The role of homeobox gene in leukaemia

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by

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Abstract

Homeobox genes are known to be active during development and they are turned off after the early stages of developmental life. The *HLXB9*/*MNX1* gene is a homeobox gene localized on human chromosome 7 and is involved in the development of pancreas and the nervous system. However, some leukaemia research groups have reported an over-expression of *HLXB9* in leukaemia patients who carry the t(7;12) and in the GDM-1 cell line that carries the t(6;7). The mechanisms of leukaemogenesis in t(7;12) patients are still unclear. The t(7;12) is one of the recurrent cytogenetic abnormalities that is associated with infant acute myeloid leukaemia (AML) patients and has been linked to poor prognosis. The aim of this study was (i) to determine the involvement of *HLXB9* in cell lines known to express this gene at the transcript level and (ii) to investigate the position on *HLXB9* in AML patients with abnormalities of chromosome 7. This aim was achieved through a series of experiments involving the use of both conventional and molecular cytogenetics.

In the first place, the chromosomal abnormalities in leukaemia and lymphoma cell lines (GDM-1, K562 and Pfeiffer) have been analysed using G-banding and Multiplex FISH (M-FISH) techniques.

Furthermore, FISH using whole chromosome painting technique was performed on 7 AML patients to investigate chromosome 7 rearrangements.

Thirdly, the involvement of the homeobox gene *HLXB9* has been investigated in the acute myeloid leukaemia (AML) derived cell line GDM-1 and in 4 AML patients. Fluorescence in situ hybridization (FISH) analysis was carried out using a specific probe for the *HLXB9* gene on the AML patients in single and dual colour FISH in combination with an additional probe distal to *HLXB9* on the GDM-1 cell line. FISH analysis showed no involvement of the *HLXB9*

gene in any rearrangement or breaks at chromosomal level on the AML cell line (GDM-1) and AML patients. Nevertheless, a breakpoint either proximal or distal to *HLXB9* has been identified.

In particular, the breakpoint in the GDM-1 cell line has been confirmed on between the two probes used. This thesis poses the basis for further studies to investigate the mechanisms of oncogenesis in leukaemias with over-expression of *HLXB9* in relation to possible breakage of chromosome 7 in the vicinity of the gene.

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List of Abbreviations

AML	Acute myeloid leukaemia
bp	Base pairs
BSA	Bovine serum albumin
C^{o}	Degree centigrade
cm	Centimetre
CO_2	Carbon dioxide
Cy3	Cyanine 3
DAPI	4, 6-Diamidino-2-phenylindole
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleoside triphosphates
dTTP	Deoxythymidine triphosphate
dUTP	Deoxyuridine trinucleotide phosphate

EDTA	Ethylenediamenetetra- acetic acid
FAB	French-American-British
FCS	Foetal calf serum
FISH	Fluorescence in situ hybridisation
FITC	Fluorescein isothyiocynate
KCL	Potassium chloride
Mb	Millions of base pairs
µg	Microgram
MGCL2	Magnesium Chloride
ml	Millilitres
µl	Microlitre
MNX1	Motor neuron and pancreas homeobox1
NAAC	Sodium acetate
No.	Number
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RPM	Rotations per minute
SSC	Sodium Saline Citrate
Tris	Tris (hydroxymethyl) methanamine

Tween 20	Polyoxyethylene sorbitan monolaurate
UV	Ultraviolet
v/v	Volume / volume

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CHAPTER 1

INTRODUCTION

1. Introduction

1.1 Normal haematopoiesis and leukaemia

Haematopoiesis is the creation of all types of the blood cells. Blood cells are produced primarily during early embryogenesis in the yolk sac (embryonic haemopoiesis) and in liver and spleen at later stages. During adult life, haemopoiesis is a continuous and active process in the bone marrow (Ebdon et al., 2010).

Stem cells play a significant role in haemopoiesis. These cells are able of self-renewal and differentiate into multiple cell lines depending on physiological needs (Ebdon et al., 2010). (Figure 1.1).

All various types of blood cells are created from one type of blood cells that is called the haematopoietic stem cell (HSC). The HSC cells divide to generate more stem cells or differentiate into myeloid or lymphoid progenitors (Hoang, 2004).

Some leukaemia cases are caused by genetic mutations during blood stem cells production in the foetus (Greaves et al., 2003). Scientists believe that they are the main cellular targets for leukaemogenesis in haemopoiesis and in addition, dysregulation in the normal blood cell formation system could result in haematological disorders.

Apoptosis is another important element that affects leukaemogenesis. Apoptosis maintains the blood homeostasis and keeps the population of blood cells in suitable numbers (Knowles et al., 2005).

Several studies suggest that certain vital genes, such as the HOX Genes that control cell division, proliferation and apoptosis are defective. As a result, surviving abnormal cells can constitute a clone of defective cells that can initiate the development of leukaemia (Greaves et al., 2003).

Chromosomal translocations are likely to happen at a very early stage during embryo development in some cases of childhood leukaemia (Greaves et al., 2003). These genetic abnormalities produce a pre-leukaemia or malignant cell clone, which only turns into leukaemia cells that could be activated later in life by additional mutations, such as gene deletions or external factors. This hypothesis assumed that leukaemia develops under a multistep mechanism (Figure 1.2) (Greaves, 2007; Knudson, 1971).

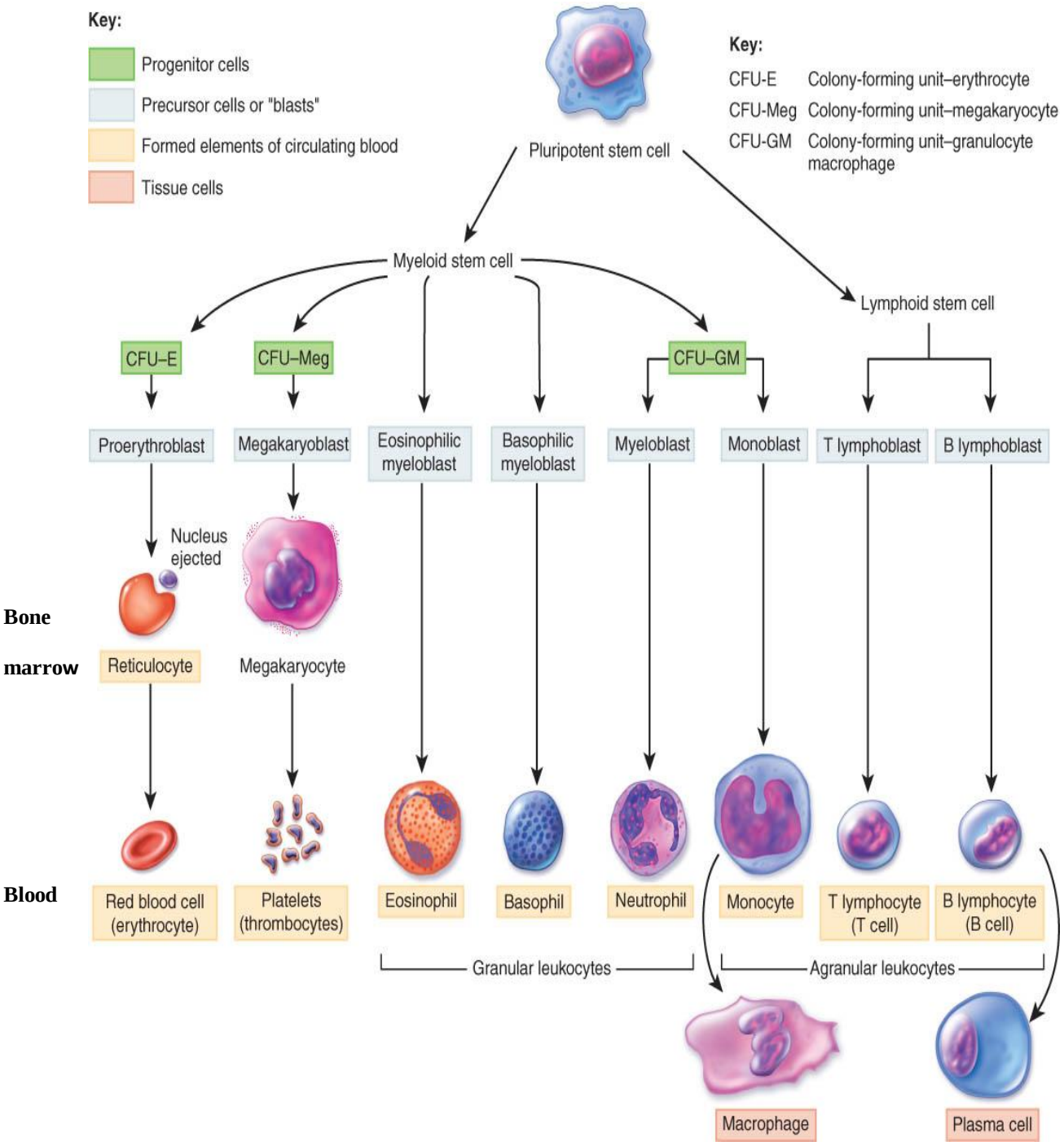

Figure 1.1: Schematic representation shows haemopoiesis process with all the precursors in the bone marrow and the mature blood elements in the peripheral blood (Morrell, 2009).

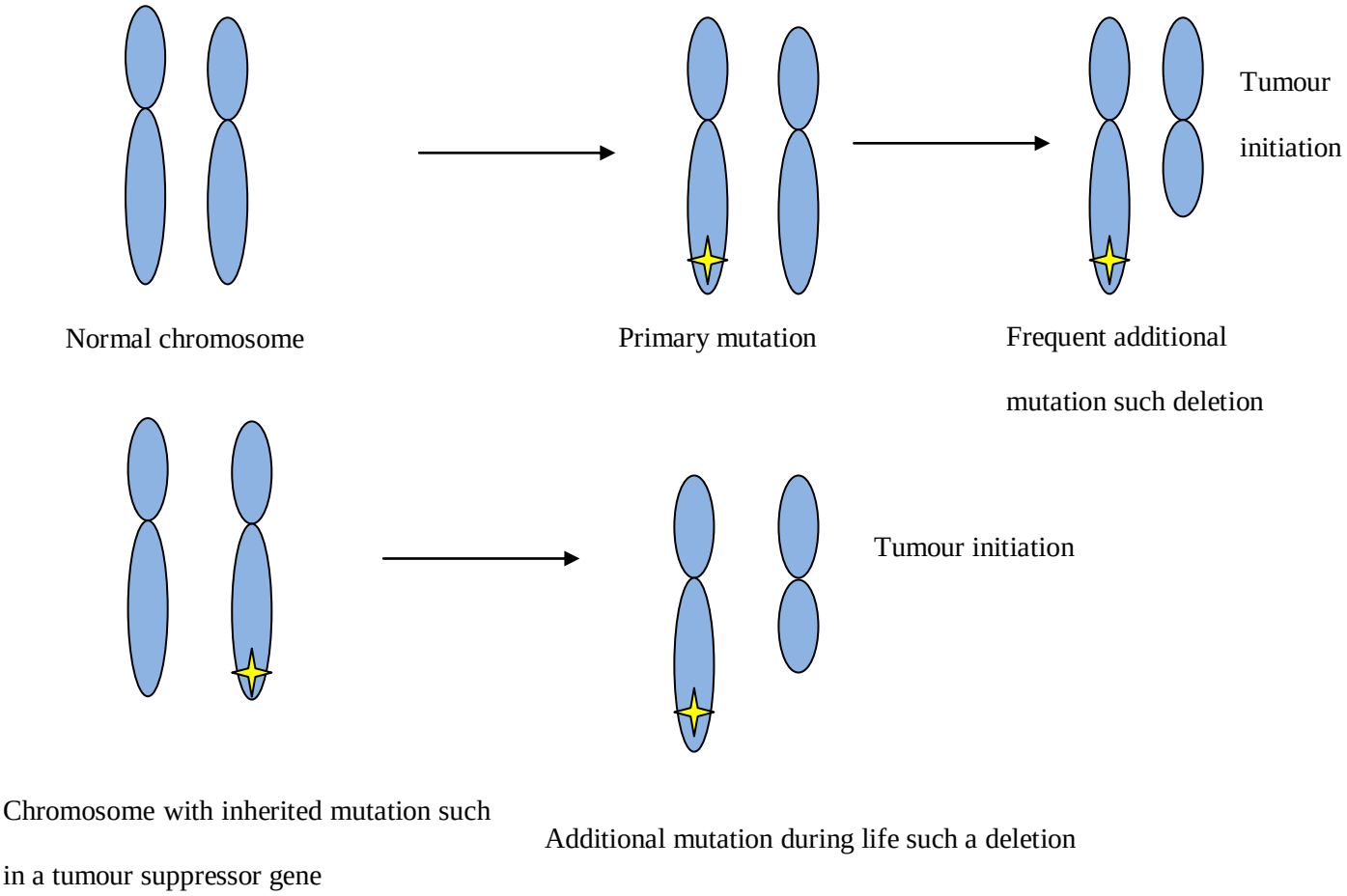

Figure 1.2: Schematic representation of the two hits hypothesis in cancer (Knudson, 1971) that required two genetic changes on both alle to progress to cancer.

1.2 Leukaemia

1.2.1 Definition

Leukaemia is the cancer of bone marrow and blood. It is defined as an increase or decrease in the number of immature white blood cells in the blood and bone marrow depending on which stage of the cell differentiation is blocked. The cancer cells cannot respond to normal control mechanisms and they continue their abnormal cell division. As a result, an abnormal white blood cell count is found in blood and bone marrow of the leukaemia patients. The cancerous cells are formed basically in the bone marrow, and then move into the bloodstream (Ludwig, 2009). It is thought leukaemia is a result of haematopoiesis dysregulation of the blood stem cell.

1.2.2 Classification

Leukaemia is classified into two groups depending on the cell origin: myelogenous or lymphocytic both of which can be acute or chronic (Ludwig , 2009). Thus there are four major types of leukaemia: acute myelogenous leukaemia (AML), acute lymphocytic leukaemia (ALL), chronic myelogenous leukaemia (CML) and chronic lymphocytic leukaemia (CLL) (Ludwig, 2009).

Initially, classification systems of acute and chronic leukaemia relied on cytomorphological and cytochemical findings. Moreover, current classification systems of acute and chronic leukaemias mainly depend on cytomorphology, cytochemistry, immunophenotyping, immunogenetics and molecular cytogenetics (Szczepanski et al., 2003).

The French-American-British (FAB) group that was established in 1976 has proposed a classification of leukaemia based on morphological and cytochemical features. Two main forms of acute leukaemia have been standardized by that organization: acute myeloid leukaemia (AML) and acute lymphoblastic leukaemia (ALL) (Bennett et al., 1976).

Modern classification systems have integrated immunophenotyping for a more accurate description of the haematopoietic lineage and differentiation stage of different kinds of leukaemias. Actually, immunophenotyping is essential for classification of lymphoid malignancies and also critical for the characterization of several subtypes of AML (Szczepanski et al., 2003).The present World Health Organization classification (WHO) of leukaemia and lymphomas includes cytogenetic and molecular characteristics (Szczepanski et al., 2003).

Lymphoid Leukaemia	**Myeloid Leukaemia**
L1 Small monotonous lymphocytes. **L2** Mixed L1- and L3-type lymphocytes. **L3** Large homogeneous blast cells.	**M0** Undifferentiated acute myeloblastic leukaemia. **M1** Myeloblasts without maturation. **M2** Myeloblasts with maturation. **M3** Hypergranular promyelocytic leukemia. **M4** Myelomonocytic leukocytes. **M5** Monocytic, subtype. **M6** Erythroleukemia. **M7** Megakaryocytic leukaemia Pleomorphic undifferentiated cells with cytoplasmic blebs.

Table 1.1: FAB-Classification, The French-American-British (FAB) group that was established in 1976 has proposed a classification of leukaemia based on morphological and cytochemical features.

1.2.3 Childhood leukaemia

Leukaemia forms one-third of all childhood cancer in Great Britain and more than 400 cases of childhood leukaemia are diagnosed every year (Swerdlow et al., 2001).

The most common type of leukaemia in children is Acute Lymphoblastic Leukaemia (ALL), which represents 12% of all leukaemia and 80% in childhood leukaemia (Redaelli, 2005). According to the Leukaemia and Lymphoma Research Organization, ALL is the most common in children of 2- 4 years old and males have a higher risk of being affected. In contrast, AML is less common in children (Table1.2). However, one out of 2000 children has a risk to have acute leukaemia under the age of 15 years (Kersey, 1997).

Acute leukaemia	Chronic leukaemia
Acute lymphoblastic (lymphoid) leukaemia (ALL): 80% of cases of childhood leukaemia	**Chronic myeloid leukaemia (CML):** less than 5% of childhood leukaemia
Acute myeloid leukaemia (AML): most of the remaining cases 17% -15%	**Chronic lymphoblastic leukaemia (CLL):** is unknown in children

Table 1.2: The most common types of leukaemia in childhood according to children with cancer organization, 2012,UK.

1.3 Chromosomes and genetic aberrations in leukaemia

1.3.1 Normal human karyotype

Chromosomes are located in the nuclei of eukaryote cells and contain the inherited materials (DNA) that carry the genetic instructions for living organisms (Strachan and Read, 2004). A normal human cell contains 46 chromosomes that are divided into 23 pairs; 22 pairs of autosome (non–sex) chromosomes and one pair of sex chromosome (XX or XY) for female and male respectively (International System for Human Cytogenetic Nomenclature-ISCN, 2009). The homologous chromosomes (each chromosome in one pair) come from each parent.

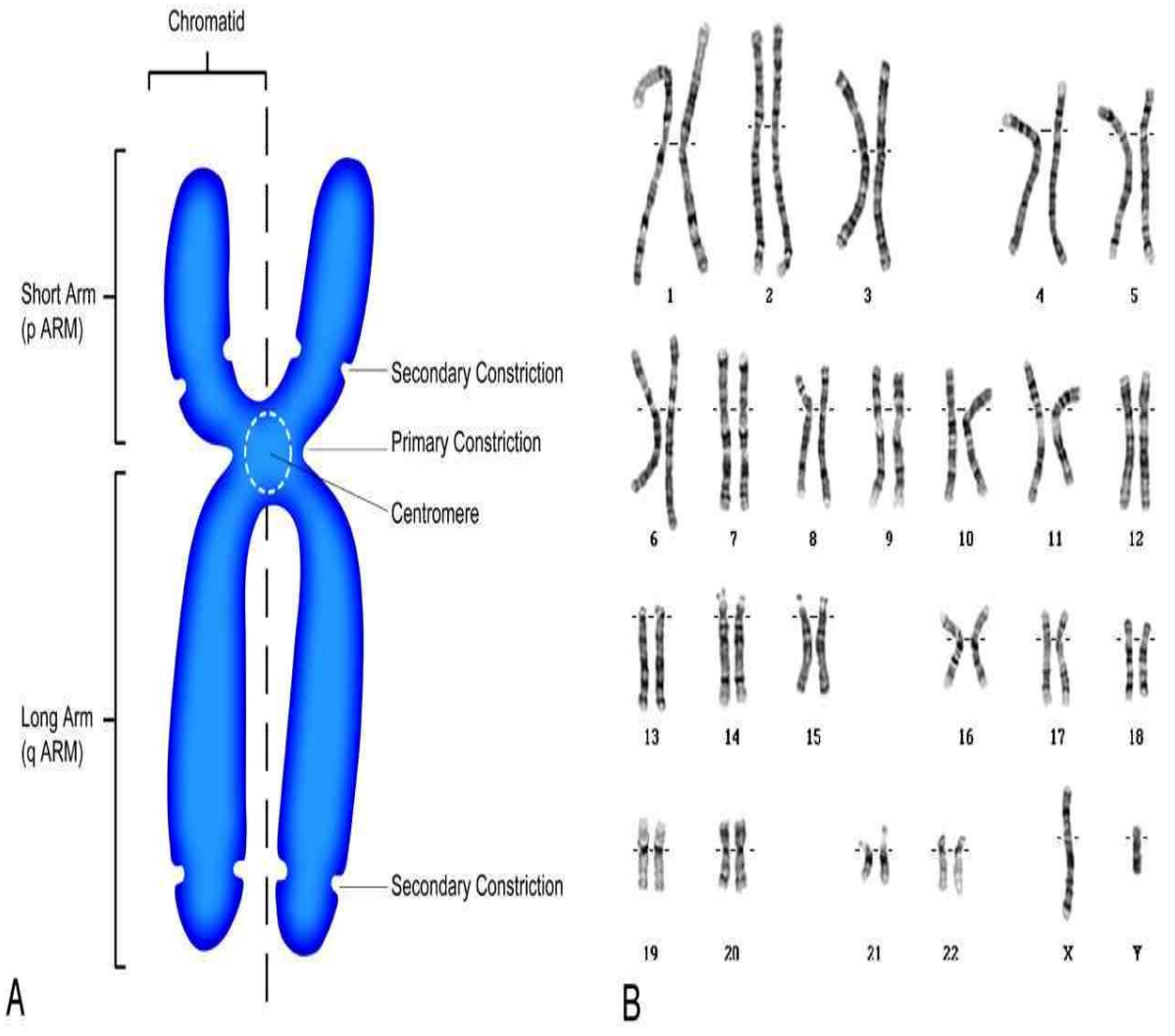

Figure 1.3: (**A**) Schematic drawing of metaphase human chromosome. (**B**) An example of a male normal human karyotype including 22 autosome and a sex pair of (XY) (Wippold and Perry, 2007).

1.3.2 Chromosomal abnormalities

Aberrations in the number and structure of chromosomes are frequently found in tumours (Macdonald and Ford, 1997). A single genetic change is rarely sufficient for the development of a malignant tumour, and evidence suggests a multiple process of accumulating genetic alterations (Croce, 2008). The International System for Human Cytogenetic Nomenclature (ISCN) defines how abnormal and normal chromosomes are described (2009), and examples of terminology of abnormalities that are common as described below, so that chromosome bands are identified by a suggested terminology.

Various chromosomal abnormalities have been associated with haematological disorders (Chetverina and Chetverin, 2010) as seen in (Table1.4).

1.3.2.1 Numerical abnormalities

Numerical chromosomal aberrations are involved in any changes in chromosome numbers in a karyotype. Numerical chromosomal abnormalities can be divided into main groups according to Rooney (2001):

(i) Polyploidy that referred to the gain of whole sets of chromosomes, such as cells with 69 chromosomes (triploid) or 92 chromosomes (tetraploid).

