IDENTIFICATION AND CHARACTERIZATION OF NOVEL GENETIC ABNORMALITIES IN ACUTE MYELOID LEUKAEMIA AND THEIR PROGNOSTIC IMPACT ON PATIENTS OUTCOME

Thesis

Submitted for Partial Fulfillment of
M.D Degree in Clinical & Chemical Pathology

BY

Nervana Mahmoud Ahmed Abou-Zeid
M.B.B.Ch., M.Sc.(Ain Shams University)
Assistant Lecturer of Clinical & Chemical Pathology
Ain Shams University

Supervised by

Professor Doctor/ Nehayet Mahmoud Azmy
Professor of Clinical & Chemical Pathology
Faculty of Medicine - Ain Shams University

Doctor / Michael John Griffiths
Consultant Clinical Cytogeneticist -Deputy Director &
Head of Oncology Department, WMRGL
Birmingham Women's Hospital
Honorary Senior Lecturer, Division of Cancer Studies
University of Birmingham, UK

Professor Doctor/ Sahar Samir Abdel Maksoud
Professor of Clinical & Chemical Pathology
Faculty of Medicine - Ain Shams University

Doctor/ Dina Aziz Khattab
Assistant Professor of Clinical & Chemical Pathology
Faculty of Medicine - Ain Shams University

Doctor/ Mahira Ismail El Mogy
Lecturer of Clinical & Chemical Pathology
Faculty of Medicine - Ain Shams University

Faculty of Medicine - Ain Shams University

Cairo, 2010

تحديد وتمييز الجينات الوراثية الحديثة الشاذة فى مرض سرطان الدم النخاعى الحاد و وقعهم التنبؤى على مآل المرضى

رسالة توطئة للحصول على درجة الدكتوراه
فى الباثولوجيا الإكلينيكة و الكيميائية
مقدمــة مـــن

الطبيبة/ نرفانا محمود أحمد أبوزيد
بكالوريوس الطب والجراحة-ماجستير الباثولوجيا الإكلينيكية و الكيميائية
مدرس مساعد الباثولوجيا الإكلينيكية و الكيميائية
كلية الطب- جامعة عين شمس

تحت إشراف

الأستاذ الدكتور/ نهايت محمود عزمى
أستاذ الباثولوجيا الإكلينيكية و الكيميائية
كلية الطب – جامعة عين شمس

الدكتور/ مايكل جون جريفث
إستشارى البحوث الجينية الإكلينيكية
و رئيس قسم الأورام بوحدة البحوث الجينية
بمستشفى برمنجهام
و مدرس بوحدة أبحاث السرطان
جامعة برمنجهام- إنجلترا

الأستاذ الدكتور/ سحر سمير عبد المقصود
أستاذ الباثولوجيا الإكلينيكية و الكيميائية
كلية الطب – جامعة عين شمس

الدكتور/ دينا عزيز خطاب
أستاذ مساعد الباثولوجيا الإكلينيكية و الكيميائية
كلية الطب – جامعة عين شمس

الدكتور/ مهيره إسماعيل الموجى
مدرس الباثولوجيا الإكلينيكية و الكيميائية
كلية الطب – جامعة عين شمس

كليـة الطـب- جامعـة عيـن شمـس
القاهرة ٢٠١٠

INTRODUCTION

The majority of patients diagnosed with acute myeloid leukaemia (AML) display acquired cytogenetic aberrations at presentation. Numerous recurring chromosomal abnormalities have been and continue to be identified in AML. In many instances, genes altered by these aberrations have been cloned, providing insights into the mechanisms of leukaemogenesis and paving the way to designing novel therapeutic strategies that target specific genetic abnormalities in leukemic blasts ***(Japanese Society of Hematology, 2000).***

Studying AML patients revealed that leukaemic cells of each major known prognostic subtype had a specific "signature" of gene expression. The exact signature depended on the underlying genetic mutation that contributed to the formation and growth of the leukaemic cells; and it corresponded to over- or under-expression of sets of genes from leukaemic cells as compared to their expression in normal white blood cells ***(James, 2006).***

Moreover, karyotypic abnormalities, whether molecularly characterized or not, are among the most important independent prognostic factors in AML and are being used in the classification and clinical management of AML patients ***(Balkhi et al., 2006).***

Current cytogenetic methods (e.g., G-banding and multicolor chromosomal painting) allow detection of translocation events but lack the resolution to (a) locate the breakpoints precisely at the chromosome band level or (b) discriminate balanced translocations from translocations with copy number alterations not previously reported, or imperfectly balanced translocations ***(Watson et al., 2006).***

