A., Dewald, G., Garson, O.M. & et al. (1994). Long-term survival in acute myelogenous leukemia: a second follow-up of the Fourth International Workshop on Chromosomes in Leukemia. *Cancer Genet Cytogenet*, **73,** 1-7.

Tallman, M., Hakimian, D., Shaw, J., Lissner, G., Russell, E. & Variakojis, D. (1993). Granulocytic sarcoma is associated with the 8;21 translocation in acute myeloid leukemia. *Journal of Clinical Oncology*, **11,** 690-697.

Taylor, P.R., Reid, M.M., Bown, N., Hamilton, P.J., Proctor, S.J. (1995). De novo acute myeloid leukaemia in patients over 55-years-old. A population-based study of incidence, treatment and outcome. *Leukemia*, **9**, 231-237.

Thomas, E., Storb, R., Clift, R. & et.al. (1975). Bone-marrow transplantation. *New England Journal of Medicine*, **292**, 835.

Tosi, S., Giudici, G., Rambaldi, A., Scherer, W., Bray-Ward, P., Dirscherl, L., Biondi, A. & Kearney, L. (1999). Characterisation of the human myeloid leukemia-derived cell line GF-D8 by multiplex fluorescence *in-situ* hybridization, subtelomeric probes, and comparative genomic hybridization. *Genes, Chromosomes and Cancer*, **24**, 213-221.

UKCCCR. (2000). Position paper by the UKCCCR elderly cancer patients in clinical trials working group. *British Journal of Cancer*, **82**, 1-3.

Veldman, T., Vignon, C., Schrock, E., Rowely, J.D. & Thomas, R. (1997). Hidden chromosome abnormalities in haematological malignancies detected by multicolour spectral karyotyping. *Nature Genetics*, **15**, 406-410.

Vidal, C., Baer, M.R. & Bloomfield, C.D. (1999). Uncommon patterns of presentation of leukemia. *Hematology and Oncology*, **17**, 11-29.

Virchow, R. (1846). Weisses Blut und Milztumoren. *Med Z*, **15**, 157.

Wall, A.M., Gajjar, A., Mahmoud, H., Pui, C.H. & et.al. (2000). Individualized methotrexate dosing in children with relapsed acute lymphoblastic leukemia. *Leukemia*, **14**, 221-5.

Walters, R.S., Kantarjian, H.M., Keating, M.J., Estey, E.H., McCredie, K.B. & Freireich, E.J. (1987). Intensive treatment of acute leukemia in adults 70 years of age and older. *Cancer*, **60**, 149-55.

Wilkens, L., Tchinda, J., Burkhardt, D., Nolte, M., Werner, M. & Georgii, A. (1998). Analysis of hematologic diseases using conventional karyotyping, fluorescence in situ hybridization (FISH), and comparative genomic hybridization (CGH). *Human Pathology*, **29,** 833-9.

Yunis, J.J., Bloomfield, C.D. & Ensrud, K. (1981). All patients with acute non-lymphocytic leukemia may have a chromosomal defect. *New England Journal of Medicine*, **305,** 135-9.

Zhang, F.F., Murata-Collins, J.L., Gaytan, P., Forman, S.J., Kopecky, K.J., Wilman, C.L., Appelbaum, F.R. & Slovak, M.L. (2000). Twenty-four-colour spectral karyotyoping reveals chromosome aberrations in cytogenetically normal acute myeloid leukemia. *Genes, Chromosomes and Cancer*, **28,** 318-328.

Zojer, N., Konigsberg, R., Ackermann, J., Fritz, E., Dallinger, S., Kromer, E., Kaufmann, H., Riedl, L., Gisslinger, H., Schreiber, S., Heinz, R., Ludwig, H., Huber, H. & Drach, J. (2000). Deletion of 13q14 remains an independent adverse prognostic variable in multiple myeloma despite its frequent detection by interphase fluorescence in situ hybridization. Multicenter investigation of bone marrow transplantation for Sickle cell disease. *Blood*, **95,** 1925-30.

Zubrod, C.G. (1979). Historic milestones in curative chemotherapy. *Seminars in Oncology*, **6,** 490-505.

Zwiebel, J.A. (2000). New agents for acute myelogenous leukemia. *Leukemia*, **14,** 488-490.

Harrison, C.J., Gibbons, B., Yang, F., Cheung, K.L., Kearney, L., Dircherl, L., Bray-Ward, P., Gregson, M., Ferguson-Smith, M. (2000). Multiplex fluorescence in situ hybridisation and cross species color banding of a case of chronic myeloid leukemia in blastic crisis with complex Philidelphia translocation .*Cancer Genetics and Cytogenetics*,**116,** 105-110.