(ii) Aneuploidy (the most common numerical abnormalities) is the l loss or gain of one or a few copies of chromosomes. Common examples of aneuploidy are monosomy (-) chromosome and trisomy (+) chromosome (Rooney, 2001).

1.3.2.2 Structural chromosomal abnormalities and possible mechanisms of leukaemogenesis

Chromosomal Deletion

Chromosomal deletions involve the loss of a segment of a chromosome. The deletions are divided into three main types: terminal, interstitial and microdeletions. Both types of deletions (terminal and interstitial) have been described in detail in chapter 4 of this thesis. Microdeletions are very small interstitial deletions (less than 3 MB of DNA in size) and approximately include one single band within a chromosome only. The chromosomal microdeletions are not detectable by conventional cytogenetic method (G-banding) and can only be detected by FISH using a specific locus probe (Rooney, 2001).There are two possible mechanisms of leukaemogenesis associated with chromosomal deletions (i) gene dosage effect haploinsufficiency and (ii) via tumour suppressor genes.

Haploinsufficiency

Abnormalities of the human Genome are various types such as gain, loss or re-orientation of DNA region contain dosage-sensitive genes. One group of genomic disorder occurs by hemizygous deletions causing a haploinsufficiency (lose of a gene copy) of a single or several genes (O'Driscoll, 2008).

Tumour suppressor genes

Tumour suppressor genes (TSG) are present in normal cells and control cell division, DNA repair and apoptosis. Mutations in tumour suppressor genes are a result of uncontrolled cell growth which can cause a cancer. Numerous tumour suppressor genes have been identified in cancer, for example *TP53* (*p53*) that is altered in more than 50% of human cancers (Komarova, 2003), *RB1* (retinoblastoma susceptibility gene) and *BRCA1* and *BRCA2* that are mutated in familial breast cancer (Knudson, 2001) and *APC* that is involved in sporadic colorectal cancer (Nishisho et al., 1991). The two copies of the gene have to be inactivated to develop a cancer (Knudson, 2001) (Figure 1.2).

Chromosomal Inversions

Chromosomal inversions involve two breakpoints within a chromosome resulting in inverting the chromosomal segment between these two breaks, and inserting it back to its same position. The inversion is paracentric in the case of both breakpoints on the same arm and pericentric in case of two breakpoints in different arms (Rooney, 2001). The most common type of inversion in AML is inversion 16 inv(16) (Table 1.4).

Chromosomal insertions

Insertions occur when a segment of a chromosome is removed and inserted at a different point on that chromosome or different chromosome (Rooney, 2001).

Chromosomal duplication

Duplications happen when a segment within a chromosome is duplicated next to itself. Direct duplication is when the chromosomal segment keeps the original orientation and inverted duplication is when the segment has altered its orientation (Rooney, 2001).

Ring chromosomes

A ring chromosome is formed when a deletion takes place in both arms of a chromosome resulting in connecting the two ends of that chromosome to each other (Rooney, 2001). Philadelphia chromosome is an example of the ring chromosome and it is a result of t(9;22) in AML.

Marker chromosomes

Marker chromosome is a chromosome that is unable to be identified within the karyotype (Rooney, 2001).

Chromosomal translocations

Chromosomal translocations, whether balanced or unbalanced, are described in detail in chapter 4 of this thesis. This section will focus more on the possible mechanisms of leukaemogenesis arising from chromosomal translocations. According to Rabbitts (1994), chromosomal translocations contribute to two main consequences, such as the creation of a fusion gene, when within a gene on each chromosome, breaks occur, that leads to a chimeric protein being encoded. The other consequence is when the juxtaposition of a coding region from a gene to the promoter of another gene induces ectopic or aberrant expression of a proto-oncogene.

Formation of fusion gene with production of a chimeric protein

The first human chromosomal aberration that was linked to a human disease specifically was the Philadelphia (Ph) chromosome, and this fusion gene has been revealed in over 95% of CML cases (Dreazen et al., 1987; Rowley, 1980; Nowell and Hungerford, 1960). The fusion

gene *BCR-ABL* is formed by t(9;22) as chromosome 9 is translocated to chromosome 22. This translocation position (*ABL)* oncogene that localize at chromosome 9 beside the breakpoint cluster region *(BCR*) of Philadelphia gene on chromosome 22 results in tyrosine kinase activation (Rabbitts, 1994).

Although tyrosine kinases are targets of AML associated chromosomal translocations, other common targets are dysregulation of transcription factors, which has a role that is critical during haematopoiesis. This is shown by over 12 chromosomal translocations that target subunits within the core binding factor (CBF), where *CBFB* and *RUNX1* (*AML1*) form the composition of a heterodimeric transcription

The loss of function of CBF is due to chromosomal translocations, for example, translocations include t(12;21) (Romana et al., 1995), inv(16) (Liu et al., 1993) and t(8;21) (Erickson et al., 1992), which led to expression of *ETV6/AML1*, *CBFB/MYH11* and *AML1/ETO* fusion proteins, (Barba et al., 1993; Golub et al., 1999).

HOX genes family members are reported to be expressed persistently as a result of chromosomal translocations in haematological malignancies. For example, several HOX genes have been fused to *NUB98* such as *HOXA 9* gene (Borrow et al., 1996), *HOXD13* (Egilmez et al., 1998; La Starza et al., 2003), *HOXA11* (Fujino et al., 2002; Taketani et al., 2002).

Chromosome abnormality	**Disease**	**Frequency**	**Fusion gene**
t(8;21)(q22;q22)	AML-M2	18% (30%)	*AML1-ETO*
t(15;17)(q21-q11-22)	AML-M3	10% (98%)	*PML-RARα*
t(11;17)(q23,q21)	AML-M3	Rare	*PLZF- RARα*
Inv(16) or t(16;16)	AML-M4E0	8% (~100%)	*CBFβ-MYH11*
t(9;11)(p22;q23)	AML-M4	11% (30%)	*MLL-AF9*
t(6;11) t(10;11) t(11;17) t(11;19) t(4;11)	AML-M5	11q23 abnormalities are detected in~35% of all	*AML-M5* *ALL-AF6/AF6q21* *MLL-AF10;CALM-AF10* *MLL-AF17/AF17q25* *MLL-ENL/ENL/EEN* *MLL-AF4*
t(6;9)(p23;q34)	AML-M1,M2, M4, M5	1%	*DEK-CAN*
t(16;21)(p11;p22)	AML	‹ 1%	*TLS(FUS)-ERG*
t(16;21)(p24;p22)	t-AML, MDS	‹ 1%	*AML1-MTG16*
t(3;21)	AML	‹ 1%	*AML1-EV11*

			AML1-EPA *AML1-MDS1*
t(7;11)(p15;p15)	AML-M2, M4	‹ 1%	*NUP98-HOXA9*
t(1;11)(q23;p15)	AML-M2		*NUP98-PMX1*
t(8;16)(p11;p13)	AML-M4, M5	‹ 1%	*MOZ-GBP*
Inv(8)(p11,p13)	AML-M0, M1, M5	‹ 1%	*MOZ-TIF2*
t(8;22)(p11;p13)	AML-M5	‹ 1%	*MOZ-P300*
t(12;22)(p13;p23)	AML-M4, CML	‹ 1%	*TEL-MN1*
t(5;12)(q33;p12)	CMMOL	2-5%	*TEL-PDGFRβ*
t(1;19)(q23;p13)	AML-M7	‹ 1%	*OTT-MAL*

Table 1.3: The most common fusion genes in acute myeloid leukaemia and myeloidsplastic disorder resulting from chromosomal translocations as described by Rego (Rego, 2002).

Proto-oncogenes: Aberrant Expression

According to Kuppers and Dalla-Favera (Kuppers and Dalla-Favera, 2001) in their study of lymphomas and lymphoid leukaemia, activation of proto-oncogenes is often a result of chromosomal translocations, which involves coding regions becoming associated with immunoglobulin or T-cell receptor gene-regulatory elements, which results in these proto-oncogenes being expressed inappropriately. In Burkitt's lymphoma cases, around 90% reveal t(8;14)(q24;q32) that places the *MYC* proto-oncogene in juxtaposition to the immunoglobulin heavy chain gene promoter and enhancer (IgH). In another study by Showe et al. (1985), the findings revealed that *MYC* could also be involved with t(2;8) and t(8;22) variant translocations, as these translocate into the immunoglobulin κ and λ light chain loci. However, the coding region is not altered for *MYC* in these translocations, and suggests that over-expression or under-expression could contribute to its oncogenic activity (Rawat, 2006).

According to Rawat (2006) research into the role of malignant transformation in myeloid leukaemia has not yet revealed that chromosomal translocation could induce ectopic and aberrant expression of a proto-oncogene, which contrasts with research into lymphoblastic leukaemia and lymphomagenesis. Point mutations or aberrant expression of proto-oncogenes could contribute to the development of leukaemia, as in animal models, some fusion genes fail to induce leukaemia, and over 50% of cases with acute myeloid leukaemia lack obvious cytogenetic abnormalities.

Chromosomal abnormality	**Genes involved**	**Frequency in given leukaemia type, %**			
		AML	**ALL**	**CML**	**CLL**
t(8;21)(q22;q22)	*AML1-ETO*	5-12			
inv(16)(p13q22)/t(16;16)(p13;q22)	*CBFB-MYH11*	3-10			
t(15;17)(q22;q21)	*PML-RARA*	6-15			
t(11q23)	*MLL*	5-8	7-10		
del(5q)		1-11			
del(7q)		1-7			
Trisomy of 8th chromosome		3-10			
t(9;22)(q34;q11.2)	*BCR-ABL1*	1-2	5-25	90-95	
t(12;21)(p13;q22)	*TEL-AML1*		10-25		
t(1;19)(q23;p13.3)	*TCF3-PBX1*		2-5		
t(17;19)(q22;p13)	*TCF3-HLF*		1		
t(8;14), t(2;8), t(8;22)	*MYC*		1-2		
Trisomy 12					13
del(13q14)					45
del(11q22-q23)					15
del(17p13)	*TP53*				6
del(6q21)					5

Table 1.4: A list of common chromosomal abnormalities in haematological malignancies. AML (acute myeloid leukaemia); ALL (acute lymphoid leukaemia); CML (chronic myeloid leukaemia); CLL (chronic lymphoid leukaemia) (Chetverina and Chetverin, 2010).

1.4 The role of homeobox genes in leukaemia

1.4.1 Homeobox genes

Homeobox genes are involved significantly in embryonic development, cell differentiation, proliferation, apoptosis (Cillo et al., 1999; Tupler et al., 2001), as well as normal and malignant haematopoiesis (Bach et al., 2010). These genes are found in the genomes of all animals, plants and fungi and are extremely conserved during evolution (Lappin et al., 2006). Homeobox genes are distributed in four paralogous clusters on chromosomes 2, 7, 12 and 17 (Borrow et al., 1996).

Although now known in most eukaryotic species, it was during the 1980s that the homeobox (HB) was revealed as a sequence motif shared by drosophila homeotic genes (HOM-C complex), and was shown to contribute to embryonic differentiation along the anterior-posterior (ap) axis (Stein et al., 1996). Tupler et al. (2001) suggested that around 200 homeobox genes are contained within the human genome, but only 39 were shown to be part of the HOX family. The constitution of homeobox genes is characterised as a gene family with a 183-nucleotide sequence that is highly conserved that encodes a 61-amino acid domain, known as the homeodomain (HD), which has DNA binding activity that is sequence specific, and is related structurally to the helix-turn-helix motif of prokaryotic DNA-binding (Buske and Humphries, 2000).

1.4.2 Leukaemic and normal haematopoietic cells: HOX gene expression

In mammalian adult normal and neoplastic tissues, as well as embryonic tissue, HOX genes are expressed, which has been widely reported in research studies, such as haematopoietic cells (Antonchuk et al., 2002; Argiropoulos and Humphries, 2007), human colonic mucosa (Wang et al., 2001), kidney (Barba et al., 1993) and normal and neoplastic skin (Care et al., 1996). Findings from these studies show that HOX gene expression is very different when comparing early progenitors and more mature differentiated hematopoietic cells, and is also strictly regulated in normal haematopoiesis. According to Buske and Humphries (2000), in early CD34+ haematopoietic progenitors, HOX genes of the A, B and C cluster are expressed, but there is no *HOXD* gene transcription in this progenitor pool. However, in the more mature CD34- compartment, some HOX genes of A, B and C clusters are substantially down-regulated or absent (Lawrence et al., 1996).

Knockout mouse models have been used to investigate the function of HOX genes in haematopoiesis. Perkins et al. (1990) found when studying mice, over expression of *HOXB8/IL-3* resulted in an aggressive transplantable leukaemia, which contrasted to the results when transplanting mice with only IL-3. Nakamura et al. (1996) found that in the BXH-2 mouse line, over expression of *HOXA9* and *HOXA7* can initiate AML.

Studies of patients with acute myeloid leukaemia (AML) found that leukemic blasts revealed an aberrant pattern of *HOXA10* expression, which suggests that dysregulated HOX gene expression could be an overall feature of this malignancy (Kawagoe et al., 1999).

Golub et al. (1999) showed that expression of *HOXA9* is the only single gene expression marker from over 6800 cDNAs after DNA micro-array analysis, as well as a diagnostic marker of AML in humans that is shown to be the most consistent. According to Armstrong et al. (2002), an important element of leukaemogenesis fuelled by *MLL* translocations is

aberrant expression of *HOXA9*. Molecular analysis studies of translocations t(7;11) and t(2;11) provide evidence that HOX genes *HOXD13* and *HOXA9* are targets of leukaemia-associated genetic alterations. Particular homeobox gene families are known as leukaemogenic and mutations of homeobox genes are have been observed in leukaemia (Raimondi et al., 1999; Look et al., 1997). As well as the aberrant expression of wild-type HOX genes in AML (Beverloo et al., 2001; Iwasaki et al., 2005; Kroon et al., 2001; Von Bergh et al., 2006).

1.4.3 Motor neuron and pancreas homeobox1 (*HLXB9-MNX1*) gene and reported mechanism of leukaemogenesis

MNX1 (HLXB9) is a member of homeobox genes family. It is expressed in neuronal cells during differentiation (Liu and Joyner, 2001). In addition, *HLXB9* is involved in pancreas differentiation (Harrison et al., 1999). Dysregulation of *HLXB9* causes Currarino Syndrome (Ross et al., 1998). *HLXB9* gene is localized on chromosome 7 at band q36. An over-expression of this gene has been observed in leukaemia cells that carry the translocations t(7;12)(Beverloo et al., 2001; Von Bergh et al., 2006) and t(6;7) (Nagel et al.,2005).

The t(7;12) and *HLXB9/ETV6* fusion transcript

It has been reported that *HLXB9* gene on 7q36 is a partner gene for *ETV6* gene in the t(7;12)(q36;p13) (Beverloo et al., 2001). Beverloo and her group identified two paediatric patients with AML and an *HLXB9/ETV6* fusion transcript. The chimeric fusion gene is created by the *HLXB9* promoter and it is predicted to code for a protein that contains the N-terminal 231 amino acids of *HLXB9* and almost the complete *ETV6* protein including the

pointed domain and the ets domain. The homeodomain of *HLXB9* is not included in the fusion.

The *ETV6* gene on 12p13 is a member of the ETS family that encodes transcription factor. More than 40 chromosomal translocations are confirmed to be involved with the *ETV6* gene in several types of leukaemia (Bohlander et al., 2005).

Beverloo et al. (2001) found that in a study on paediatric patients, *HLXB9/ETV6* fusion transcript was detected in around 20% of cases, and suggested that transformation for these fusions could have been due to *HLXB9/ETV6* proteins interacting through the pointed domain with the wild-type *ETV6*, which would interfere with normal *ETV6* function.

However, studies of various breakpoints in the *HLXB9*/7q36 region have shown that the t(7;12) rearrangement does not always result in the formation of a fusion transcript. However, the presence of an ectopic expression of the non rearranged *HLXB9* gene is detected in all t(7;12) leukaemias. This would suggest that the mechanism of leukaemogenesis does not necessarily involve the generation of a chimeric protein in some t(7;12) cases (Von Bergh et al., 2006).

The t(6;7) rearrangement and an ectopic expression of *HLXB9* by juxtaposition of the *MYB* gene on the GDM-1 cell line

The function of ectopically expressed *HLXB9* in hematopoietic cells is unknown (Nagel et al., 2005). However, Nagel has confirmed successfully in his study an ectopic expression of *HLXB9* due to juxtaposition of this gene with regions upstream of *MYB* in the GDM-1 cell line via the t(6;7) rearrangement.

Nagel's is based on expression analysis using RT-PCR of genes flanking the breakpoint region *C7ORF2*, *C7ORF3*, *HLXB9*, and ubiquitin protein isopeptide ligase *E3*, at 7q36. All genes at 7q36 were expressed in all hematopoietic cell lines, whether neoplastic or normal except *HLXB9* that was only expressed in the GDM-1. The *HLXB9* gene expression was unnoticeable in 19 other AML cell lines, in 17 T-cell lines, and in 22 additional hematopoietic cell lines.

Interestingly, 3 of 8 non- haematopoietic (prostate and cervix carcinoma) cell lines expressed *HLXB9*. This finding might support an oncogenic role for this gene.

RT-PCR analysis using two different oligonucleotide primer pairs for *HLXB9* (7q36) and for *HBS1L* (centromeric to the 6q23 breakpoint) revealed no fusion. These data pin down *HLXB9*, at 7q36, as the only possible particular target of the t(6;7) in GDM-1 cell line (Nagel et al., 2005).

Recently, *MYB* was recognized as a second-hit target in AML (Castilla et al., 2004), supporting cooperative roles for *HLXB9* and *MYB* in this situation.

Apparently, the most likely neoplastic role for t(6;7) in GDM-1 is ectopic transcription of *HLXB9* by juxtaposition with regulatory elements of an activated *MYB* allele, perhaps chromosomally mediated, though cytogenetic release of *HLXB9* from upstream repressors cannot be excluded (Nagel et al., 2005).

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1.5 Aims

The main aim of my project is to verify the involvement of homeobox genes in the biology of leukaemia. In particular, my interest focuses on the *HLXB9* gene, located on chromosome 7 and reported to be involved in some types of leukaemia.

In order to achieve this goal, my objectives are:

(i) Verify the involvement of chromosome 7 through the study of chromosomal abnormalities in leukaemia and lymphoma cell lines. This will be achieved by G-banding analysis and 24 colour karyotyping methods (Chapter 3)

(ii) Verify the involvement of chromosome 7 in a series of patients with leukaemia using chromosome painting (Chapter 4)

(iii) Verify the involvement of *HLXB9* in a series of patients with leukaemia using single locus probes (Chapter 5).

CHAPTER 2

MATERIALS AND METHODS

2. Materials and Methods

2.1 Preparation of target genetic materials

2.1.1 Origin of patients' materials

Patients' materials of bone marrow were delivered in a form of archival methanol-acetic acid fixed chromosome suspensions stored at -20ºC. The G-banding analyses of the samples were carried out previously in the diagnostic lab that provided the samples. The patients' samples were provided by Professor Jochen Harbott, Children's University Hospital, Giessen, Germany.

2.1.2 Cell lines

In this study, three cell lines were used. Two cell lines were derived from myeloid leukaemia and one cell line was derived from lymphoma.

2.1.2.1 Cell line GDM-1

The GDM-1 is an acute myeloid leukaemia (AML) cell line was established from the peripheral blood of a 65-year-old female patient who had acute monoblastic leukaemia prior to her death (Ben-Bassat et al., 1982). This cell line was commercially obtained by the National Institute for Cancer Research, Genova, Italy.

2.1.2.2 Cell line K562

The K562 is a chronic myeloid leukaemia (CML) cell line that established from the pleural effusion of a patient with Philadelphia chromosome positive (Ph +) chronic granulocytic

leukaemia (Lozzio & Lozzio, 1975). The cell line was provided in a form of fixed cells suspension by a collaborator (University of Oxford).

2.1.2.3 Cell line Pfeiffer (CRL-2632)

The Pfeiffer (CRL-2632) cell line is a lymphoma cell line. It was derived from the lymphoblast cells of a patient in the leukemic phase of diffuse large cell lymphoma (DLCL) with cleaved and non-cleaved nuclei (Gabay et al., 1999). The cell line was purchased in a form of live cells culture (ATCC, UK).

2.1.3 Cell culture

The GDM-1 cell line was cultured in 80% RPMI 1640 medium (Gibco) supplemented with 20% (v/v) foetal calf serum (FCS) (Gibco), 2% (v/v) penicillin and streptomycin antibiotics (Gibco) and 2mM L-glutamine (Gibco). All reagents used for the cell culture were warmed to 37ºC in a water bath before being used. The cells were maintained at 37ºC in an incubator containing 5% CO_2. The cells were then seeded in a T25 tissue culture 25 ml flask (Thermo scientific, UK). The medium was changed twice a week until the growth of cells was obtained, and then passages were started. The growth of the cells was checked every 2 days using a 10X lens of a phase contrast light microscope. The cells were treated with Colcemid (0.05mg/ml) for 1 hour before harvesting to arrest mitotic chromosomes.

2.1.4 Harvesting of cell cultures

The cells were centrifuged at 1200 RPM (MSE, Centaur2E centrifuge, UK) for 5 minutes. The supernatant was discarded and the pellet was re-suspended in 10 ml of hypotonic solution (0.075M potassium chloride-KCL) at 37ºC. After incubation for 15 minutes, the cells were centrifuged again for 5 minutes at 1200 RPM. The supernatant was then discarded and the pellet was re-suspended in a 10 ml of fresh fixative solution containing three parts methanol and one part acetic acid. After incubation for 15 minutes at room temperature, the cells were centrifuged as described previously. Fixative solution washes were carried out twice. The fixed chromosomes and cells were re-suspended in a small amount of fresh fixative solution. The cells suspension was stored at -20ºC until required.

2.1.5 Preparation of slides

The fixed cell suspensions were centrifuged at 3000 RPM for 5 minutes and the supernatant was discarded. The cell pellets were re-suspended in a small amount of fresh methanol: acetic acid fixative solution usually 150µl to achieve an appropriate concentration. Eight µl of fixed cells suspension were dropped from a height (8cm) onto the middle of a dry clean slide (Superfrost, UK). The slides were air-dried and the quality of metaphases and chromosome spreads were checked by a contrast inverted microscope. The slides were aged by leaving them on the bench for 2 days at room temperature. The slides were ready for FISH investigation at this stage.