Fluorescence in situ Hybridization (FISH) is a molecular cytogenetic technique that can detect chromosomal

abnormalities that cannot be appreciated by standard chromosomal analysis or when mitotic cells are not available for chromosomal analysis also it was demonstrated to be more sensitive than G-banding analysis in the detection of some rearrangements in acute myeloid leukaemias ***(Cavazzini et al., 2006).*** It can clarify suspected abnormalities identified in banded karyotypes, identify the presence of complex or cryptic cytogenetic abnormalities, or provide cytogenetic information even when banded karyotype data is not available ***(Shali et al., 2006).***

Recent molecular analysis of leukaemic blasts with application of gene-expression profiling has also identified a gene-expression signature that appears to separate the cytogenetically normal AML patients into prognostic subgroups ***(Mrozek et al., 2007).*** Single nucleotide polymorphism (SNP) microarrays attempt to identify DNA sequence variants in specific genes or regions of the human genome that are responsible for a variety of phenotypic traits and also identify chromosome copy number aberrations at high-resolution independent of chromosome morphology quality, loss of heterozygosity, uniparental disomy and determining parental origin of alleles in families ***(Nusrat and Terence, 2006).***

Recently the West Midlands Regional Genetic Laboratory and others have established that acquired isodisomy (AI), sometimes more loosely called acquired uniparental disomy, occurs frequently in myeloid leukaemia. AI identifies sites of duplication of cancer causing gene mutations, which are not detectable by karyotype or FISH analysis, but may be assessed using Affymetrix SNP arrays ***(Griffiths et al., 2005; Raghavan et al., 2005).***

AIM OF THE WORK

The aim of our work is to:

- Detect cytogenetic aberrations in AML patients by Conventional Cytogenetic Analysis (G- banding technique).
- Identify novel genetic abnormalities in those patients by Single Nucleotide Polymorphism Microarrays (SNP) to be confirmed by Fluorescence In Situ Hybridization technique (FISH).
- Assess the relation of each novel abnormality to the clinical status of the disease.

ACUTE MYELOID LEUKAEMIA

Acute myeloid leukaemia is a rapidly progressing disease that affects mostly cells that are unformed or immature yielding them unable to carry out their normal functions.

It is an extremely heterogeneous disorder and occurs at any time of life, with average incidence being 2.3 per 100,000 people per year. It usually occurs (80%) in adults with the majority of cases occurring in people over the age of 60 ***(Greer et al., 2004).***

AML is more common in men than in women; however, it can occur in children especially if there is a predisposition for leukaemia. One third of all childhood cancers are leukaemia with approximately 400 new cases occurring each year in the United Kingdom, most of these cases suffer from acute lymphoblastic leukaemia (ALL). Similarly, among Egyptian patients ALL is the most common childhood cancer accounting for approximately 80% of leukaemias ***(Khalifa et al., 1999)*** with a higher incidence in males than females (male to female ratio 1.7: 1 to 2.1: 1) ***(Gadalla, 1996)*** while less than a quarter of these cases are AML.

Predisposing Factors:

Although several factors have been implicated in the causation of AML, most patients who present with de novo AML have no identifiable risk factor.

Antecedent Haematologic Disorders (AHD):

Myelodysplastic syndrome (MDS) is the most common among this category, especially high risk MDS (eg, refractory anemia with excess blasts type-2). Other AHD that predispose to AML include aplastic anemia, myelofibrosis, paroxysmal

nocturnal haemoglobinuria, and polycythemia vera ***(Appelbaum et al., 2006).***

Genetic factors:

It includes genetic defects and marrow failure syndromes *(Table 1).*

Table (1): Genetic disorders implicated in the pathogenesis of AML

Congenital defects	*Marrow failure syndromes*
Down syndrome	Fanconi anaemia
Bloom syndrome	Dyskeratosis congenital
Monosomy 7 syndrome	Shwachman-Diamond syndrome
Klinefelter syndrome	Amegakaryocytic thrombocytopenia
Turner syndrome	Blackfan-Diamond syndrome
Neurofibromatosis	Kostmann agranulocytosis
Congenital dysmorphic syndromes	Familial aplastic anaemia

(Greer et al., 2004).