2.2 Staining and banding of chromosome slides

2.2.1 G-banding

In a coplin jar, slides were treated with Trypsin solution (3ml of 0.25% stock Trypsin with 47 ml of 0.85% Sodium Chloride NaCl) for 20 seconds at room temperature (RT). Some leukaemia cell lines slides were treated longer with Trypsin up to 60 seconds as slides were aged over a week. The slides were transferred to a coplin jar containing 0.9% Sodium Chloride Saline (9g of NaCl dissolved in a litre of pure water) for 20 seconds at RT. Next, slides were transferred to a coplin jar of Gurr buffer solution (6.8pH, 1 tablet of Gurr buffer dissolved in a litre of distilled water) for 20 seconds at RT. The staining solutions were prepared in a coplin jar by mixing 3 mls of each Giemsa's and Leishman's stains. The coplin jar was topped up with 6.8PH Gurr Buffer. The slides were left to stain for 7 minutes at RT. Then, were washed with cold water and dried on a hot plate at 58ºC for 15 minutes. The slides were protected by 22X40 mm cover slips and sealed with Eukite glue. The quality of chromosomes staining was assessed by an inverted microscope.

Over-trypsinized chromosomes were very pale with the loss of a few chromosomal segments. In contrast, under-trypsinized chromosomes were very dark without an appearance of the light bands within a chromosome.

The over-stained chromosomes were destained by treating slides with a fresh fixative solution (3Methanol:1 acetic acid) for a few seconds, then re-stained for a shorter period of 5 minutes.

2.3 Preparation of DNA probe

2.3.1 DNA isolation of P1 artificial chromosome (PAC) clones

The P1 artificial chromosomes were preserved in bacterial single colonies in the P1 derived host. The PAC was provided in a form of glycerol stock at -70 by Dr. Tosi, Brunel University, UK. The bacterial cells containing the DNA sequences of the gene of interest were grown in 2ml of LB broth medium (LB Broth Miller pre-Buffered capsule, Fisher Scientific, UK) supplemented with 12.5µg Kanamycin antibiotic in 15 ml-polypropylene tubes (Fisher Scientific , UK) overnight at 37ºC while shaking. The cells were centrifuged at 3000 RPM for 5 minutes. The supernatant was discarded and the pellet was resuspended vigorously in 300 µl of P1 solution (15mM Tris pH 8.0, 10Mm EDTA and 100 µg/ml RNase A). The suspension was transferred into a 2ml micro centrifuge tube containing 300 µl of P2 solution (0.2 M NaOH and 1% SDS). The suspension was mixed gently by inverting the tube several times and incubated for 5 minutes at room temperature. Then, 300 µl of P3 solution (3M Potassium acetate KOAc, pH 5.5) were added gradually. The tube was centrifuged at 10,000 rpm for 10 minutes at 4°C. . The supernatant was transferred into a new Eppendorf tube that already contained 800 µl of ice cold isopropanol. The tube was inverted several times and incubated over night at -20°C. After the incubation, the tube was centrifuged at 10,000 RPM for 15 minutes at 4°C. The supernatant was discarded and 500 µl of ice cold 70% ethanol was added and the tube was inverted several times to wash the pellet. The tube was centrifuged in a micro centrifuge at 10,000 RPM for 10 minutes at 4°C. The supernatant was discarded and the pellet left to dry for 2 hours at room temperature. The DNA pellet was re-suspended in 20µl of water once it completely dried. The pellet was left for 2 hours at the bench with tapping the tube gently to dissolve. The pellet was stored at -20ºC.

2.3.2 Agarose gel electrophoresis

The purified PAC DNA was run on mini gel electrophoresis (7x10cm) to validate the DNA size. The DNA sample was diluted with water to achieve a final volume of 5µl to be mixed with 1µl of DNA dye. The mixture was loaded in 1% agarose gel with DNA size marker III (PeQlab, Erlangen, Germany). The gel electrophoresis was run in TBE buffer (0.089 M Tris, 0.089 boric acid and 2Mm EDTA, Ph8.0) at 80 volts for 1 hour. The Gels were stained with ethidium bromide (0.5µg/ml) and visualised by a UV transilluminator (Alpha Innotech Corporation, California, U.S.A).

2.3.3 Measurement of DNA concentration

The concentration of purified DNA was calculated by using a Nano Drop Spectrophotometer (Nano Drop 2000C, Thermo scientific, and UK). A sample of 2µl DNA was loaded into the machine. The machine had to be cleaned with 2µl of water twice before loading the DNA sample. The reading of DNA concentration was given in ng/µl.

2.4 Labelling of DNA probes

2.4.1 Nick translation

Nick translation system was used to label DNA probes with biotin to be used in FISH investigation (BioNick™ DNA Labelling System, Invitrogen, UK). In a small Eppendorf tube, 1 µg of DNA probe (5µl of PAC1121A15) was mixed with 5µl of 10xdNTP mix (0.2 mM each dCTP, dGTP, dTTP, and 0.1mM dATP and biotin-14-dATP) and 5µl of the enzymes mix (DNA polymerase I and DNase I). In order to obtain a final volume of 50 µl, 35 µl of pure water were added. The mixture was mixed and incubated at 16ºC for 2 hours. The

reaction was stopped by placing the DNA probe on ice or using stop buffer (0.5 M EDTA, pH 8.0).

2.4.2 Agarose gel electrophoresis

Obtaining a labelled DNA fragment between 100-500 bp in size is essential for efficient FISH hybridisation. After successful nick translation reaction the labelled fragment should be around 300 bp in size. In order to confirm the size of DNA fragment, a labelled DNA probe was run in 2% agarose gel and DNA size marker XIII (PeQlab, Erlangen, Germany). The gels were run in TBE buffer at 80 Volts for 45 minutes. The gels were stained with Ethidium Bromide (0.5µg/ml) and visualised with a UV transilluminator (Alpha Innotech Corporation, California, U.S.A).

2.4.3 Purification of labelled probes

Unincorporated nucleotides of the labelled probe were removed using microSpin G-50 Columns (Illustra MicroSpin G-50 Columns, GE health care life sciences, UK). The labelled probe obtained by nick transaction (approximately 50µl) was placed at the top of the column. The column was centrifuged at 6000 RPM for 3 minutes then discarded. The purified DNA probe was collected and was mixed with 5µl of salmon sperm (11mg: 1g), 10µl of Sodium Acetate (NAAC) and 2.25 volumes of ice- cold 100% Ethanol. The labelled DNA probe was precipitated at -70ºC for an hour. The mixture was centrifuged at 13000 RPM for 30 minutes at 4ºC. The supernatant was discarded and the pellet was washed in ice-cold 70% Ethanol. The tube was centrifuged again at 13000 RPM for 15 minutes at 4ºC. The supernatant was discarded and the pellet left to dray at room temperature for 2 hours. The pellet was re-suspended in 20µl of pure water for overnight at 4ºC. The purified labelled DNA probe was stored at -20ºC until needed.

2.5 Fluorescence in situ hybridization

2.5.1 Denaturation of the target DNA

The aged slides were washed in saline-sodium citrate (2XSSC, pH7.0) while shaking for five minutes (SSC buffer in 20Xconcentration, Sigma, UK). They were then dehydrated through an alcohol series (70%, 90% and 100% ethanol) followed by air-drying for five minutes. The slides were denatured in 70% formamide denaturing solution containing 2XSSC at 70^{o} C for 5 minutes. Following the denaturation, the slides were instantly plunged into ice-cold 2XSSC for five minutes, then dehydrated again through an alcohol series (70%, 90% and 100% ethanol) and air-dried at room temperature. The denatured slides were ready to hybridize with the denatured probe at this stage.

2.5.2 Competitive in situ suppression

In order to block the repetitive sequences of the genomic DNA, PAC probes were annealed with a DNA competitor before the hybridization. In a small Eppendorf tube, 5µl of the purified labelled DNA probe was annealed with 5 µl of human COT 1 DNA (Roche Diagnostics GmbH, Germany). The mixture was dried in a speed vacuum dessicator for 10 minutes (Speed Vac® Plus SC110A, Sanvant Instruments Inc., Farmingdale, NY). Then, 12µl of commercial hybridisation buffer was added. The hybridisation mixture was denatured at 80ºC for 10 minutes, placed on ice immediately and incubated at 37ºC for 15 minutes.

2.5.3 Hybridization

The denatured DNA probe was added to denatured slides. The slides were covered with 22 X22 mm glass coverslips (VWR international, UK). The slides were sealed with rubber solution and incubated in a humid chamber at or 37ºC for overnight. After the hybridisation the rubber solution was removed and the slides were washed in 2XSSC while shaking for 5 minutes to remove the coverslip. The slides were washed in 0.4XSSC at 72ºC for 5 minutes then in fresh 2XSSC for 5 minutes to remove unbind probe. Finally, the slides were washed in phosphate-buffered saline (PBS) for five minutes while shaking (phosphate-buffered saline, Sigma, UK). For DNA visualization, 15µl of counterstained diamidino-2-phenylindole (DAPI) solution (Cambio, UK) was added for each slide. The slide was covered with a 22X40 mm coverslip and sealed with nail polish.

2.5.4 Detection of hybridized, labelled probes

Biotin-labelled probes

Biotinylated probes were detected with avidin conjugated CY3 dye. The slides were treated with 100 µl blocking solution of 4% bovine serum albumin (w/v) (BSA in 4XSSC, 0.05%Tween20) (Sigma, UK) for 20 minutes at 37ºC to block non-specific protein binding sites. The antibodies used in the detection were diluted in BSA.

On each slide, 75µl of Streptavidin - CY3 conjugated were added, covered with a piece of parafilm and incubated in a humid chamber for 20 minutes at 37ºC. After the incubation the slides were washed 3 times in 4XSSC, 0.05%Tween20 while shaking for 5 minutes each. The second layer was 75µl of biotin- anti avidin conjugated. The slides were covered with parafilm and incubated for 20 minutes at 37ºC. The slides were washed 3 times in 4XSSC,

0.05%Tween20 while shaking for 5 minutes each. In order to amplify the weak biotinlated probe hybridized signal, a third layer of 75µl Streptavidin - conjugated CY3 was added to the slides. The slides were covered with parafilm and incubated in a humid chamber for 20 minutes at 37ºC. The slides were washed in 4XSSC containing 0.05%Tween20 for 5 minutes shaking. Finally, the slides were washed finally in phosphate-buffered saline (PBS) for 5 minutes shaking. The slides were mounted in anti fading medium supplemented with DAPI (Vectashield, Vector laboratories, UK).

2.6 Commercial painting probes

The hybridisation using the whole chromosome 7 painting probe directly labelled with fluorescein isothiocyanate (FITC) was carried out according to the manufacturer's instructions. The human chromosome 7 paint probe was mixed with the hybridisation buffer (1 part probe: 4 parts hybridisation buffer) to make a final volume of 15µl for each slide. The hybridisation mixture was denatured at 65ºC for 10 minutes and incubated at 37^{o}C for 10 minutes to allow re-annealing of repetitive sequences. The denatured probe (15 µl for each slide) was applied to the denatured slide and covered with a 22X22 mm glass coverslip. The coverslip was sealed by rubber cement glue and left to hybridise in a moist chamber at 37^{o}C overnight. After hybridisation, the glue was gently removed and the slide was washed in 2XSSC for five minutes while shaking in order to remove the coverslip. The slide was then washed in pre-warmed 0.4XSSC for five minutes in a water bath at 72^{o}C (diluted from 20X SSC buffer, Sigma, UK). The slide was washed in 2XSSC for five minutes at room temperature while shaking. It was then washed in phosphate-buffered saline (PBS) for five minutes while shaking (phosphate-buffered saline, Sigma, UK). For each slides 15µl of diamidino-2-phenylindole (DAPI) solution (Cambio, UK) was added. The slide was covered with a 22X40 mm coverslip and sealed with nail polish.

2.7 Multiplex FISH (M-FISH)

The method here described has been used according to the instruction given by the manufacturer of M-FISH probe-set (Metasystem , Altlussheim, Germany).

2.7.1 Aging of the slides

The slides were incubated in 3:1 methanol: acetic acid for an hour and then, dehydrated in Ethanol series 70%, 90% and 100% for 2 minutes each. The slides were baked at 65° C for 20 minutes, and then placed in acetone for 10 minutes, and then air-dried.

2.7.2 Pre-treatment of slides with RNase

The slides were treated with RNase (100µg/ml in 2XSSC) for 1 hour at 37° C. The slides were washed in 2XSSC then in phosphate buffered saline (PBS).

2.7.3 Formaldehyde fixation

The slides were washed in (PBS) containing Magnesium Chloride (50 mM of $MgCl_2$) for 5 minutes. Then, the slides were incubated in a solution of PBS containing 50mM ($MgCl_2$) and 1% paraformaldehyde for five minutes at room temperature. Finally the slides were washed in 1X PBS for five minutes at room temperature.

2.7.4 Slide denaturation:

The slides were washed in 0.1XSSC for 1 minute at room temperature, and then denatured with 70% formamide in 2XSSC at 70°C for 30 minutes. The slides were then allowed to cool on the bench for a proximately twenty minutes. Once slides cooled, they were sequentially washed with 0.1XSSC and 0.07 N NaOH at room temperature for 1 minute each. Followed by 0.1XSSC and 2XSSC washes at 4°C for one 1 minute each. The slides were dehydrated in ethanol series at 30%, 50%, 70% and 100% for 1 minute each and then air-dried.

2.7.5 Probe denaturation and hybridisation

Ten microliters of 24XCyte commercial painting probe (Metasystem kit, Altlussheim, Germany) was placed into an Eppendorf tube and denatured at 75° C for 5 minutes. The probe is light sensitive, therefore it was kept in the dark (Eppendorf tube was covered with aluminium foil). The probe was then placed on ice for 10-20 seconds and then incubated at 37° C for 30 minutes, and then briefly centrifuged and placed on the slide. A 22X22 mm coverslip was placed onto the probed area of denatured slide and sealed by rubber cement solution. The slides were incubated at 37° C in a humidified chamber for 3 days.

2.7.6 Post-hybridisation washes

After incubation, the rubber cement and coverslips were removed and the slides were washed in 1XSSC at 73ºC then in 4XSSC containing 0.05% tween 20 for 5 minutes respectively.

2.7.7 Detection

One µl of detection reagent was added to 50 µl blocking reagent and mixed. The mixture was applied to the probe area of the slide and covered with parafilm, then incubated at 37 C° for 15 minutes in a humidified box. The slides were washed twice in 4XSSC containing 0.05% tween 20 for 3 minutes each, then once in PBS while shaking at room temperature. The slides were air-dried. The DNA was counterstained with 20µl of DAPI. The slides were covered with 22X40 mm coverslip and sealed with nail varnish. The slide was stored at 4° C in the dark until required.

2.8 Image analysis

2.8.1 Microscopy

2.8.1.1 G-banded chromosomes

G-banded metaphases chromosomes were visualised and analysed using Zeiss Axioskop microscope (Zeiss, Oberkochen, Germany) connected to a digital camera used for capturing images at 100X objectives oil focus.

2.8.1.2 Single and dual colour FISH

For single and dual colour FISH, the slides were visualised and analysed using the Olympus BX41 fluorescence microscope and UPlanFLN 100X oil immersion lens. A minimum of twenty metaphases were analysed for cell line and minimum of five metaphases were analysed for patients. For some patients there were a limited number of metaphases due to the poor quality of the delivered materials.

2.8.1.3 M-FISH

For M-FISH metaphase analysis an 8-positions filter equipped fluorescent microscope was used (AXioplan2 imaging, Carl Zeiss, Germany). The microscope contains individual filter sets for each component fluorochrome of the XCyte probe cocktail plus DAPI (FITC, DEAC, Spectrum orange, Texas Red and CY5).

Digital FISH images were captured using a charged-coupled device (CCD) camera. Metaphases chromosomes were analysed and karyotyped by enhanced DAPI banding. Further paint analysis was achieved by assessing paint coverage of each individual fluorochrome along the entire length of each chromosome. A metaphase was considered normal if all 46 chromosomes were observed to be normal with that procedure, and consequently confirmed by the Isis Software of M-FISH analysis. At least 25 metaphases were analysed for each cell lines.

2.8.2 Specific software for analysis

2.8.2.1 G-banding analysis software

Specific G-Banding software was used to analysis the metaphases chromosomes spreads. These were CytoVision (Applied Imaging Corporation, UK) and Genikon software (Nikon, UK).

2.8.2.2 FISH analysis software

Single and dual colour FISH Metaphase images were captured using a greyscale digital camera (Digital Scientific, UK) and Smart Capture 3 software (Digital Scientific, UK).

M-FISH metaphases chromosomes were analysed and karyotyped using ISIS software (Metasystem Isis, Altlussheim, Germany).

CHAPTER 3

CHARACTERIZATION OF CHROMOSOMAL ABERRATIONS IN MYELOID LEUKAEMIA AND LYMPHOMA DERIVED CELL LINES USING CONVENTIONAL CYTOGENETIC TECHNIQUE (GBANDING) AND MULTIPLEX FISH (M-FISH)

3.1. Introduction

3.1.1 Complex chromosomal aberrations (CCAs)

Chromosomal abnormalities are a hallmark for disease progression and classification in cancer, including leukaemia. Recurrent chromosomal abnormalities have been identified in all leukaemia subtypes as sole aberration present or as part of a complex karyotype.

Complex chromosomal aberrations (CCAs) which involve more than two chromosomes and/or more than three breakpoints have been observed in Leukaemia. CCAs have been reported in 20% of de novo acute myeloid leukaemia (AMLs), 24% of secondary AMLs and up to 50% of therapy-related AML and MDS cases (Mauritzson et al., 2000; Rossi et al., 2000).

CCAs are associated with rather poor prognosis and respond poorly to anti-leukaemic treatment and it was suggested that some of these rearrangements contribute to drug resistance and disease progression (Lindvall et al., 2004; Schoch et al., 2001).

Cryptic chromosomal translocations (that impossible to detect by G-banding method) are common findings in leukaemia (Cherif et al., 1993; Pan et al., 2012; Tosi et al., 1996, 1997 and in this study chapter 4).

3.1.2 The GDM-1 cell line

The GDM-1 is an acute myeloid leukaemia (AML) cell line was established from the peripheral blood of a 65-year-old female patient who had acute monoblastic leukaemia (AML-M4) (Ben-Bassat et al., 1982).

The attempts of establishing the GDM-1 cell line from the patient blood peripheral were carried out several times before the growth of the leukemic cells was obtained. The patient

sample was cultured in glass flasks that contain RPMI 1640 medium supplemented with 30 % foetal calf serum and antibiotics to achieve a final cell concentration of 2-4 × 10^6 cells/ml. The cells were maintained at 37°C with 5% CO_2 in air and 80-95% relative humidity (RH) with changing the medium twice a week (Ben-Bassat et al., 1982).

The characterization of GDM-1 cell line abnormalities was carried out using conventional cytogenetic technique G-banding. It enabled the identification of various chromosomal abnormalities, such as trisomy 8, deletion of chromosome 6, del(6q), an additional genetic material on chromosome 7, add (+7q), and a deleted chromosome 12(12p). The G-banding karyotype of 47, XX, +8, del(6)(q), add(7)(q), del(12)(p) was observed in 85% of cells analysed (Ben-Bassat et al., 1982).

A more detailed G-banded karyotype of the GDM-1 cell line, that included refined breakpoints of several abnormalities, was reported as 48,XX,der(2)t(2;11)(q36;q13),t(6;7)(q23;q36),+8,del(12)(p11.2p12.2),+13,del(16)(q23) (Nagel et al., 2005). In should be noted that in this latter report a t(2;11) and a del(16) has been observed.

The GDM-1 cell line present the translocation t(6;7)(q23;q36) with an over expression of the *HLXB9* gene and distal break point at 7q36. These features make the cell line a good model to study the t(6;7) and the mechanism of leukaemogenesis that related to the activation of *HLXB9* in AML –M4 subtype in vitro.

3.1.3 Conventional cytogenetic technique (G-banding)

G-banding analysis is a first line investigation enabling a scanning of the whole DNA genome at the chromosomal level. It is a widely used technique for the routine staining of mammalian chromosomes. The aim of the technique is to produce a banding pattern within chromosomes that facilitates karyotypic analysis. Chromosomal bands are generated by staining with Giemsa's and Leishman's stains after pre-treating chromosomes with Trypsin. This causes chromosomes to stain as a series of dark G bands and pale inter-bands. Each homologous chromosome pair has a unique pattern of G-bands enabling chromosomes analysis under the microscope by eye or by using special software such as CytoVision or Genikon (Sander Operation Procedure, Paediatric Malignancy Unit, Great Ormond Street Hospital, London, UK). However, complex chromosomal aberrations (CCAa) may be difficult to interpret by banding methods and cryptic chromosomal translocations are not detected by G-banding; for example, cryptic translocations are not detected or have been misdiagnosed as deletions in conventional cytogenetic G-banding analysis (Cherif et al., 1993; Pan et al., 2012; Tosi et al., 1996 and 1997).

The limitation of G-banding approach can be overcome by combining the information obtained by G-banding analysis with further molecular cytogenetic methods, such as multiplex FISH, that obtains a 24-colour karyotype.

3.1.4 Multiplex cytogenetic technique (M-FISH)

Multiplex fluorescence in situ hybridization (M-FISH) technique allows the visualization of all human chromosomes in 24 colours by using a cocktail probe of whole chromosomes paints in a single hybridization (MetaSystems, Altlussheim, Germany).

The recognition of 24 different chromosome painting probe is obtained with five varicolored fluorochromes (FITC, DEAC, Spectrum Orange, Texas Red and Cy3). Individual paint is labelled with one of these five fluorochromes or with a unique combination of them. The 24 colour karyotype results in a mixture of these five different fluorochromes. Suitable filter sets are used to guarantee the separation of different excitation and emission spectra. This technique allows the production of a special colour for each chromosome which facilitates the analysis of numerical and structural abnormalities especially cryptic and complex chromosomal abnormalities (MetaSystem lab manual, Altlussheim, Germany).

The M-FISH analysis is facilitated by the aid of specially designed software for metasystem images analysis (ISIS) and an axioplan epifluorescence microscope (ZEISS, Germany).

3.2 Aim of the study

The aim of this chapter was to characterize the chromosomal aberrations in myeloid leukaemia and lymphoma derived cell lines that present an over-expression of *HLXB9* gene. In order to achieve this goal, information obtained by conventional cytogenetic technique (G-banding) and molecular cytogenetic technique (M-FISH) were combined.

3.3 Materials and methods

3.3.1 Cell line

The human acute myeloid derived cell line GDM-1 and chronic myeloid derived cell line K562 were used in this study. In addition, one lymphoma cell line CRL2632 has been investigated (See chapter 2 for a detailed description of the cell lines).

The GDM-1 cell line was obtained from the National Institute for Cancer Research, Genova, Italy. The culturing of GDM-1 cells, harvesting and preparations of slides was carried out as described in the materials and methods chapter (Chapter 2).

The K562 cell line was provided via collaboration with Oxford University, UK while the Pfeiffer cell line -CRL2632 was purchased from the American Type Culture Collection-ATTC.

All three cell lines were delivered in the form of live cell cultures. However, the harvesting of cells and chromosomes for GDM-1 was carried out by myself, whereas the same procedure for K562 and Pfeiffer cell lines was performed by others in the group and I handled these two cell lines in the form of fixed chromosomes and cell suspensions.

3.3.2 Probe

The Meta System 24XCyte probe containing 24 different chromosomes paints was used in this study (Metasystem kit, Illustras, Germany). The probe was kindly provided by Dr. Rhona Anderson, Brunel University, UK.

3.3.3 G-banding analysis

The aged slides were treated with trypsin and Giemsa dye according to G-banding procedure as described earlier (Chapter 2). (Stander Operation Procedure, Paediatric Malignancy Unit, Great Ormond Street Hospital, London, UK). The karyotype obtained after G-banding analysis was described according to conventional cytogenetic nomenclature (ISCN, 2009).

3.3.4 Multiplex fluorescence in situ hybridization

The hybridization was carried out according to Metasystem protocols. The whole procedure is described in details in chapter 2.

3.3.5 Image capture and analysis for G-banding

An Olympus microscope that connected to a digital camera was used for capturing images at 100 objectives oil focus. Specific G-Banding software was used for analysis such as CytoVision and Genikon. Image capture and analysis was performed by myself during my training period at the laboratory of Paediatric Malignancy Unit, Great Ormond Street Hospital, London, under the supervision of Dr Steve Chatter.

3.3.6 Image capture and analysis for M-FISH

Twenty metaphases were analysed for each cell line. The chromosomes were observed using a fluorescence microscope (ZEIZZ, AXioplan2 imaging) and MetaSystem camera. Images analysis and karyotype of metaphase chromosomes were performed using Metasystem (ISIS) software.

3.4 Result

3.4.1 G-banding and M-FISH analysis

3.4.1.1 Cell line K562

In the present work complex chromosomal abnormalities of 2 leukaemia cell lines (GDM-1 and K562) and one lymphoma cell line (Peiffeir-CRL2632) were analysed using both classic cytogenetic technique (G-banding) and molecular cytogenetic technique (M-FISH).

The G-banding analysis of the CML leukaemia cell line K562 revealed numerical and structural chromosomal abnormalities (Figure 3.1.A). The numerical aberrations consisted of 14 trisomies (+1),(+2),(+4),(+5),(+6),(+8),(+10)(+11),(+12),(+15),(+16),(+17),(+19),(+21). Moreover, 4 copies of chromosome 7 were observed. The M-FISH analysis on K562 cell line has confirmed numerical abnormalities findings and more trisomies were detectable such as (+9), (+20), and 4 copies of chromosome 15. The G-banding analysis revealed additional materials on several chromosomes add (11p) and add (21p), as well 4 markers chromosomes. These 4 marker chromosomes were identified by M-FISH karyotype analysis.

M-FISH analysis revealed several cryptic translocations that were not detected by G-banding analysis in the cell line such as t(1;21),t(5;6),t(6;18),t(3;10),t(9;17),t(11;13)and t(12;21). Moreover, add(11) and add (21) that were detected by G-banding analysis were identified as t(11;13) and t(1;21) resulting in der(11) and der(21) by M-FISH analysis (Figure 3.1, 1.B and 2.B).

Importantly, the ring Philadelphia chromosome resulting from (9;22) on the cell line was misdiagnosed by G-banding analysis and detected by M-FISH analysis. Furthermore t(3;10) was detected in70% of the cells analyzed as 30% of the leukemic blast revealed the lack of this abnormality (Figure 3.1, B.2).

3.4.1.2 Cell line GDM-1

G-banding analysis on the acute myeloid leukaemia derived cell line GDM-1 revealed trisomy 8 and 13. Moreover structural abnormalities such as add(2q), del(6q) and add(7q) were also detected (Figure3.2, A).

The del (6q) and add(7q) were refined using M-FISH analysis as a reciprocal translocation t(6;7) resulting in and der(7). The add(2q) was refined by M-FISH as t(2;11) resulting in der(2) . Furthermore, a del(16) was identified by M-FISH analysis (Figure3.2.B).

3.4.1.3 Cell line Pfeiffer-CRL2632

The lymphoma derived cell line (Pfeiffer-CRL2632) abnormalities were investigated using G-banding and single and dual colour FISH. G-banding analysis revealed trisomy 5, isochromosome(8), add(11p), del(12p) and add(17q). Moreover 5 marker chromosomes were detected by G-banding analysis (Figure3.3).

In order to identify these five markers chromosomes on the Pfeiffer cell line further FISH images were analysed. The images were kindly provided by Dr. Tosi for analysis (Figure 3.4 A, B, C, D).FISH analysis confirmed G- banding findings of Trisomy 5 and isochromosome 8. FISH using a whole chromosome paint for chromosome 5 (WCP5), was confirmed trisomy 5 (Figure 3.4, A). Moreover, FISH using whole chromosome paint for chromosome 8 (WCP8) and locus specific probe for *C-MYC* gene at 8q24 confirmed iso-chromosome 8, as we can see that the q arm is duplicated (Figure 3.4, B). FISH using WCP6 and WCP13 have confirmed derivative chromosome der(6), del(6) and der(13) (Figure 3.4, C) that was detected by G-banding analysis as markers. However, FISH using WCP12 and WCP17 has

revealed a der(12) and a der(17) resulting from t(12;17). This cryptic translocation was detected by G-banding previously as deletion of (12p) and add(17q).

3.4.2 Revised karyotypes after FISH

3.4.2.1 The cell line K562

The G-banding analysis of K562 revealed a very complex karyotype with 4 marker chromosomes. The karyotype obtained through G-banding analysis was 64,XX,+1,del(1)(p?36),+2,+4,+5,del(5)(q?)+6,+7,+8,-9,+10,+11,add(11)(p12**) ,+**12,+15,+16, +17,+19,-20,+21,add(21) (q21).

The karyotype obtained after M-FISH was 68XX,+1,del(1)(p?36), +2,+4,+5,der(5)t(5;6), der(6)t(6;18),+6,+7,+7,+8,+9,del(9),der(10)t(3:,10),der(11)t(11;13),+11,+12,der(12)t(12;21) +15,+15,+16,+17,der(17)t(9;17),der(18)t(6;18),+19,+20,der(21) t(1;21), del(22),+22. This karyotype was detected in 70% of the cells analysed while 30% of the cells revealed a karyotype of 64 chromosomes.

3.4.2.2 The GDM-1 cell line

The G-banding analysis revealed a karyotype of 48,XX,add(2)(q34?),del(6)(q23), add(7)(q36),+8,+13. The revised the karyotype after M-FISH was revised to: 48,XX,der(2)t(2;11)(q34;q?p),del(6),der(7),t(6;7)(q23;q36),+8,del(16)(q?).

3.4.2.3 The Pfeiffer cell line (CRL2632)

The G-banding analysis indicated 5 marker chromosomes and revealed a karyotype of 49,XY,+5,del(6),add(6),iso(8)(q11.1?p11.1),add(11)(p15),del(12)(p13),add(13)(q?),del(14)(q?), +16,add(17)(q25). The 5 markers were identified by FISH analysis and the karyotype was modified to 49,XY,+5,del(6),der(6)t(6;13),iso(8)(q11.1),der(12)t(12;17),der(13)t(6;13)+16,der(17)t(12;1).

A

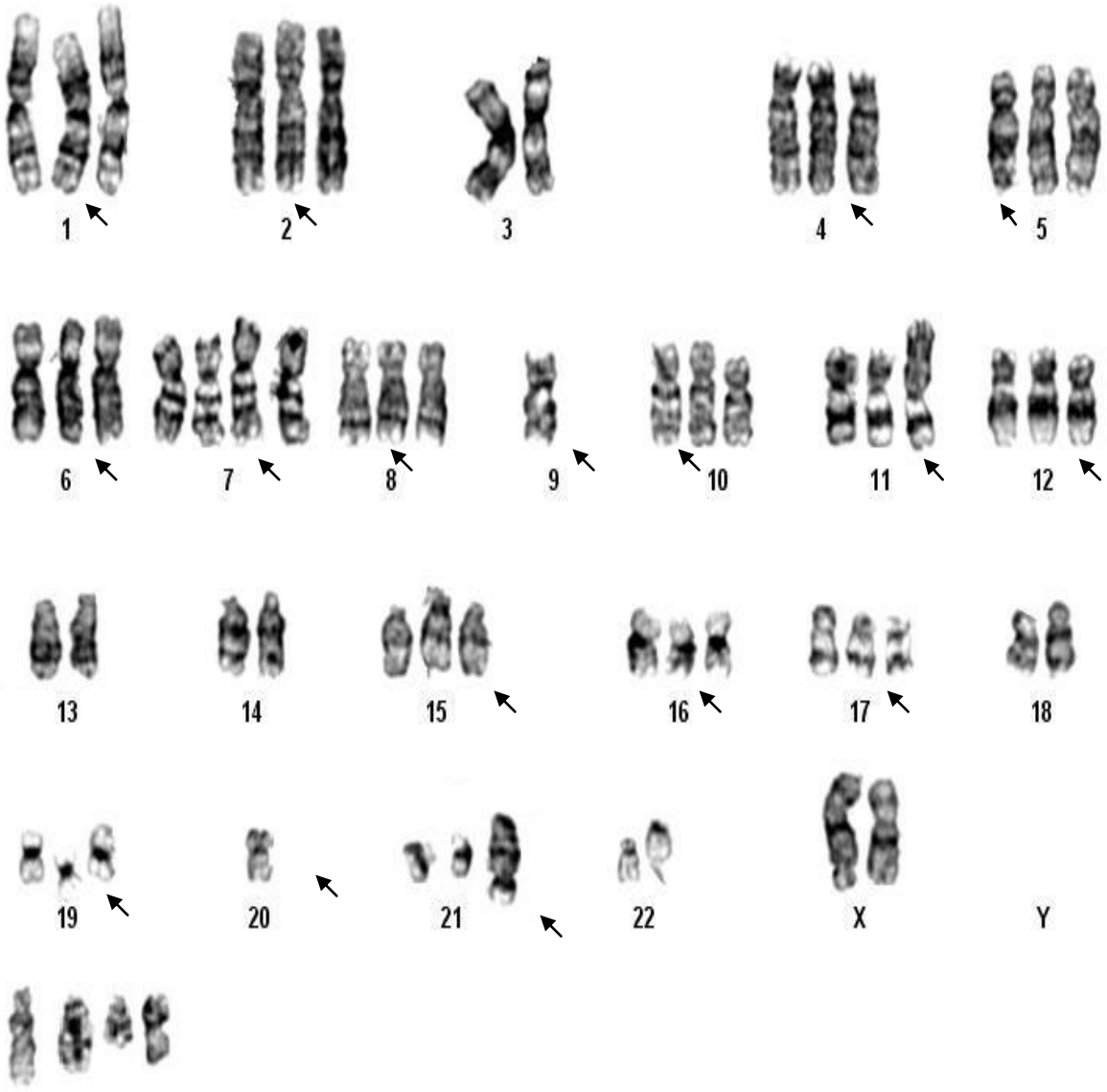

Figure 3.1 :(A) An example of G-banding karyotype of k562 cell line metaphase indicating numerical and structural abnormalities and karyotype of 64,XX,+1,del(1)(p?36), +2,+4, +5 ,del(5)(q?)+6,+7,+8,-9,+10,+11,add(11)(p12**) ,+**12,+15,+16, +17,+19,-20,+21,add(21) (q21).

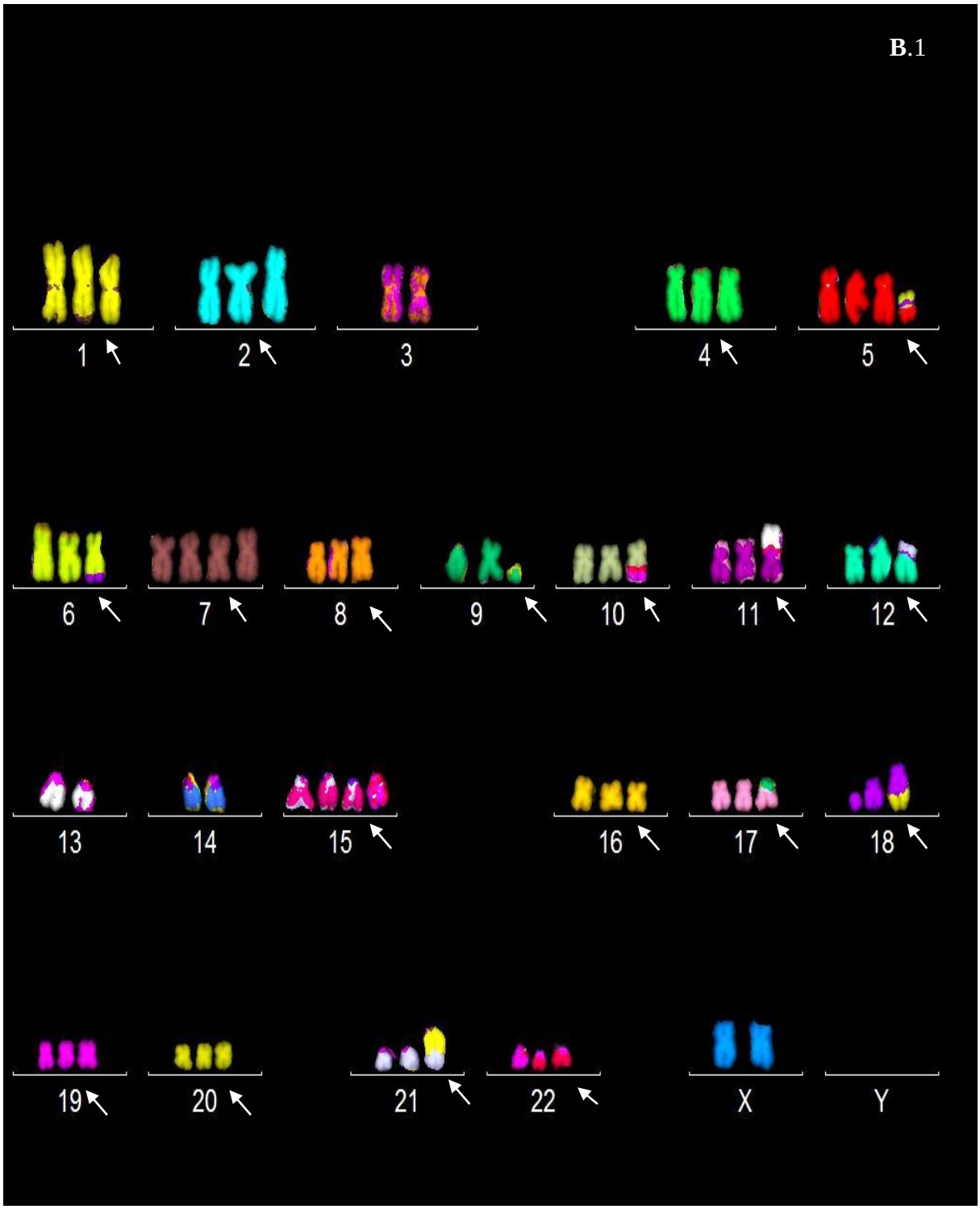

Figure 3.1: (B.1) An example of K562 M-FISH karyotypes (found in 70% of the leukaemia blasts) revealed very complex karyotype and cryptic translocations that were not detected by G-banding only. M-FISH karyotype of 68XX,+1,del(1)(p?36),+2,+4,+5,der(5)t(5;6),der(6)t(6;18),+6,+7,+7,+8,+9,del(9),der(10)t(3:,10),der(11)t(11;13), +11,+12,der(12)t(12;21)+15,+15,+16,+17,der(17)t (9;17),der(18)t(6;18),+19,+20,der(21) t(1;21), +21,del(22),+22.

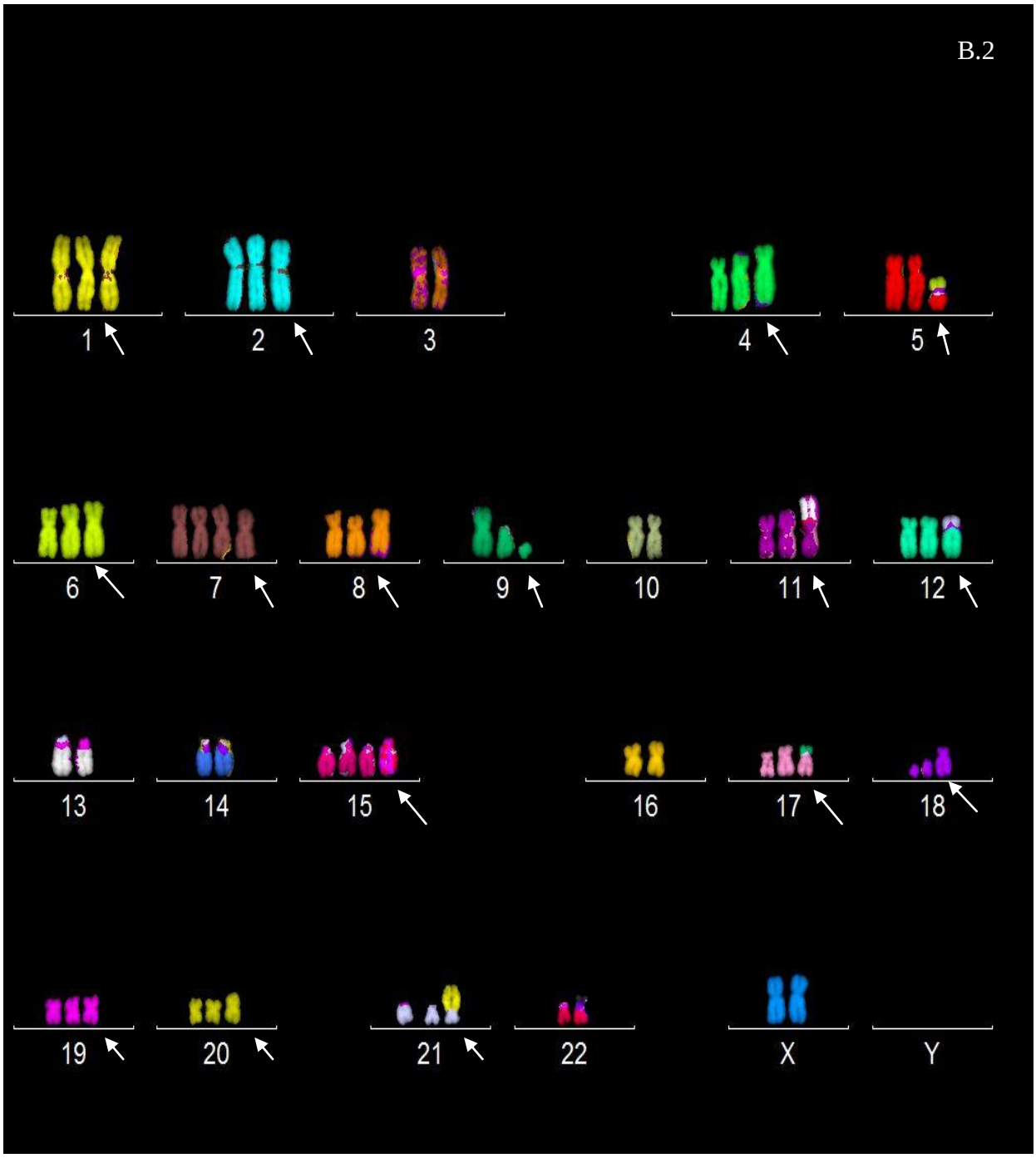

Figure3.1:(B.2) An example of M-FISH karyotype that has been found in 30% of K562 cells of 64XX ,+1,+2,+4,+5,der(5)t(5;6),+6,+7,+7,+8,del(9),+9,der(11)t(11;13),+11,+12,der(12)t(12;21)+15,+,+15,+16,+17, der(17) t(9;17)del(18),+18 +19,+20, der(21)t(1;21)+21,del(22).