Environmental factors:

These are presented in *(Table 2).*

Table (2): Environmental factors contributing to AML

Solvents (benzene)
Smoking
Ionizing radiation
- Atomic bomb exposure
- Nuclear power exposure
- Medical radiation

Non-ionizing radiation (?)
Chemotherapy
- ***Alkylating agents***
- ***Topoisomerase II inhibitors***

Other drugs: Chloramphenicol- Phenylbutazone

(Greer et al., 2004).

Diagnosis:

1-General symptoms and signs:

Most of the symptoms are due to the increased number of white blood cells replacing the normal marrow cell production. This makes the patient tired, pale, has shortness of breath, tendency for easy bruising , bleeding with minor trauma and susceptible to infections.

Early symptoms are usually non specific and similar to influenza with fever, malaise, fatigue, loss of weight, loss of appetite, bone and joint pain. Enlargement of the spleen may occur but is typically mild and asymptomatic, lymph node enlargement is rare unlike ALL, skin involvement occurs in 10% of cases in the form of leukaemia cutis ***(Greer et al., 2004)***.

Rarely, Sweet's syndrome; a paraneoplastic inflammation of the skin can occur with AML. Moreover, some patients may experience gum swelling due to infiltration by leukaemic cells, chloroma which is a solid leukaemic mass outside the bone marrow. Occasionally, a person may show no symptoms and the leukaemia is discovered accidently during routine blood film examination ***(Hoffmann et al., 1998).***

2- Laboratory diagnosis:

a) Blood picture:

The first clue to a presumptive diagnosis is abnormal blood film results. The total leucocytic count (TLC) may range from less than $1x10^3/mm^3$ to more than $200x10^3/mm^3$. Neutropenia with absolute neutrophil count less than 1000/ mm^3 is present in over half of patients at time of diagnosis ***(Miller and Daoust, 2000).*** Blasts are almost always present in peripheral blood ranging from 3-95% of total leucoctes but in

leucopenic patients they may be infrequent (aleukaemic leukaemia). Auer rods which are pathognomonic of AML are present in the blast cells of about one quarter of patients ***(Hoffmann et al., 1998).***

Anaemia is a constant feature in AML and is predominantly normocytic normochromic, however normal haemoglobin level may be present and it indicates rapidly proliferating leukaemia, very acute onset and bad prognosis ***(Braham et al., 2006).***

Thrombocytopenia is present in about 92% of AML patients at diagnosis. It is a combination of inadequate production and decreased platelet survival. Platelet count is usually $<5x10^3/mm^3$ at time of diagnosis. Thrombocytopenia may be associated with disseminated intravascular coagulopathy which is common in Acute Promyelocytic Leukaemia (APL) ***(Braham et al., 2006).***

b) <u>Bone marrow examination:</u>

Definitive diagnosis usually requires bone marrow aspiration and biopsy, marrow films are sent for leishman's and peroxidase staining and examined under light microscopy for blast type and a sample is sent to immunophenotypic studies to diagnose the type of leukaemia and classify the subtype *(Table 3).* A sample of marrow or blood should also be tested for chromosomal translocations by cytogenetic studies or FISH for specific chromosomal imbalances which affect prognosis and treatment ***(Miller and Daoust, 2000).***

According to the World Health Organization (WHO) criteria, the diagnosis of AML is established by the presence of 20% or more myeloblasts in blood and/or marrow sample. AML should be differentiated properly from preleukaemic conditions such as MDS or myeloproliferative disorders (MPDs) which are treated differently ***(Vardiman et al., 2009).***

Table (3): Immunophenotyping of AML cells

Marker	Lineage
CD13	**Myeloid**
CD33	**Myeloid**
CD34	**Early precursor**
HLA-DR	**Positive in most AML, negative in APL**
CD11b	**Mature monocytes**
CD14	**Monocytes**
CD41	**Platelet glycoprotein IIb/IIIa complex**
CD42a	**Platelet glycoprotein IX**
CD42b	**Platelet glycoprotein Ib**
CD61	**Platelet glycoprotein IIIa**
Glycophorin A	**Erythroid**
TdT	**Usually indicates acute lymphocytic leukaemia, however, may be positive in M0 or M1**
CD11c	**Myeloid**
CD117 (c-kit)	**Myeloid/stem cell**
CD56	**NK-cell/stem cell**

(Swirsky and Bain, 2001).

Classification:

The 2 most widely used classification schemata are the French – American –British (FAB) system *(Table 4)(Photos 1-7)* and the WHO system.

Table (4): FAB classification of AML

FAB category	*Criteria for FAB typing*	*Cytochemistry*
M0 (AML with minimal evidence of myeloid maturation)	• Morphologically undifferentiated blasts. • Myeloid phenotype. • Lymphoid markers negative.	• <3% blasts positive for SBB or MP0. • No Auer rods present on SBB or MP0. • Esterases negative in blast cells.

FAB category	*Criteria for FAB typing*	*Cytochemistry*
M1 (AML without maturation)	• Blasts >90% of BM non-erythroid cells (NEC) • Maturing monocytic cells <10% • Maturing granulocytes <10%	• 3-100% blasts positive for SBB or MP0. • Localized pattern of positivity. • SBB- or MP0-positive. • Chloroacetate esterase-positive cells <10%. • NSE- or BE-positive cells scanty or absent.
M2 (AML with maturation)	• Blasts 30-89% of NEC. • Maturing granulocytes >10% of NEC. • Monocytic component <20%.	• 3-100% blasts positive for SBB or MP0. • SBB- or MP0-positive Auer rods frequently present. • SBB- or MP0-negative neutrophils may be present if dysplastic. • Chloroacetate esterase-positive cells >10% (maturing granulocytes). • NSE- or BE-positive cells scanty or absent.
M3 (hypergranular promyelocytic leukemia) M3 variant (hypergranular promyelocytic leukemia)	• M3 shows marrow replacement by granular and hypergranular promyelocytes. • M3 variant shows mainly agranular basophilic cells, bilobed nuclei.	• SBB and MP0 show characteristic heavy staining filling the cytoplasm. • Multiple SBB- pr MP0-positive Auer rods present, often obscured by heavy cytoplasmic staining. • Majority of cells are chloracetate esterase-positive. • Auer rods are chloracetate esterase-positive. • Leukaemic promyelocytes show a deep pink cytoplasmic blush with PAS stain. • t(15;17) demonstrable by cytogenetics, RT-PCR or FISH.

FAB category	*Criteria for FAB typing*	*Cytochemistry*
M4 (acute myelomonocytic leukaemia)	• Blasts <30% of NEC. • Granulocyte component >20% of BM NEC. • Monocytic component >20% of BM NEC.	• Esterase stains show a mixture of chloroacetate- and NSE-positive cells, usually >20% of each. • Some cells may show both types of esterase. • 3-100% blasts positive for SBB- or MP0, localized pattern in the myeloblasts, scattered pattern in the monoblasts/monocytes. • SBB- or MP0-positive Auer rods common. • SBB- or MP0-negative neutrophils if dysplasia present.
M4E0	• Typical,large "eosinobasophils" present which define presence of inv or t(16).	• Usually conforms to M4 by conventional criteria. • inv/del/t(16) demonstrable by cytogenetics, RT-PCR or FISH.
M5 and M5b (acute monoblastic leukaemia without maturation [M5a] and with maturation [M5b])	• Blasts <30% of NEC. • <80% monocytic component of BM NEC. • M5a when monoblasts >80% of BM NEC.	• Usually >80% of BM cells show NSE or BE positivity. • Chloroacetate esterase-positive cells usually rare, but always <20%. • SBB and MP0 may be negative in the blasts/monocytes. • SBB- and MP0-positive Auer rods rare.
M6 (erythroleukaemia)	• Erythroid cells (all stages) >50% of BM nucleated cells. • Myeloid blasts <30% of BM NEC.	• Some/many erythroid precursors positive on PAS stain, rarely all negative. • Some myeloid blasts SBB- or MP0-positive.

FAB category	*Criteria for FAB typing*	*Cytochemistry*
		• SBB- and MP0-positive Auer rods occasionally present.
M7 (acute megakaryoblastic leukaemia)	• Blasts,mainly megakaryoblasts shown by,immunological methods.	• Immunological confirmation of megakaryocytic blasts required. • Trephine biopsy may be helpful. • Megakaryoblasts may show platelet-like granules on PAS stain. • Focal NSE (but not BE) positivity may be present. • Myeloid blasts may show SBB or MP0 positivity and rarely Auer rods.

NEC, non erythroid cells; *BE, butyrate esterase; MPO, myeloperoxidase; NSE, non- specific esterase; PAS, periodic acid- Sciff.*

(Swirsky and Bain, 2001).