A

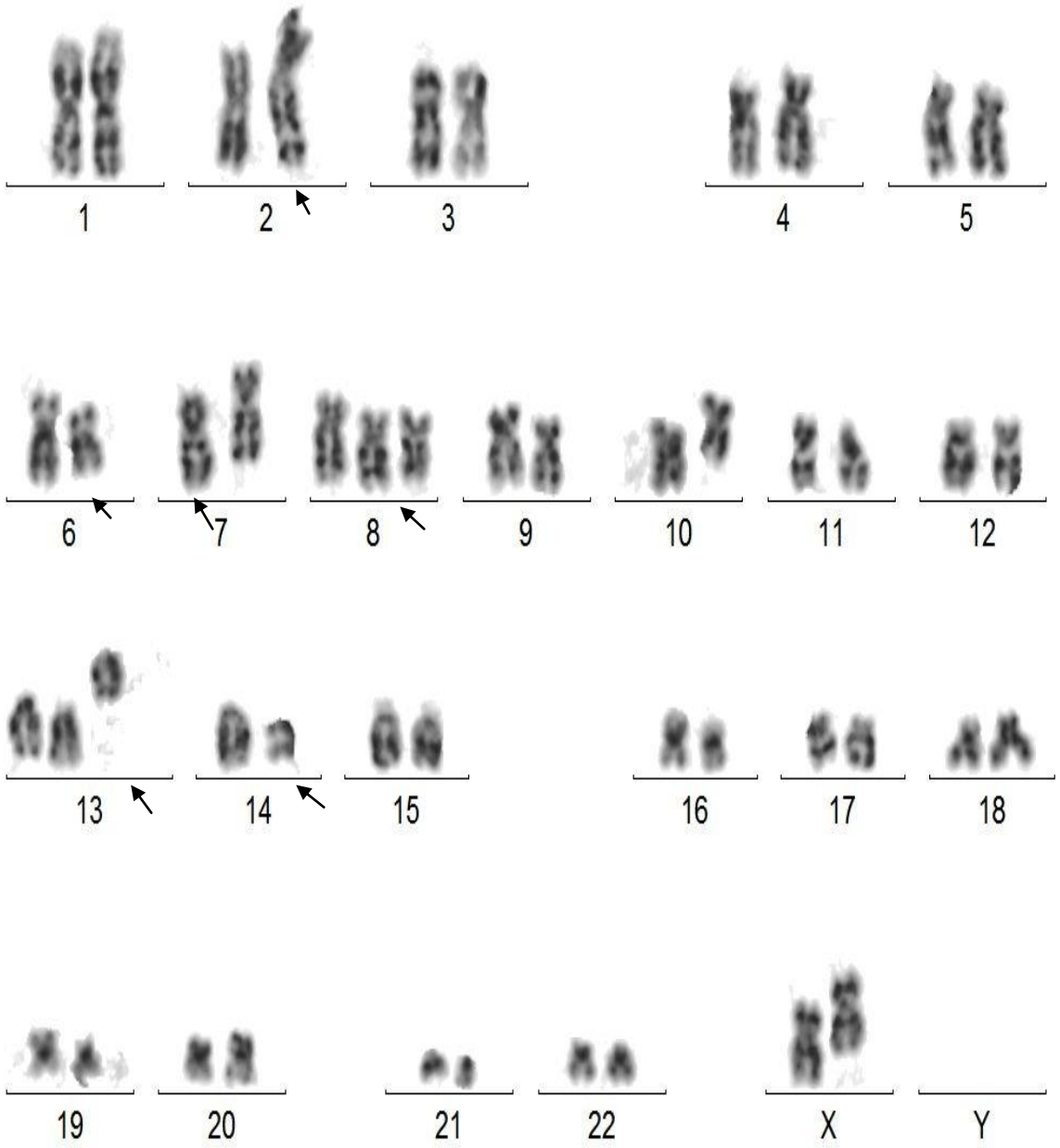

Figure3.2: (A) An example of G-banded analysis of the GDM-1 cell line metaphases indicated abnormal karyotype metaphase revealed a karyotype of 48,XX,add(2)(q34?),del(6)(q23), add(7)(q36),+8,+13.

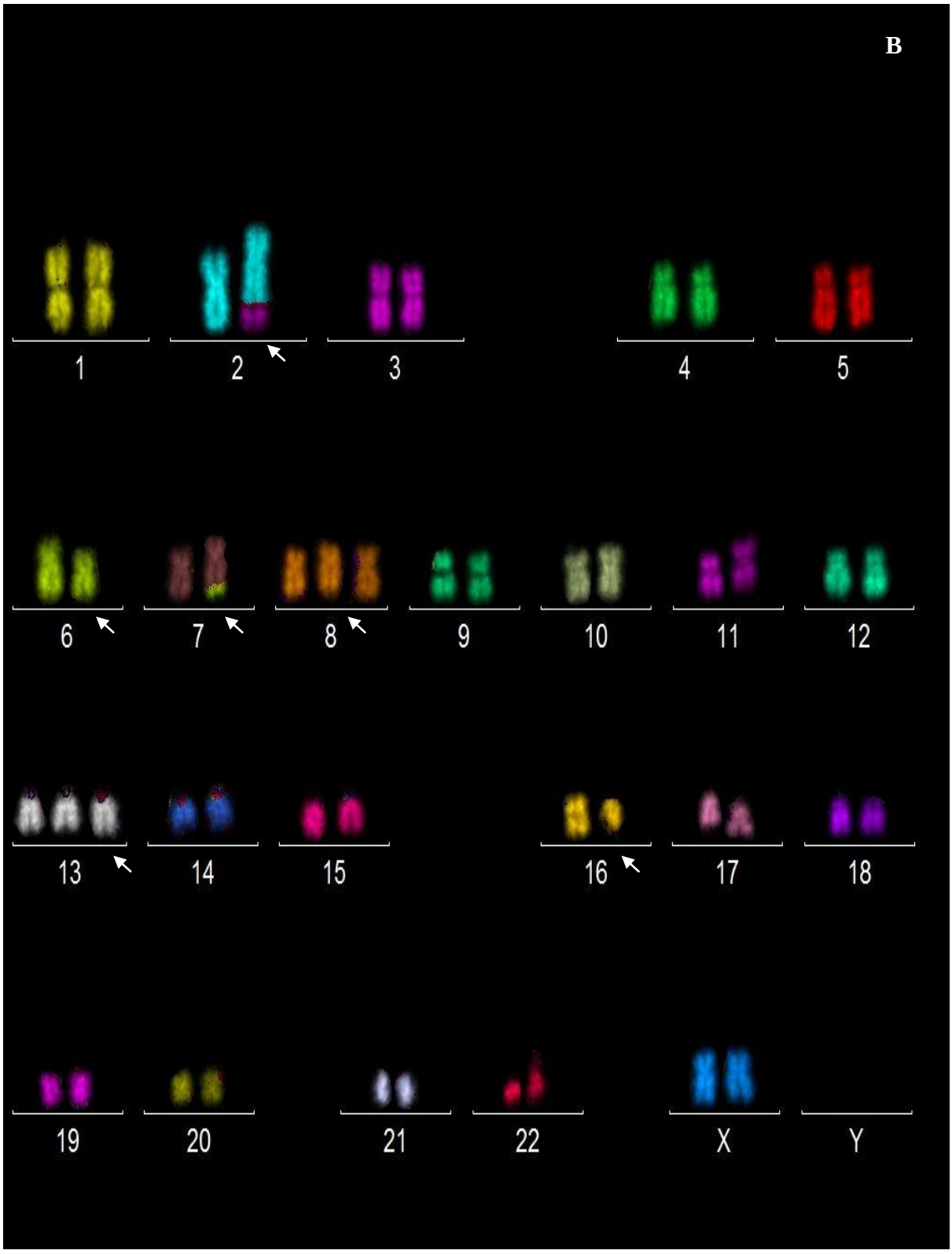

Figure3.2: (B) An example of M-FISH analysis on GDM-1 chromosomes detected numerical and structural abnormalities and a karyotype of 48XX,der(2)t(2;11),t(6;7), der(7),+8,del(12p),del(16q).

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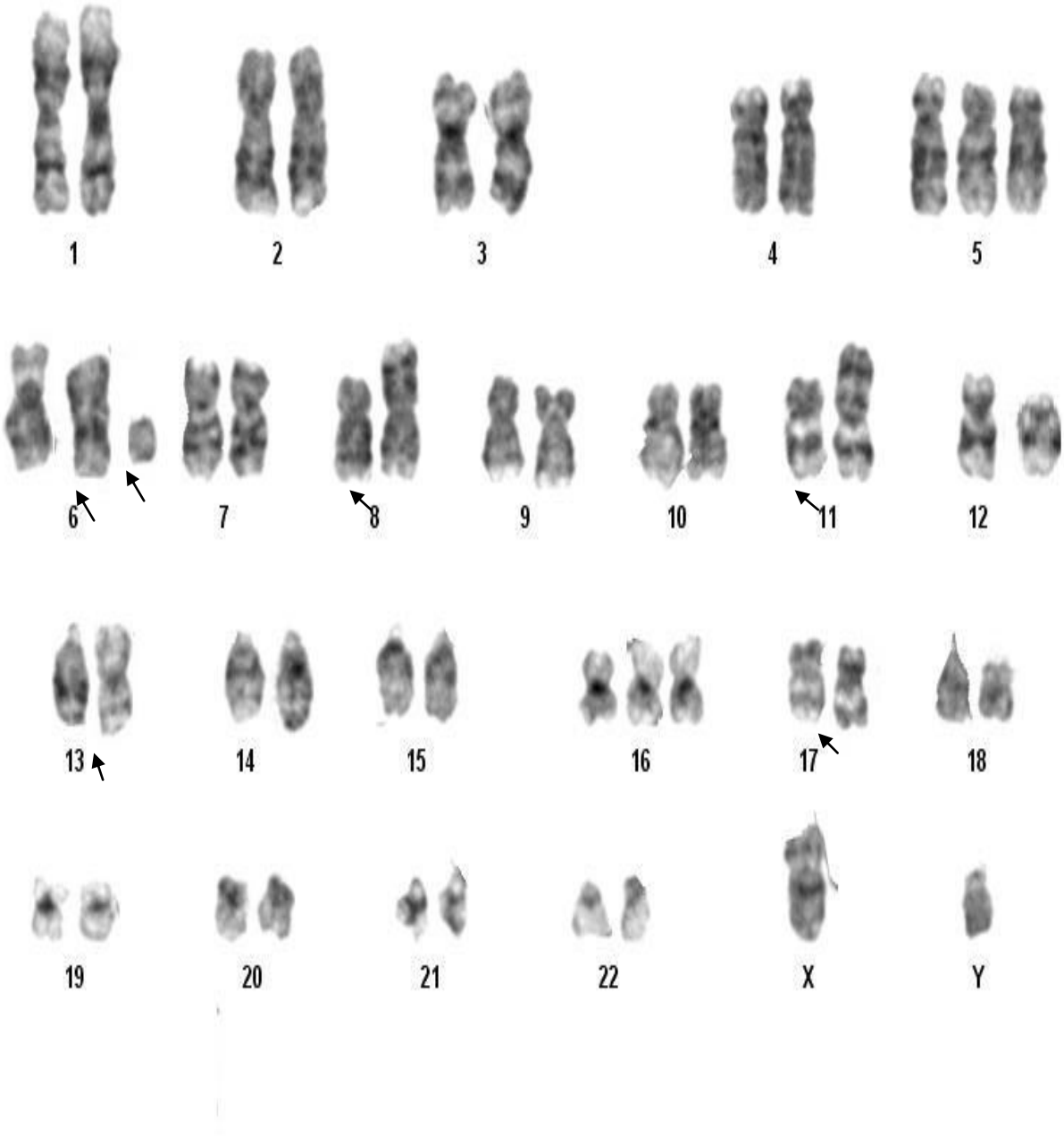
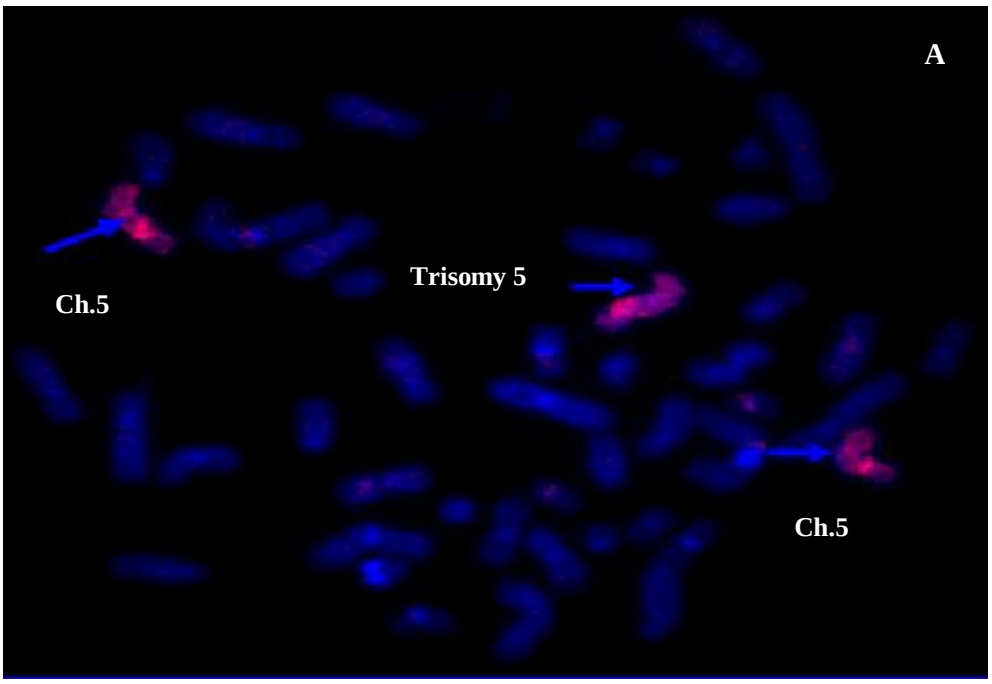
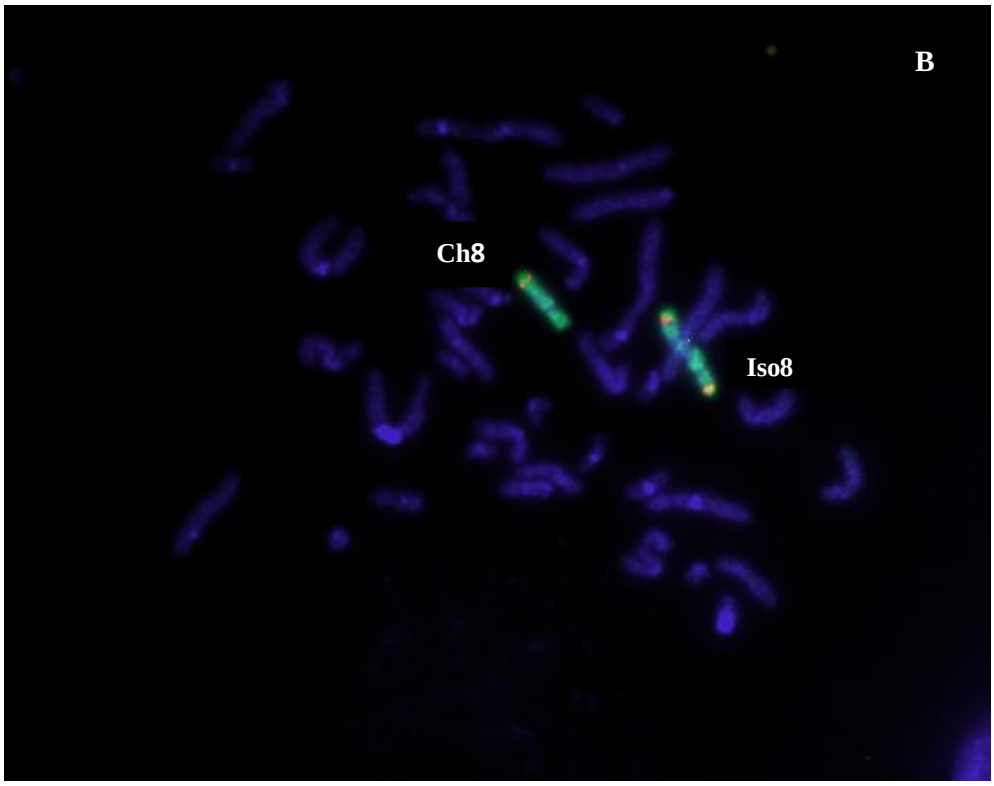
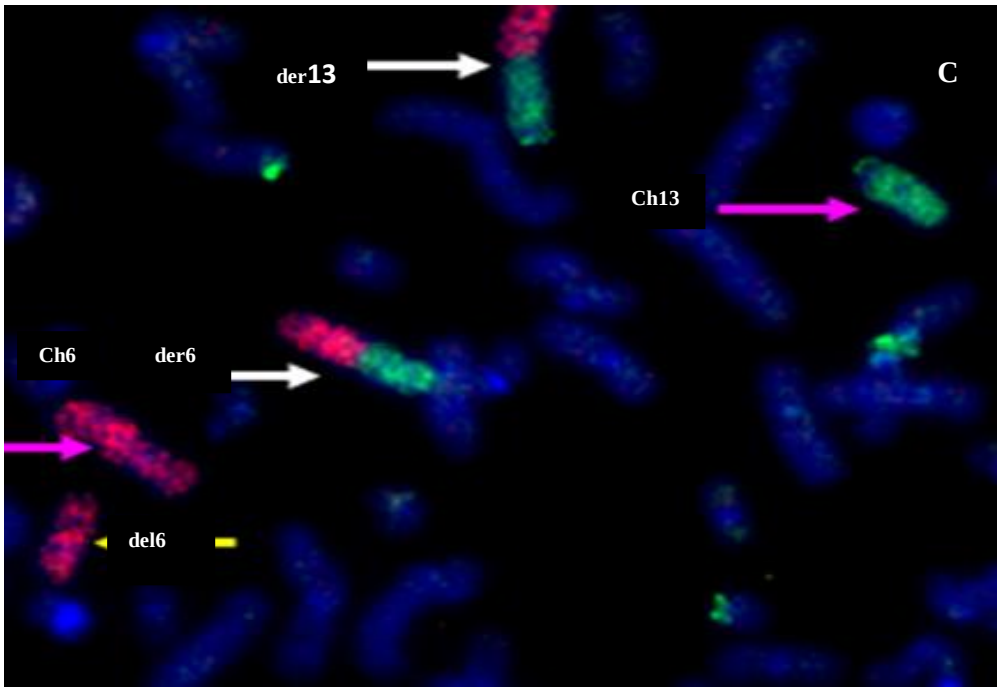


Figure 3.3: An example of G-Banding analysis on CRL2632-Pfeiffer cell line revealed chromosomal abnormalities and abnormal karyotype with five markers as the arrows indicates. The Karyotype of Pfeiffer cell line is 49,XY,+5,del(6),add(6),iso(8)(q11.1?p11.1),add(11)(p15),del(12)(p13),add(13)(q?),del(14)(q?), +16,add(17)(q25).


A
Ch.5
Trisomy 5
Ch.5


B
Ch8
Iso8



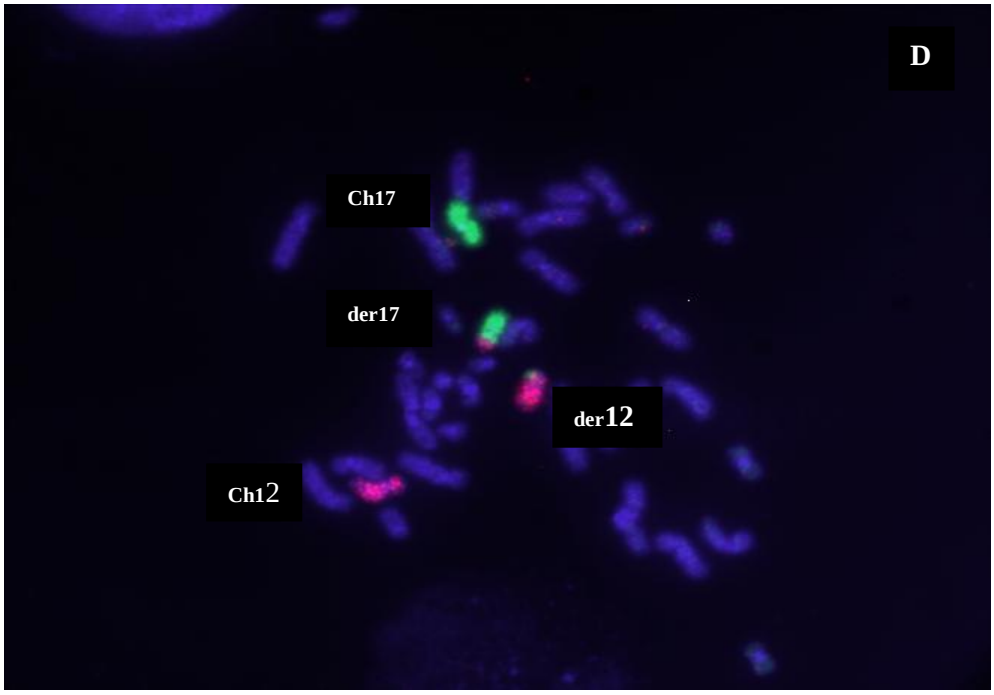


Figure 3.4: Metaphase FISH images of Pfeiffer cell line. **(A) FISH** using WCP5 in red confirmed trisomy 5. **(B)** WCP8 in green and specific locus probe for *C-YMC* gene in red confirmed iso(8)(q11.1). **(C)** Dual colour FISH using WCP6 in red and WCP13 in green confirmed der(6),der(13) and del(6). **(D) Dual colour FISH** using WCP12 in red and WCP 17 in green confirmed a reciprocal translocation t(12;17).

3.5 Discussion

Chromosomal translocations are among the most common genetic abnormalities associated with lymphomas and leukaemia (Rabbitts, 1994). Changes in chromosome 7 have been identified in leukaemia and lymphomas in the form of loss of chromosome 7 material as well as translocations of chromosome 7.

In this study conventional cytogenetic G-banding technique and molecular cytogenetic technique multiplex FISH (M-FISH), as well as single and dual colour FISH were applied to investigate very complex chromosomal abnormalities in leukaemia and lymphoma cell lines. All three cell lines have shown an over expression of *HLXB9* gene (data not shown). The human acute myeloid leukaemia (AML) cell line GDM-1 and chronic myeloid leukaemia (CML) cell line K562 were analysed, as well as a lymphoma cell line (CRL2632) also known as Pfeiffer). The use of this dual approach has helped in refining the chromosomal aberrations in these cell lines and enabled us to obtain a more accurate karyotype.

The conventional cytogenetic technique G-banding was a useful screening tool to study the whole chromosomes abnormalities and identified the breakpoints within translocations. However, M-FISH technique was a powerful method to indicate cryptic translocations that were misdiagnosed as deletions by only G-banding analysis in the cell lines (K562 and GDM-1) that have been analysed in this study. Therefore, M-FISH analysis was enabled a more accurate karyotype by identifying the origin of translocated genetic material between chromosomes.

The power of M-FISH analysis in unravelling very complex rearrangements and cryptic translocations has been reported previously by other groups (Hilgenfeld et al., 2001; Mathew et al., 2001; Naumann et al., 2001; Nordgren et al., 2001).

However, M-FISH is a low-resolution molecular technique enabling a broad screening of the gross chromosomal aberrations. The combination of M-FISH analysis and classic banding technique enables the establishment of an accurate karyotype. This is due to the M-FISH enabling the identification of the origin of chromosomes involved in a specific rearrangement, whereas the conventional cytogenetic approach enables the assigning of specific breakpoints due to the availability of a precise banding pattern.

M-FISH on K562 cell line was previously reported. This cell line is well known and widely studied as a model of CML and because it harbours a Philadelphia ring chromosome r(22) resulting from (9;22).

Naumann et al., 2001 established a complete karyotype of the K562 cell line using G-banding, M-FISH, FISH using whole chromosome painting and locus specific probes and comparative genomic hybridization (CGH). This study showed that structural abnormalities of chromosome 7 were present beside the numerical abnormalities reported before. Two abnormal copies of chromosome 9 have been indicated while our data confirm 3 copies of chromosome 9, of which two copies are abnormal. Interestingly 4 copies of chromosomes 10 and 12 (of which 2 copies of each were abnormal) and 11 (of which one copy was abnormal) were confirmed. The following trisomies of chromosomes 1, 2, 4, 6, 15, 16, 17, 19, 21 and 22 were confirmed and in agreement with the M-FISH analysis here reported. Importantly four copies of chromosome 5 have been confirmed and this matches our finding. Moreover, in Naumann study the following derivative chromosomes der(5), (10),der(11),der(12),der(17) and der(21) have been confirmed which support our data.

In the study presented here, the GDM-1 cell line was analysed using M-FISH for the first time. The M-FISH revealed a very similar karyotype to G-banding analysis that was reported by Nagel (Nagel et al., 2005). This finding showed that G-banding is a valuable tool to detect

the chromosomal abnormalities despite the difficulty of identifying the added or deleted genetic materials origin and the special training it requires.

3.5.1 Conclusions

These studies enabled more analysis of gross chromosomal abnormalities in leukaemia and lymphoma cell lines that are associated with over-expression of *HLXB9*. The GDM-1 cell line was shown previously (Nagel et al., 2005 and in this study) to carry t(6;7)(q23;q36) with breakpoint at 7q36, which makes it a good candidate to study *HLXB9* gene furthers as it localises at the same breakpoint. M-FISH was a good method to indicate gross chromosomal abnormalities. Unfortunately, the investigation of the *HLXB9* gene could not be done by M-FISH due to the lower resolution of the technique.

The characterisation of chromosomal abnormalities in cell lines is a starting point on which to base more refined studies at the gene level. The work here described here has allowed the discrimination of different types of abnormalities in cell lines characterized by over-expression of the *HLXB9* gene. As the *HLXB9* gene is localized on chromosome 7, it was expected to find abnormalities involving this chromosome. In fact, this was the case in the myeloid leukaemia cell lines K562 and GDM-1, where both numerical (K562) and structural (K562 and GDM-1) abnormalities were found. The Pfeiffer cell line has not shown any abnormality of chromosome 7 with the methodologies applied here. Therefore, the reason for *HLXB9* over expression needs to be investigated further.

3.5.2 Future work

Future work will involve investigations into the mechanisms of activation for *HLXB9* gene expression. To date, little is known about the factors that induce over-expression of *HLXB9* and there is scope for more research in this area. One of the cell lines studied here, GDM-1, has been already used in this sense and the *Myb* gene has been proposed to promote *HLXB9* over-expression being juxtaposed to *HLXB9* by virtue of the t(6;7) rearrangement(Nagel et al., 2005). The precise molecular mechanisms by which this occurs have yet to be elucidated. Similarly, more work is needed to clarify the mechanisms of *HLXB9* over-expression in the other two cell lines described here. In K562, *HLXB9* over-expression might be due to the presence of additional copies of chromosome 7. The situation might be more complex in the case of the Pfeiffer cell line, where no abnormalities of chromosome 7 have been reported. Studies at the molecular level will shed some light on these mechanisms. However, these are out of the scope of this project. In order to confirm the chromosomal abnormalities that were indicated by M-FISH, further FISH using specific probe of the *HLXB9* will be carried out. Such experiments will be shown in the GDM-1 cell line and in a series of patients in chapter 5 of this thesis.

CHAPTER 4

CHARACTERIZATION OF CHROMOSOME 7 ABNORMALITIES IN ACUTE MYELOID LEUKAEMIA (AML) DERIVED CELL LINE (GDM-1) AND AML PATIENTS USING WHOLE CHROMOSOME PAINTING

4.1 Introduction

4.1.1 Aberrations of chromosome 7 in myeloid leukaemia

Characterization of chromosomal abnormalities is essential to better understand and diagnose haematological malignancy. Two different categories of clonal chromosome abnormality are identified in acute myeloid leukaemia (AML): unbalanced rearrangements that are associated with gain or loss of chromosome materials and a balanced rearrangement without gain or loss of chromosome genetic materials (Pedersen-Bjergaard and Rowley, 1994).

Chromosomal deletions are characterized by the absence of chromosome segments. Deletions are classified as terminal if the missing genetic materials are localized by the chromosome edge (Figure 4.1 (A)), and interstitial if the deleted genetic materials are within the chromosome arms (Figure 4.1(B)) (Genetic home reference hand book, 2012).

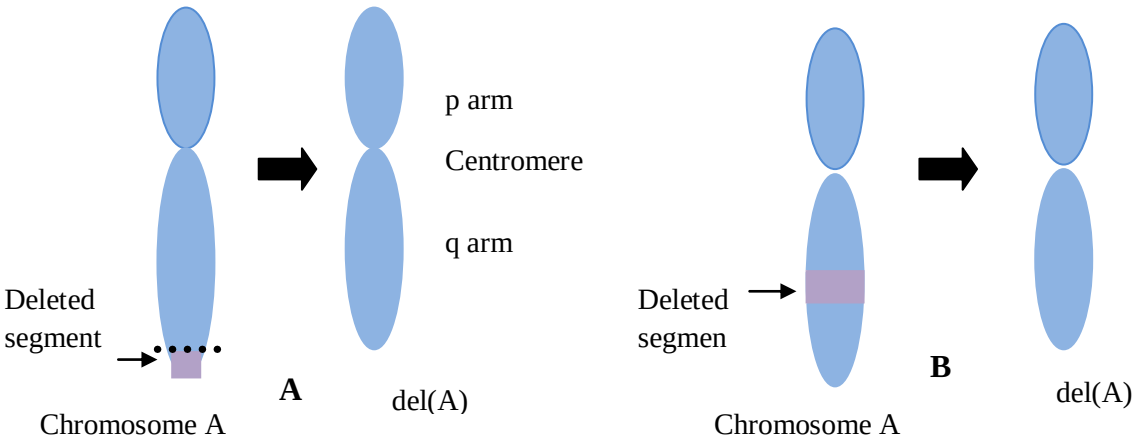


Figure 4.1: (**A**) An example of ideogram illustrates terminal deletion that occurs on the chromosome end and affected telomere region. (**B**) An example of ideogram illustrates interstitial deletion that localizes within the chromosome long or short arms and occurs between 2 breakpoints at the same arm of a chromosome.

Chromosomal translocations are one of the most common abnormalities associated with lymphomas and leukaemia (Rabbitts, 1994). Translocations are characterized by the exchange of DNA segments between two different chromosomes. This is balanced when there is no loss or gain of chromosome material (Figure 4.2) and unbalanced when there is a gain or loss of chromosomes material (Figure 4.3) (Genetic home reference hand book, 2012).

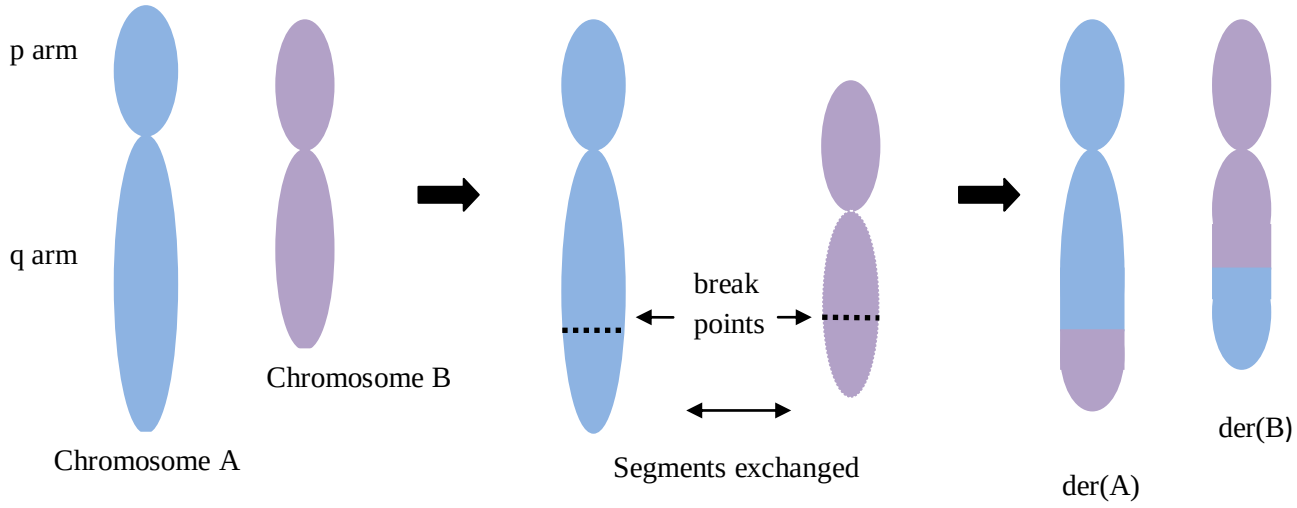


Figure 4.2: An example of ideogram illustrates the balanced translocation with exchange between two segments on two different chromosomes (A) and (B). The derivative chromosomes der(A) and der(B) show the translocated genetic material on each.

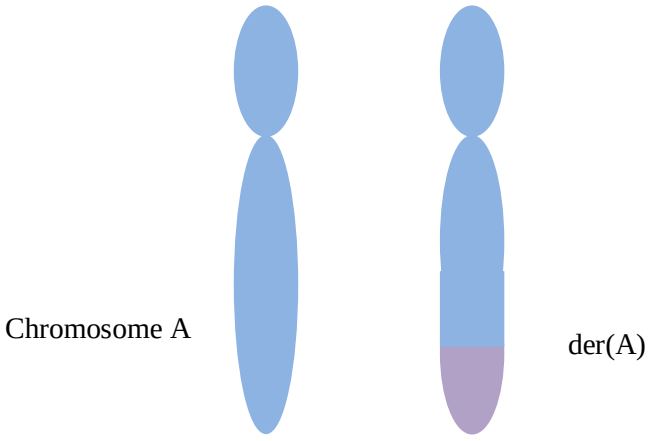


Figure 4.3: An example of ideogram illustrates unbalanced translocation that shows a normal chromosome (A). In addition the derivative of chromosome (A) der (A) with the presence of additional material derived from another chromosome and the deletion of the chromosome long arm genetic material.

The identification of genetic alterations in AML patients has a significant importance in classification, prognosis and response to the treatment.

Numerous chromosomal translocations have been associated with a specific subtype of AML. Various chromosomal regions that frequently show gains or losses have been identified in leukaemia. In addition, the involvement of some genes in leukaemogenesis has been established as shown in (Table 4.1) (Zutven, 2005).

Clonal abnormality	**Phenotype**	**Genes involved**
11q23 rearrangements	AML and other leukaemia	*MLL*-partner gene
t(15;17)(q22;q12)	AML	*PML-RARA*
t(8;21)(q22;q22)	AML	*AML1-ETO*
-5/del(5q)	AML and other leukaemia	
-7/del(7q)	AML and other leukaemia	
11p15 rearrangements	AML and other leukaemia	*NUP89*
t(7;12)(q36;p13)	AML and very rare in other leukaemia	*ETV6/HLXB9*

Table 4.1: The most frequent chromosomal abnormalities associated with AML and other types of leukaemia with some genes that have been involved. This table modified from (Zutven, 2005).

Several chromosomal abnormalities are associated with good or poor prognosis; for example, t(15;17)(q22;q12), t(8;21)(q22;q22) and t(16;16)(p13q22) are connected with favourable outcomes and a high rate of patient survival (Von Neuhoff, et al., 2010). However, the deletion of 7q is associated with poor prognosis.

Loss of chromosome 7 material, whether of the whole chromosome or part of it, is a common finding in myeloid disorders (Beau et al., 1996).

Myeloid leukaemia disorders involving the long arm of chromosome 7, del(7q), are clinically associated with a short survival time, poor prognosis and poor response to chemotherapy (Bernstein et al., 1984).

Several deleted segments on 7q have been identified in myeloid leukaemia. The deletion of 7q22 has been reported previously (Curtiss et al., 2005; Le Beau et al., 1996; Fischer et al., 1997) and 7q31–q32 and 7q33 (Tosi et al., 1999). In addition, the deletion of 7q32-33 segment is also a known feature (Beau et al., 1996) and the deletion of 7q35-q36 region (Döhner et al., 1998)

However, the interstitial and terminal deletions of 7q that was described earlier in this introduction (Figure 4.1 (A) and (B)) have been identified previously by FISH using WCP7 and specific probes for chromosome 7 centromere and telomere regions (Tosi et al., 1996). Tosi's study was able to identify five patients with interstitial deletions of 7q and three patients with terminal deletions of 7q.

4.1.2 Chromosomal Translocations involving 7q36

The chromosomal translocations associated with loss of the whole or main material of 7q were detected by FISH in leukaemia patients (Bernstein et al., 1984).

The acute myeloid leukaemia cell line, GDM-1, is characterized by the presence of t(6;7)(q23;q36) with a break point in the 7q36 region. The translocation resulted in the

MYB/HLXB9 rearrangement and over expression of the *HLXB9* gene on the GDM-1 cell line. The translocation has been confirmed by conventional cytogenetic technique G-banding (Ben-Bassat et al., 1982) and detailed fluorescence in situ hybridization studies (Nagel et al., 2005).

The 7q36 region is observed to be rearranged in the GDM-1 cell line and particular AML patients with t(7;12)(q36;p13) and indicated a breakpoint within the 7q36 region where the *HLXB9* gene is localized. An over expression of *HLXB9* has been associated with these chromosomal rearrangements in the GDM-1 cell line and such patients.

The presence of t(7;12) (q36;p13) is associated with infant leukaemia. It has been reported in one third of AML patients under the age of two years with poor outcomes (Tosi et al., 2003; Von Bergh et al., 2006). The t(7;12) has resulted in the rearrangement of the *ETV6/HLXB9* genes. However, the *ETV6- HLXB9* fusion transcript has been found in approximately 50 per cent of t(7;12) patients with over-expression of *HLXB9* (Tosi et al., 2003; Von Bergh et al., 2006).

4.1.3 Fluorescence in situ hybridization using the whole chromosome painting (WCP) approach in Leukaemia studies

Fluorescence in situ hybridization (FISH), using whole chromosome painting (WCP), is an accurate tool that allows the visualization of each individual chromosome's origin by staining the length of chromosome in metaphase spreads and interphase cells (Chevret et al., 2000).

Two decades ago, chromosome painting technique was established by two independent research groups at (Pinkel et al., 1988; Cremer et al., 1988). The groups had taken advantage of the accessibility of cloned DNA libraries that were derived from flow-sorted human chromosomes (Gray et al., 1975).

The principle of this method is based on the hybridization of previously labelled a nucleic acid probe to target DNA sequences of fixed cells or tissues. Following hybridization, the hybridized signals can be visualized using special filters and a fluorescence microscope.

FISH, using whole chromosome painting, is a powerful technique to reveal cryptic unbalanced translocations that are not detected or have been misdiagnosed as deletions in conventional cytogenetic G-banding analysis (Bennour et al., 2012; Cherif et al., 1993; Pan et al., 2012; Tosi et al., 1996 and 1997).

However, the limitations of chromosome painting probes are apparent. The detection of interchromosomal structural abnormalities, such as translocations, is greatly higher than intrachromosomal aberrations such as deletions or inversions. The latter abnormalities are difficult to detect because the genetic material involved in the rearrangement would be stained with the same fluorochrome that stains the rest of the chromosome. The combination of conventional cytogenetic techniques such as G-banding and FISH analysis help to fill up this methodological gap (Lurie et al., 1980).

4.2 Aims of the study

The aim of this work was to characterise the chromosomal rearrangements involving chromosome 7 in an AML cell line and patients in order to discriminate between deletions and translocations using the whole chromosome 7 painting technique.

In order to achieve this, FISH with the WCP7 will be used on the AML cell line (GDM-1) and on a series of AML patients. The chromosome 7 abnormalities were defined by G-banding analysis previously in the GDM-1 cell line (Nagel et al., 2005) and in AML patients by the diagnostic lab (Children's University Hospital, Giessen, Germany).

4.3 Materials and methods

4.3.1 Cell line and patients

The human AML derived cell line, GDM-1, and seven AML patients that were characterized by the presence of chromosome 7 abnormalities were analysed to investigate the 7q rearrangement in this study. The patients' materials were provided kindly by Professor Jochen Harbott at the Children's Hospital, Giessen, Germany. The clinical and cytogenetic details of these patients are provided in (Table 4.2).

Pt	Sex/Age	Hematologic disease	Reported karyotype (by G-banding)	Revised karyotype (by FISH)
1	M /5Years	AML-M4	46,XY,del(7)(q33~34)	46,XY,add(7)(p11)
2	F /11Years	AML-Mixed	46,XX,del(7)(q22)	_
3	F /3Years	AML	45,XX,add(7)(q3?4),-9	_
4	M /80Years	AML	41~43,XY,del(7)(q?)	43,XY,add(7)(p11)
5	F /15Years	AML-M4	46,XX,del(7)(q22)	_
6	M /13Years	AML-M4	46,XY,del(7)(q22~q31)	_
7	F /15Years	AML-M4	46,XX,del(7)(?q22q31)	46,XX,add(7)(q?)

Table 4.2: Clinical and Cytogenetic data of patients with Chromosome 7 rearrangement in this study. (-) means the karyotype was not revised by FISH and remained the same as reported by G-banding analysis.

4.3.2 Cell culture

The GDM-1 cell line was cultured as described in part 2.1.3 of chapter 2.

4.3.3 Harvesting of cells

The GDM-1 cell line was harvested as described earlier in part 2.1.4 of chapter 2.

4.3.4 Preparation of slides

The GDM-1 cell line and AML patients' chromosome suspension were spread as described in part 2.1.5 of chapter 2.

4.3.5 Probe

A whole chromosome 7 painting probe (WCP7) that was directly labelled with FITC and commercially obtained (Cambio, Cambridge, UK) was used to detect chromosome 7 abnormalities in this study.

4.3.6 Fluorescence in situ hybridization

The aged slides were washed in saline-sodium citrate (2XSSC pH=7.0) while shaking for five minutes (SSC buffer in 20Xconcentration, Sigma, UK). They were then dehydrated through an alcohol series (70%, 90% and 100% ethanol) followed by air-drying for five minutes. The slides were denatured in 70% formamide denaturing solution containing 2XSSC at 70^{o} C for five minutes. Following the denaturation, the slides were instantly plunged into ice-cold 2XSSC for five minutes, then dehydrated again through an alcohol series (70%, 90% and 100% ethanol) and air dried at room temperature. The slides were ready to hybridize with the probe. The human chromosome 7 paint probe was mixed with the hybridisation buffer (1 part probe: 4 parts hybridisation buffer). The probe was denatured at 65ºC for 10 minutes and incubated at 37^{o}C for 10 minutes to allow re-annealing of repetitive sequences. The 15µl of denatured probe was applied to the denatured slide and covered with a 22X22 mm coverslip. The coverslip was sealed by rubber cement glue and left to hybridise in a moist chamber at 37^{o}C overnight. At post hybridisation, the glue was gently removed and the slide was washed in 2XSSC for five minutes while shaking in order to remove the coverslip. The slide was then washed in pre-warmed 0.4XSSC for five minutes in a water bath at 72^{o}C (diluted from 20X SSC buffer, Sigma, UK). The slide was washed in 2XSSC for five minutes at room

temperature while shaking. It was then washed in phosphate-buffered saline (PBS) for five minutes while shaking (phosphate-buffered saline, Sigma, UK). For DNA detection, 15µl of diamidino-2-phenylindole (DAPI) solution (Cambio, UK) was added for each slide. The slide was covered with a 22X40 mm coverslip and sealed with nail polish.

4.3.7 Image capture and microscope analysis

The slides were visualised and analysed using the Olympus BX41 fluorescence microscope and UPlanFLN 100Xoil immersion lens. Metaphase images were captured using a greyscale digital camera (Digital Scientific, UK) and Smart Capture 3 software (Digital Scientific, UK). A minimum of twenty metaphases were analysed for GDM-1 cell line and minimum of five metaphases were analysed for patients. In some patients there were a limited number of metaphases due to the poor quality of the material.

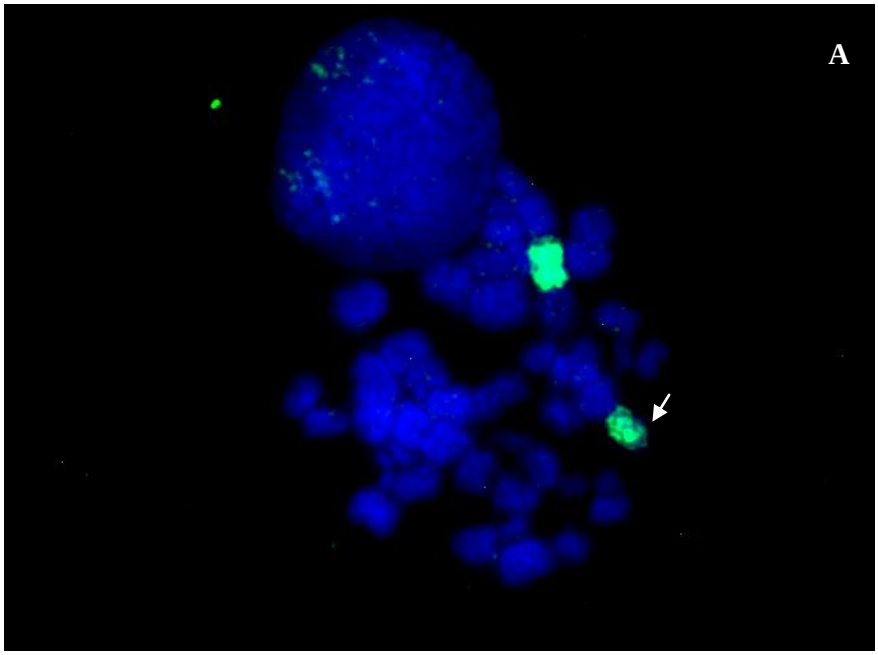
4.4 Results

The GDM-1 cell line and seven AML patients with 7q abnormalities were analysed in this study. FISH using the whole chromosome 7 paint (WCP7) was used to validate the karyotype obtained previously by G-banding. Chromosome 7 painting analysis confirmed chromosome 7 rearrangements in two main categories: deletions and translocations.