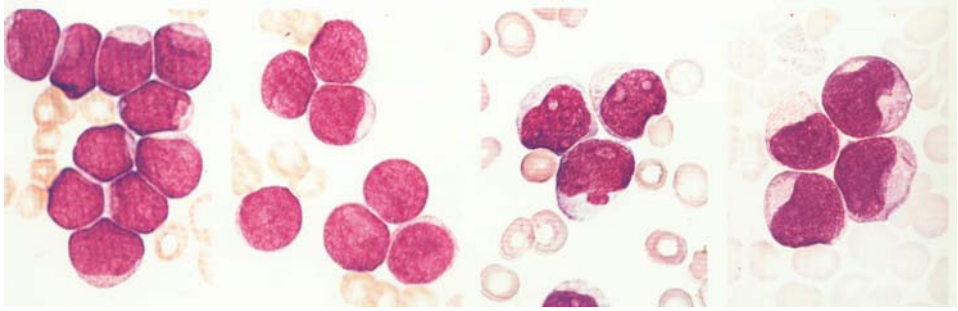

Photo (1): AML, M0/M1 subtype

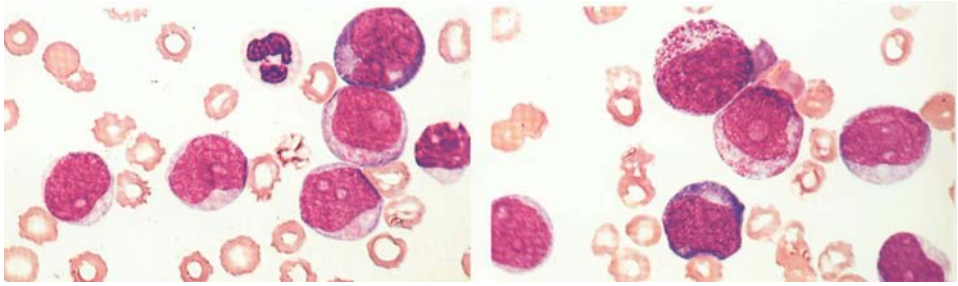

Photo (2): AML, M2 subtype

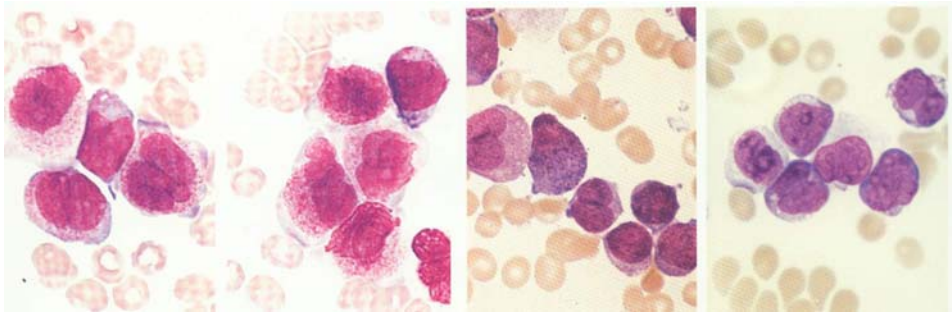

Photo (3): AML, M3 subtype

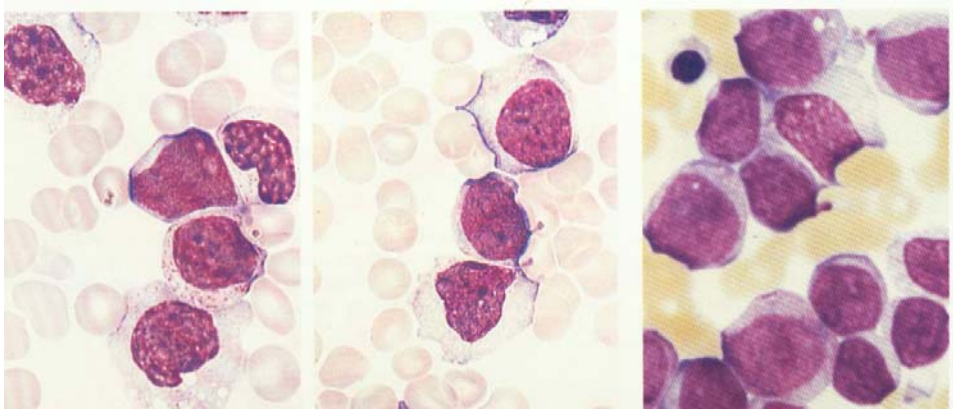

Photo (4): AML, M4 subtype