The G-banding findings of del 7q were confirmed in 3 patients (Patient nos. 2, 5 and 6) However, the G-banding abnormal karyotype was modified by FISH in 3 patients (Patient nos. 1, 4 and 7) as shown in (Table 4.2).

4.4.1 Deletions of 7q

Based on the combination of both G-banding analysis and FISH using WCP7 analysis we were able to confirm 3 deletions of 7q in patients (Patient nos. 2, 5 and 6). In these patients, the normal chromosome 7 and del(7) were completely painted. FISH analysis images were taken in two patients, with del(7)(q22) (Patient nos. 2 and 5) (As shown in Figure 4.4 and Figure 4.5) and one patient (Patient no. 6) with del(7)(7q22~q31) (Figure 4.6).



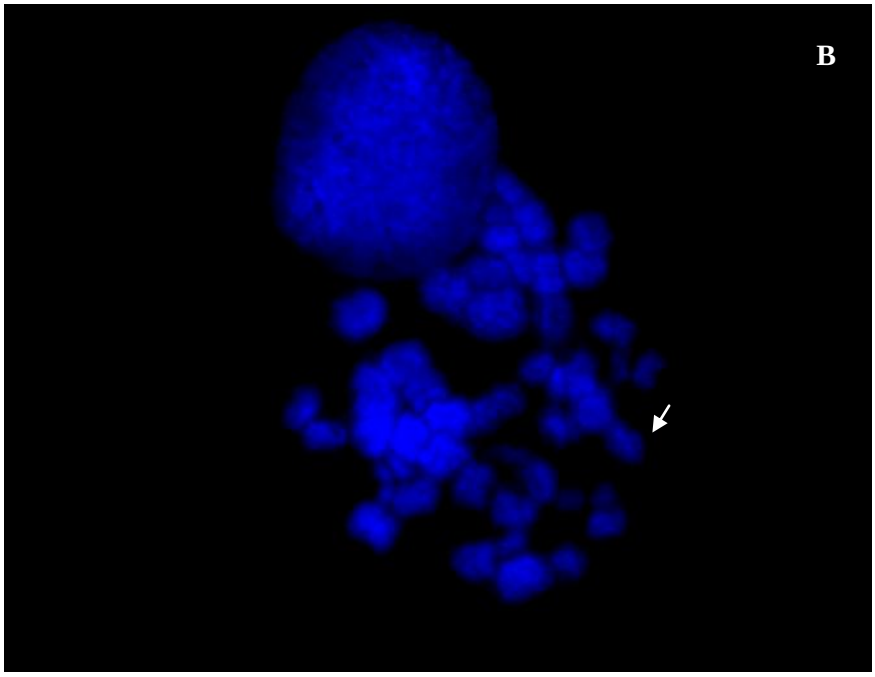


Figure 4.4: (**A**) An Example of FISH performed on metaphase chromosome obtained from patient No. 2 using WCP7 FITC (visible in green). The white arrow indicates del(7q) that shows the deletion of 7q22 materials. (**B**) With the same image as (A) showing DAPI only.

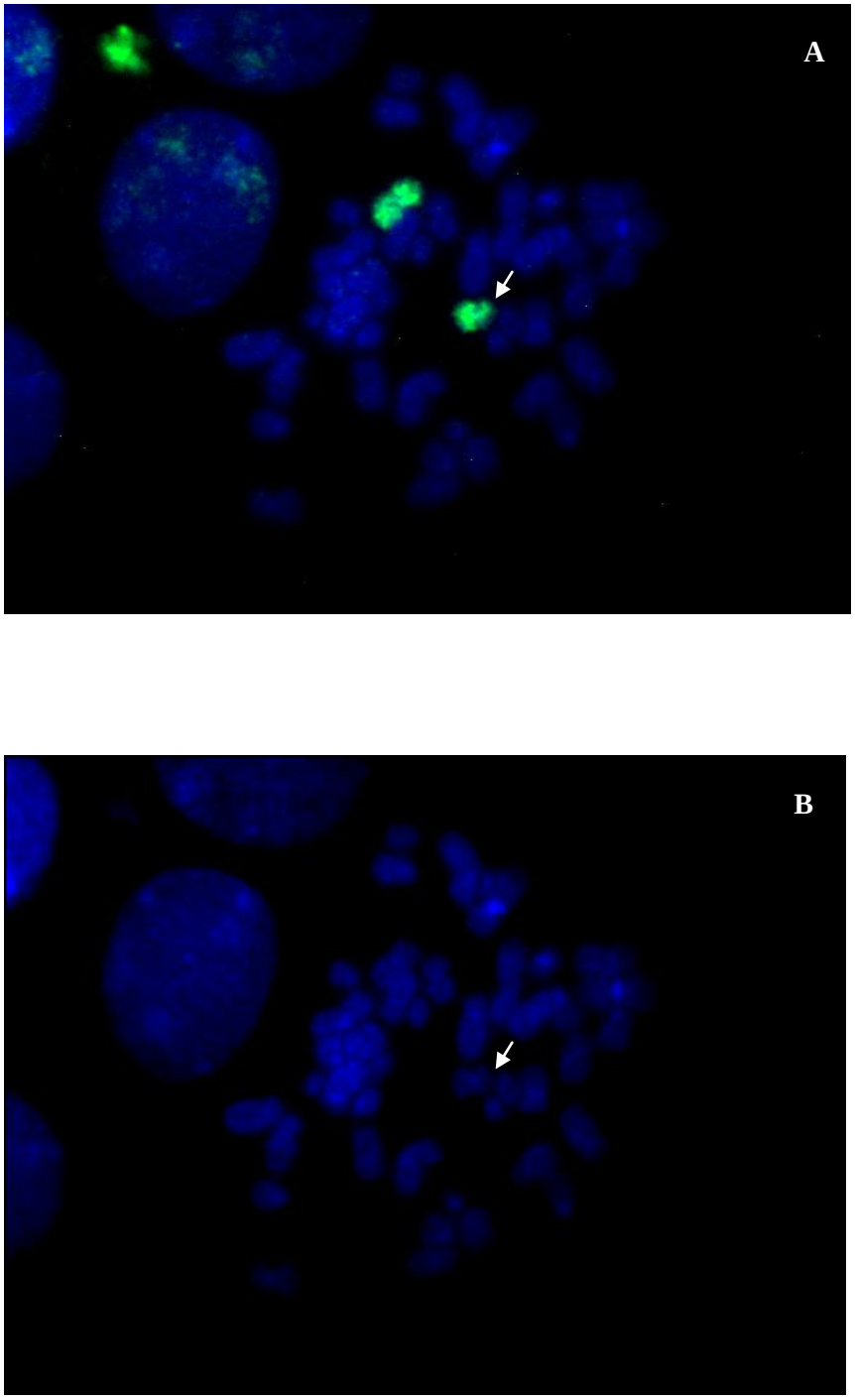
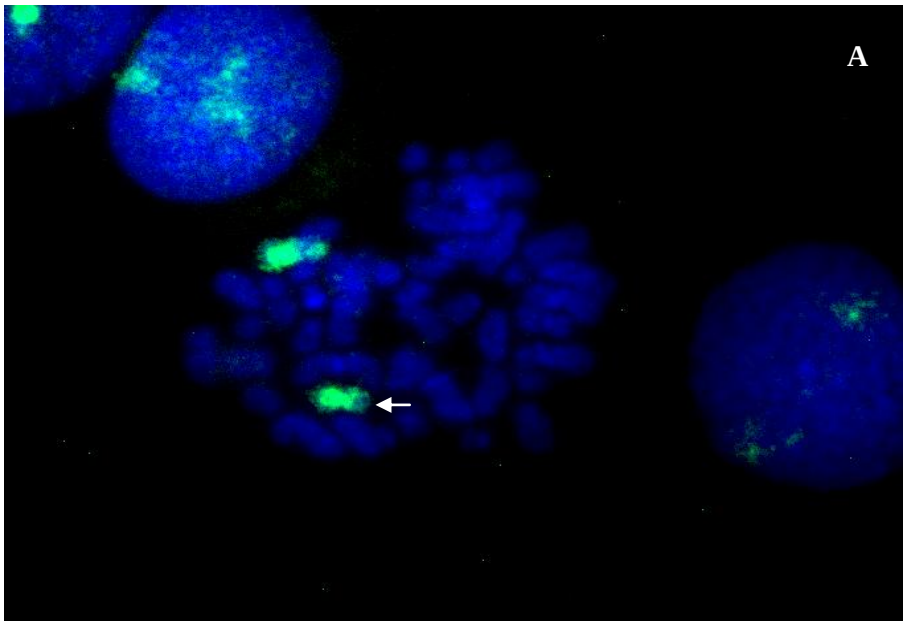


Figure 4.5: (**A**) An Example of FISH performed on metaphase chromosome obtained from patient No. 5 using WCP7 FITC (visible in green). The white arrow indicates del(7) that shows a shorter chromosome 7 with deletion. (**B**) The same image as (A) with DAPI only.



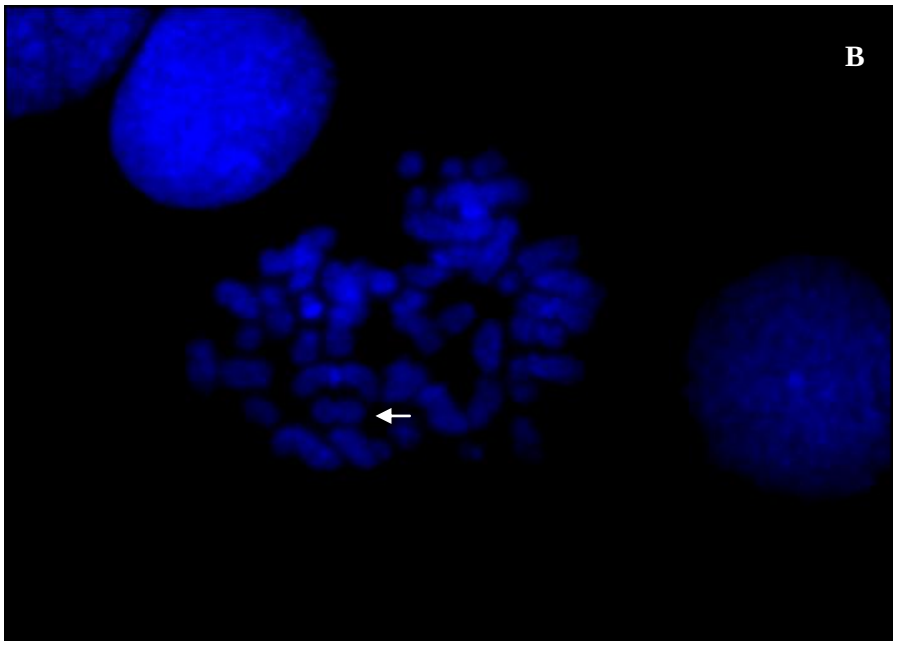


Figure 4.6: (**A**) An example of FISH performed on metaphase chromosomes obtained from patient No.6 using WCP7 FITC (visible in green). The white arrow indicates del(7) that shows deleted bands of chromosomes 7(q22~q31). (**B**) The same as image (A) with DAPI only.

4.4.2 Balanced translocation in the GDM-1 cell line

FISH using WCP7 analysis on the GDM-1 cell line confirmed a chromosomal translocation involving chromosome 7. FISH images analysis showed normal chromosome 7 that was fully painted and derivative 7, der(7), which was partially painted, confirming an additional DNA material was translocated from another chromosome (Figure 4.7). Unfortunately, the translocated part of chromosome 7 was not detected by FISH using WCP7, as the region is quite small and not detectable by that approach. The balanced translocation t(6;7)(q23;q36) of the GDM- cell line was studied in details (Nagel et al., 2005).

4.4.3 Unbalanced translocations involving chromosome 7 in AML patients

FISH using WCP7 successfully detected three unbalanced translocations in AML patients that were reported previously as deletions of 7q in G-banding karyotype (patient nos. 4, 7 and 1). In these patients, normal chromosome 7 was fully painted and the abnormal chromosome 7, der(7), was partially painted with the presence of additional genetic materials. These unbalanced translocations are shown in (Figures 4.8, 4.9 and 4.10).

Furthermore, a translocation on one patient (patient No. 3) was detected by G-banding analysis previously and was also confirmed by FISH analysis (Figure 4.11).

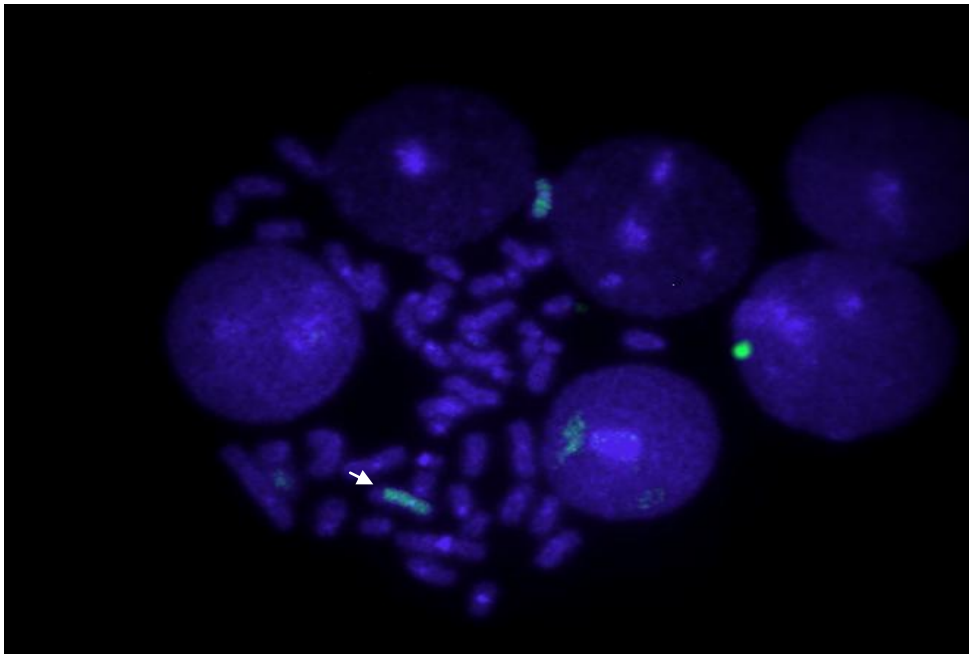
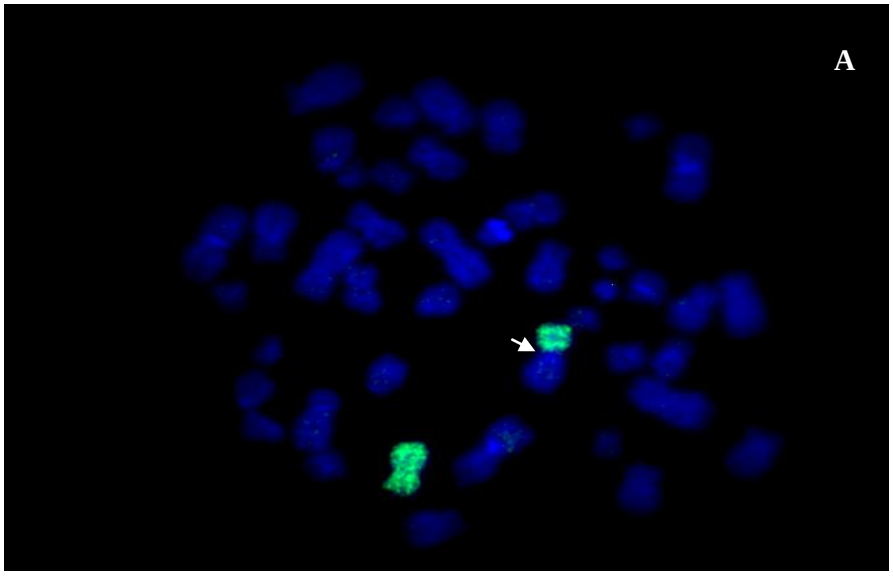


Figure 4.7: An example of FISH experiment using WCP7 (FITC) in green on GDM-1 cell line chromosomes. The arrow shows additional material on derivative 7 delivered from other chromosomes that confirms a translocation on the GDM-1 cell line.



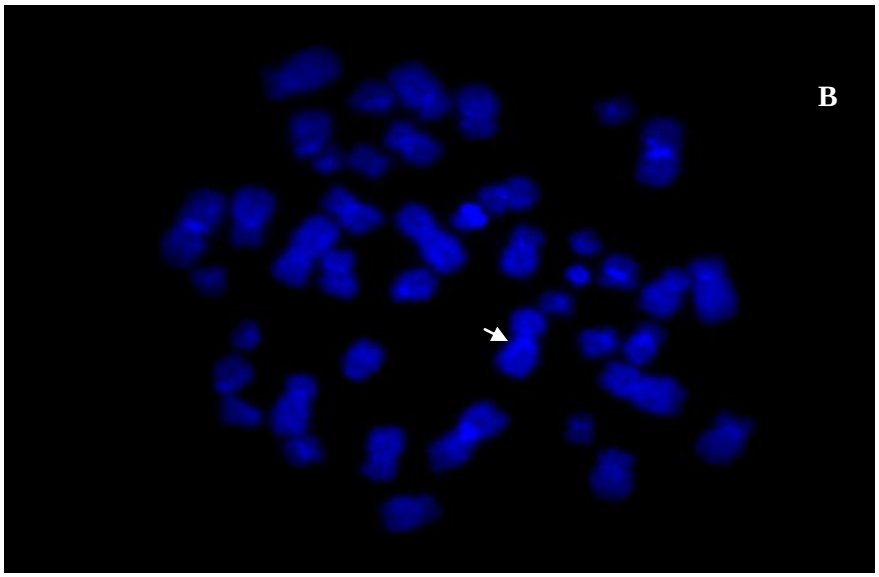


Figure 4.8: (**A)** An example of FISH performed on metaphase chromosome obtained from patient No. 4 using WCP7 FITC (visible in green). The white arrow indicates der (7) that shows additional materials on 7p11. (**B)** With the same showing DAPI only.

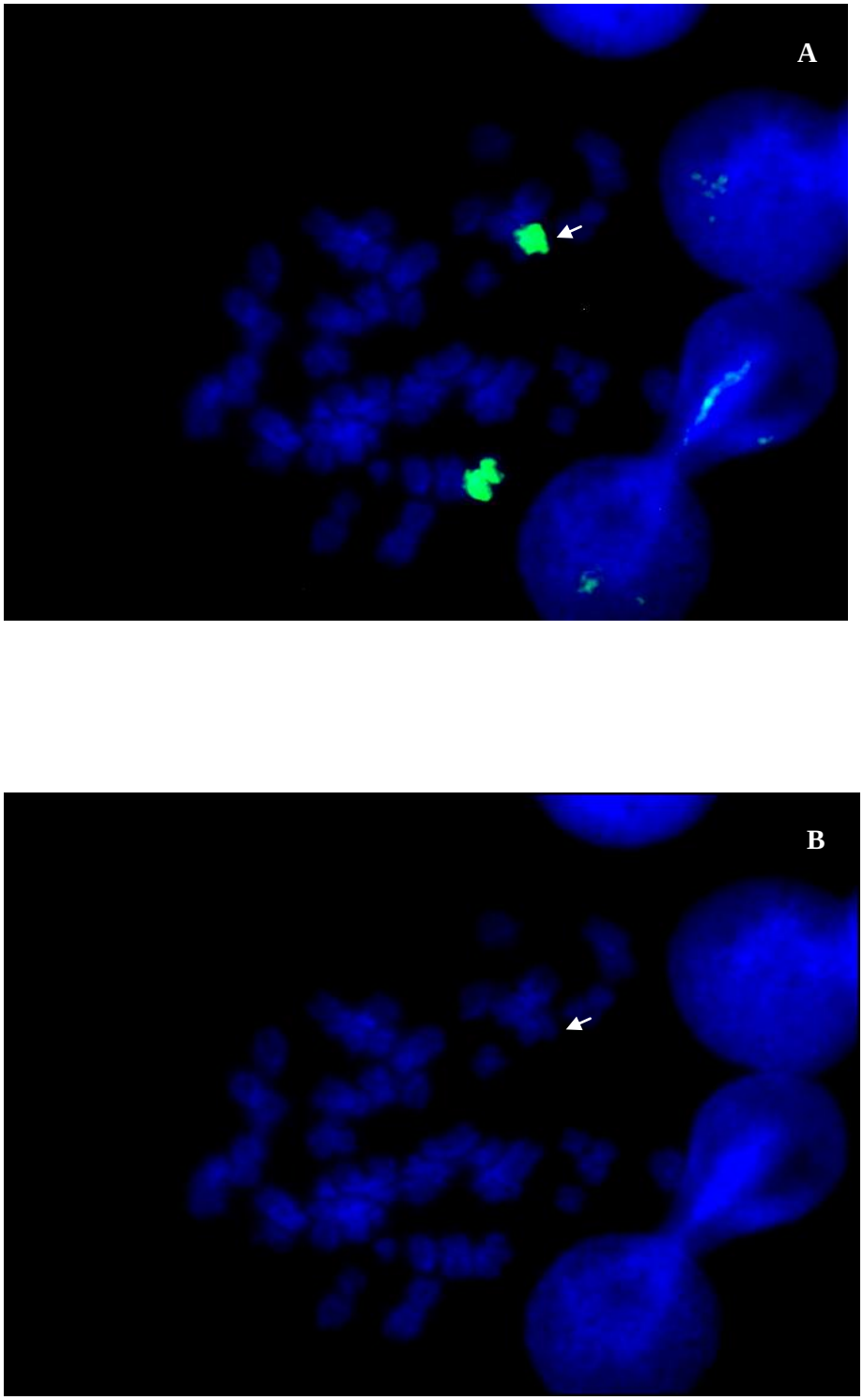
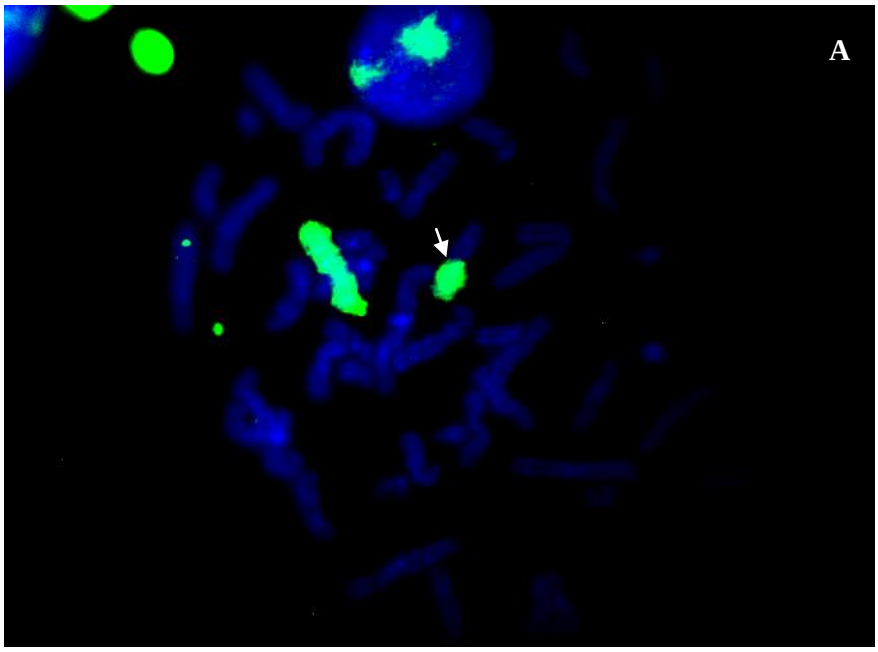


Figure 4.9: (**A**) An Example of FISH performed on metaphase chromosomes obtained from patient No.7 using WCP7 FITC (visible in green). The white arrow indicates der(7) that shows additional materials on derivative chromosome 7. (**B**) The same image as (A) with DAPI only.



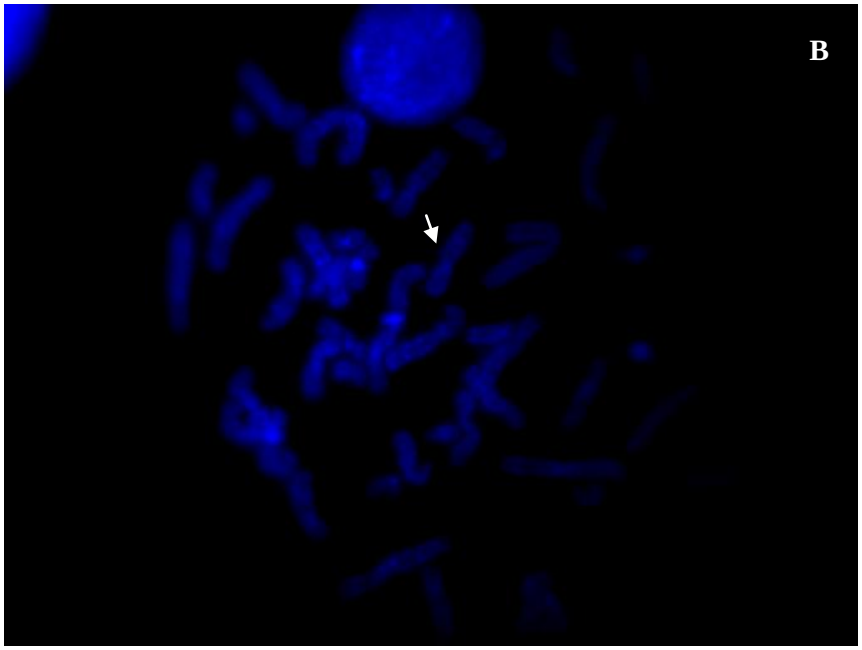


Figure 4.10: (**A**) An example of FISH performed on metaphase chromosomes obtained from patient No1 using WCP7 FITC (visible in green). The white arrow indicates der(7)(q) chromosome with additional genetic materials. (**B**) The same image as (A) with DAPI only.

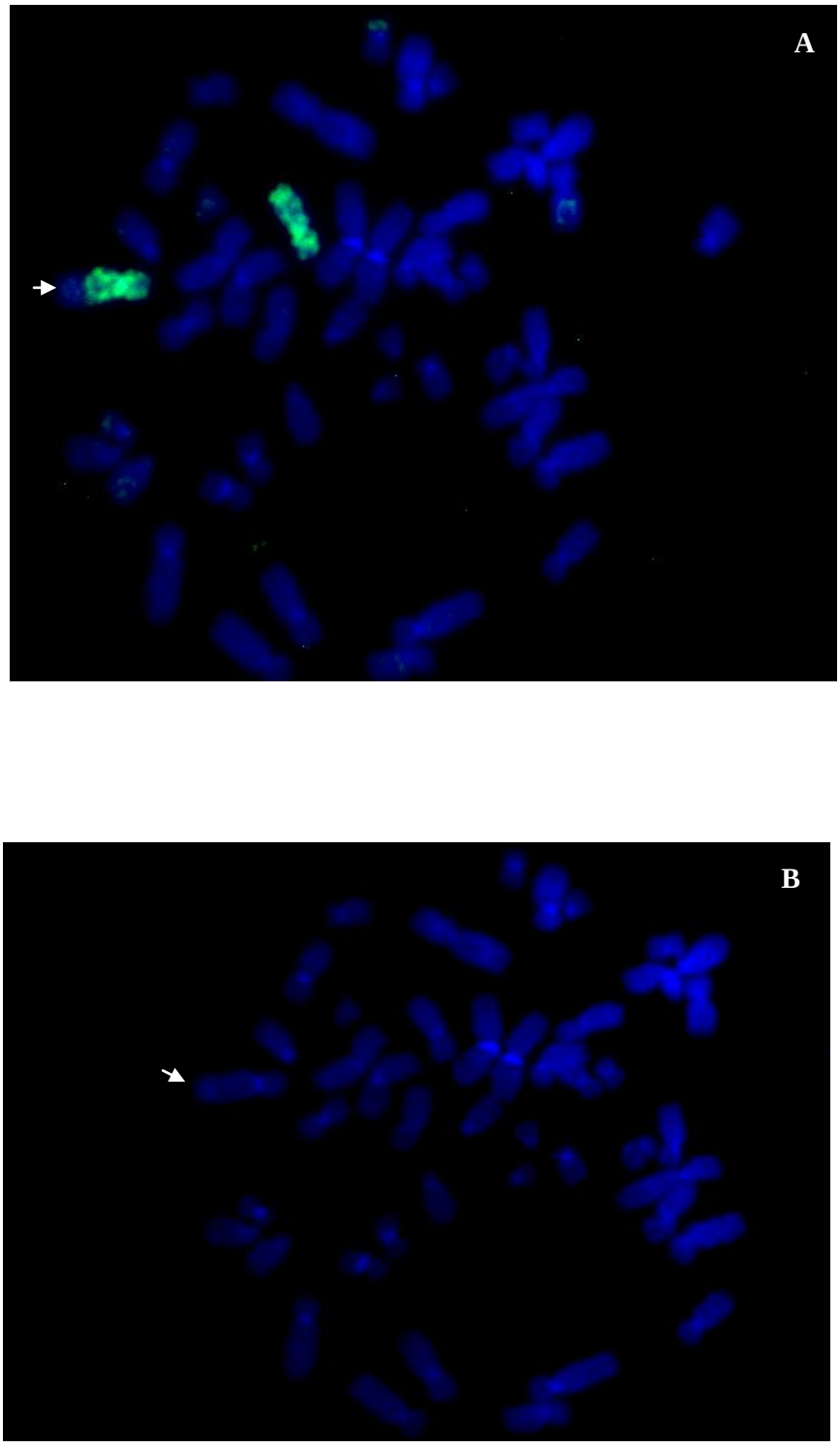


Figure 4.11: (**A**) An example of FISH performed on metaphase chromosome obtained from patient No. 3 using WCP7 FITC (visible in green). The white arrow indicates der(7) that shows the presence of additional material derived from another chromosome. (**B**) The same image as (A) showing DAPI only.

4.4.3 The revised patients' karyotype by FISH

FISH was used with WCP7 to modify the G-banding investigation analysis in 3 patients as follows:

Patient 1

Patient 1 was a five-year-old boy. The diagnostic lab G-banding karyotype was 46,XY,del(7)(q33~34). This karyotype was revised after FISH analysis, as FISH using WCP7 defined a chromosomal translocation with a deletion of the entire chromosome 7 long arm del(7q) as shown in (Figure 4.10).

Patient 4

Patient 4 was an 80-year-old with G-banding karyotype of 41~43,XY,del(7)(q?). This patient's G-banding karyotype revealed a deletion of chromosome 7 long arm del(7)(q) with no breakpoint specification. FISH using WCP7 confirmed unbalanced translocation and the karyotype was revised to 43,XY,add(7)(p11) for this patient. We were able to identify the breakpoint of this patient at p11 as it is possibly within the chromosome 7 centromere (Figure 4.8).

Patient 7

Patient 7 was a 15-year-old with G-banding karyotype of 46,XX,del(7)(?q22q31). G-banding analysis revealed 7q deletion for this patient. FISH was used with WCP7 to revise the chromosome 7 q deletion to an unbalanced translocation. It was not possible to identify the breakpoint for this patient by using FISH with whole chromosome painting as WCP7 paint is not an accurate tool to investigate the breakpoints (Figure 4.9). However, from the FISH

image the breakpoint could be estimated around 7q22. Therefore, the revised abnormality in this case could be add(7) (q22).

4.5 Discussion

In this study, the whole chromosome painting technique was used to classify the chromosome 7 rearrangements as deletions or translocations in the AML patients which had been analysed previously by conventional cytogenetic technique G-banding.

FISH using WCP7 confirmed (del)(7q) that was detected previously by G-banding in 3 patients. Two segments were approximately identified by G-banding and FISH analysis del(7) (q22) and del(7)(q22~q31). This finding is in agreement with Fischer's study of fluorescence in situ hybridization (FISH) in leukaemia patients (Fischer et al., 1997). The analysis on 7q deletions has defined 7q22 and 7q22~31 previously as common deleted regions (Fischer et al., 1997).

In Fischer's study, deletion of 7q22 was defined in 2 cases of chronic myeloid leukaemia and deletion of 7q22~q31 was reported in the AML cases. However, in the present study we reported two cases of 7q22 deletions and one case of 7q22~q31 deletion in AML patients.

Le Beau's and colleagues have used a series of YAC probes in FISH specifically to study the 7q22 breakpoint on myeloid patients, this study has identified 7q22 region as a common deleted region in twenty-seven patients (Le Beau's et al., 1996). Additionally, that study has identified breakpoints on 7q22 on other nine AML patients with balanced translocations.

All these results, and in the agreement with some other studies (Curtiss et al., 2005; Kratz et al., 2001) which have suggested that some 7q regions such as 7q22 or 7q31 might contain a tumour suppressor gene (TSG), which plays an important role in preventing normal cells developing into cancer cells. However, a recent study ,Wong's study, using a chromosome engineering to model a segment 7q22 deletion in myeloid leukaemia in a vivo (mouse) does not support that theory (Wong el al., 2010).

On some occasions, the whole chromosome painting proved to be a more accurate technique than G-banding to detect cryptic unbalanced translocations. FISH analysis using WCP7 re-defined some rearrangements from deletions as previously reported by G-banding to be unbalanced translocations with the omission of chromosome 7 long arm materials (Patient nos.1 and 4). The importance of the whole chromosome painting in detecting cryptic translocation which is not detected by G-banding was reported previously (Pan et al., 2012; Tosi et al., 1996 and 1997).

FISH using WCP7 on the GDM-1 cell line has confirmed chromosomal translocation. The study of t(6;7)(q23;q36) has been reported (Nagel et al., 2005) using G-banding analysis and FISH using specific probes for the 6q23 and 7q36 regions.

The whole chromosome painting is a powerful application to investigate cryptic translocations; in the meantime it showed a limitation in the identification of the exact break point by only FISH analysis. However, the combination of G-banding and FISH analysis enabled us to give an approximate estimate of the breakpoints in some cases in this study. Further FISH investigations using specific probes along the different regions of chromosome 7 are required to confirm the breakpoints.

4.5.1 Conclusions

In this study, WCP7 analysis was used to identify chromosome 7 rearrangements in AML cell line and patients. Three patients with 7q deletions have been reported. Moreover, four patients were associated with unbalanced translocation. Three of these translocations were cryptic and not visible in G-banding analysis.

4.5.2 Future work

The GDM-1 cell line that present add(7q) along with the AML patients with a deletion of 7q will be investigated in a further detail by using a FISH study, which applies a specific probe for the *HLXB9* gene at 7q36 as will be described in the next chapter (Chapter5). This will determine the exact breakpoint in the cell line and whether the 7q deletions on patients are intersetial.

The four AML patients with the unbalanced translocations will not be investigated by FISH using specific probe for *HLXB9* at 7q36 region, as the breakpoints in these patients are proximal to 7q36. This means that the 7q36 band has been deleted and *HLXB9* gene is lost.

The study outlined in this chapter served the purpose of selecting those patients with 7q deletions and discarded those patients with unbalanced translocations that involved the loss of the telomeric region of chromosome 7, and because of the loss of a large segment in 7q, we can assume that in these cases the *HLXB9* gene is also lost.

Further studies (as described in chapter 5 in this thesis) will focus on a selected series of patients with del(7q), hoping that these are interstitial, to check whether *HLXB9* gene is present. This will be done by FISH using a specific locus probe for *HLXB9* gene.

The GDM-1 cell line carries the t(6;7)(q23;q36) with over-expression of the *HLXB9* gene indicated distal breakpoint to *HLXB9* on 7q36 region, which makes it a model cell line to study t(6;7) in AML (Nagel et al., 2005). The breakpoint is within band 7q36 and close to the *HLXB9* gene and might the breakpoint resulting in an over-expression of the *HLXB9* gene.

However, *ETV6* is a transcription factor that has been identified as a partner gene of *HLXB9* in t(7;12) (q36;p13)((Beverloo et al., 2001). Interestingly the involvement of *MYB* on 6q23 with *HLXB9* via t(6;7) has been established by Nagel (2005). *MYB* is a proto oncogene that is involved in leukaemogenesis and has been reported to be over-expressed in acute leukaemias

(Sinclair et al., 2005). However, the involvement of *MYB* with *HLXB9* has been so far reported only in the GDM-1 cell line (Nagel et al., 2005). Further studies to support this finding will be plausible.

CHAPTER 5

FISH STUDIES OF THE *HLXB9* GENE IN MYELOID DISORDERS

5.1 Introduction

5.1.1 The t (7;12) rearrangements and the association with childhood leukaemia and *HLXB9* over expression

The t(7;12) (q36;p13) is a common chromosomal translocation associated with infant leukaemia and clinically poor outcome (Tosi et al., 2003; Von Bergh et al., 2006). It is found approximately in one third of infant AML leukaemia. In contrast, it is rare in infant ALL and older AML patients. To date, it has never been reported in adult AML cases (Tosi et al., 2003; Von Bergh et al., 2006). According to von Bergh and her group's study in 2006 that covered 59 AML patients, 18 of them were infants. The study indicated that six cases carried the t(7;12)(q36; p12) and five of them were infants aged between 0 and 12 months, and one was 18 months old. This finding suggested that the t(7;12) is found in about 30% of infant AML. In contrast, only one case carried t(7;12) (q36;p13) in all 290 ALL patients.

The t(7;12) results in the rearrangement of the *ETV6/TEL* gene at (12p13) (Tosi et al., 2003; Von Bergh et al., 2006). Alteration of the *ETV6* gene occurs at its 5' end and involving fusion with chromosome 7 at various breakpoints such as 7q22 and 7q36 (Tosi et al., 2003; Von Bergh et al., 2006). Dr. Tosi and her group's study, which is based on fluorescence in situ hybridization (FISH) and Southern Blotting analysis, reported an interesting case of t(7;12) (q22;p13) that also included a breakpoint at 7q36 genomic region (Tosi et al., 2003).

Furthermore, the breakpoints in the 7q36 region in the t(7;12) cases are heterogeneous (Simmons et al., 2002; Tosi et al., 2003). Studies of the breakpoints in the t(7;12) support an involvement of *HLXB9* as partner of *ETV6* gene (Beverloo et al., 2001). However, the *ETV6-HLXB9* fusion transcript has been found in approximately 50% of t(7;12) cases. The over-expression of *HLXB9* has been reported in all t(7;12) patients reported to date (Ballabio et al., 2009; Von Bergh et al., 2006).

5.1.2 The over-expression of *HLXB9* gene in acute myeloid leukaemia derived cell line GDM-1 via t(6;7) rearrangement

Activation of expression of *HLXB9* has been reported in translocations other then the t(7;12). This is the case of the myeloid leukaemia derived cell line GDM-1 where it has been shown that the balanced chromosomal translocation t(6;7)(q23;q36) is the cause of over-expression of *HLXB9* due to the juxtaposition of the *MYB* gene on 6q23 (Nagel et al., 2005).

5.2 Aim of the study

The main aim of this study was to localise the *HLXB9* gene in cases with reported abnormalities of chromosome 7 in order to define whether the gene was deleted, disrupted or located on a derivative chromosome.

The following are the specific objective for this study:

(i) To analyse the status of *HLXB9* by FISH in a series of patients with reported del(7), in order to assess whether this gene is lost (as a consequence of the deletion) or retained (because it is outside of the deleted region).

(ii) To determine the 7q36 breakpoint on the GDM-1 cell line in relation to *HLXB9*.

5.3 Materials and methods

The material presented in this chapter was provided to me by my supervisor in the form of FISH images. Experiments were performed by others.

5.3.1 Cell line and patients sample

The human acute myeloid leukaemia derived cell lines (GDM-1) and 4 acute myeloid leukaemia (AML) patients were analysed in this study. The cell line and patients shared the presence of chromosome 7 abnormalities, add(7q) in GDM-1 cell line and del(7q) in AML patients. The cell line carries t(6;7)(q23;q36) and breakpoint distal to *HLXB9* gene on 7q36 t(6;7)(q23:q36) (Nagel et al., 2005). The patients carried deletions with breakpoint within chromosome 7 away of 7q36 (telomeric region of chromosome 7) as shown in table 5.1.

The genetic materials	Ch.7 abnormalities	karyotype
GDM-1 cell line	add(7)(q36)	47,XX,+8,del(6)(q),add(7)(q),del(12)(p)
Patient 94-20	del(7)(q22)	46,XY,del(7)(q22)
Patient 94-2086	del(7)(q?)	46,del(7q)
Patient C	del(7)(q22)	45,XX,del(7)(q22),-21
Patient K	del(7)(q22)	46,XY,del(7)(q22)

Table 5.1: Chromosome 7 abnormalities and the karyotype of GDM-1 cell line as reported by Ben-Bassat et al., 1982, and AML patients as reported by the diagnostic lab.

5.3.2 Probes

In order to investigate the *HLXB9* gene at 7q36 in the GDM-1 cell line and AML patients 2 probes were used:

(i) A whole chromosome 7 paint commercially available and directly labelled with FITC visible in green (Cambio, Cambridge, UK). This probe (WCP7) was used to identify the origin of chromosome 7 genetic materials.

(ii) In house prepared locus specific probe PAC H_DJ1121A15 for the *HLXB9* gene at 7q36. The PAC probe was labelled with biotin and detected with streptavidin conjugated with CY3 visible in red as described in details in chapter 2 of this thesis.

In order to study the breakpoints of GDM-1 cell line further, 2 probes were hybridised simultaneously:

(i) PAC clone of H_DJ1121A15 for *HLXB9* gene at 7q36. The probe was labelled with digoxigenin and detected with FITC visible in green.

(ii) PAC clone of 48H15 for distal region of *HLXB9* gene. The probe was labelled with biotin and detected with CY3-avidin visible in red.

All PAC clones used in this study were kindly provided by Professor Steve Scherer, Department of Genetics, Hospital for Sick Children, Toronto, Canada.

The labelling system method of the probe was described in details in chapter 2 in this thesis.

5.3.3 Fluorescence in situ hybridization

FISH protocol for the whole chromosome painting was carried out as recommended by the manufacturer and is described in materials and methods of chapter 4. Moreover, the FISH protocol for using PACs was described in details on materials and methods chapter (Chapter 2) of this thesis.

5.4 Results

5.4.1 Dual colour FISH investigation using WCP7 and specific PAC for the *HLXB9* gene

Dual colour FISH analysis of the AML patients and the GDM-1 cell line revealed no abnormalities of the *HLXB9* gene at 7q36.

FISH using WCP7 and unique sequences probe for the *HLXB9* gene on the 4 AML patients indicated normal chromosome 7 and del(7q). The normal chromosomes 7 and del(7q) on the all AML patients were fully painted in green, although del(7q) appeared shorter which confirmed the deletions. Two red FISH signals for the *HLXB9* gene were detected in all of the 4 patients on both normal chromosome 7 and del(7q) (Figures 5.2, 5.3, 5.4 and 5.5).

In the GDM-1 cell line, FISH using the whole chromosome painting WCP7 and PAC H_DJ1121A15 for *HLXB9* showed normal chromosome 7 fully painted in green and derivative chromosome 7, der(7), partially painted in green with the presence of the *HLXB9* gene red signals in both (one on each chromatid within a chromosome) as shown in (Figure 5.1).

5.4.2 Dual colour FISH investigation using specific probes for the *HLXB9* gene (PAC 1121A15) and distal region (PAC48H15)

This investigation was performed on the GDM-1 cell line only in order to achieve a detailed localization of the breakpoint in 7q36 in the cell line. The metaphase chromosomes and interphase nuclei were analysed. The FISH images analysis of GDM-1 metaphases showed green signals of the *HLXB9* gene on both normal chromosome 7 and derivative der(7).

However, the red signals specific for the probe distal to *HLXB9*, were only observed on the normal chromosome 7. The simultaneous visualization of green and red FISH signals showed that the normal chromosome 7 harboured both, whereas the der(7) harboured only green signals. No red signals for the PAC 48H15 were seen on the der(7). Interestingly, a red FISH signal for PAC 48H15 was localised on another chromosome (Figure 5.6). This chromosome is most likely to be chromosome 6 according to published data (Nagel et al., 2005). The analysis of the GDM-1 cell line interphase nuclei showed a fused green-red signal, one single green signal and one single red signal. The analysis on both metaphase and interphase cells showed that the chromosome 7 breakpoint in the der(7)t(6;7) is in the region between PAC 1121A15 and PAC48H15 (Figure5.7).

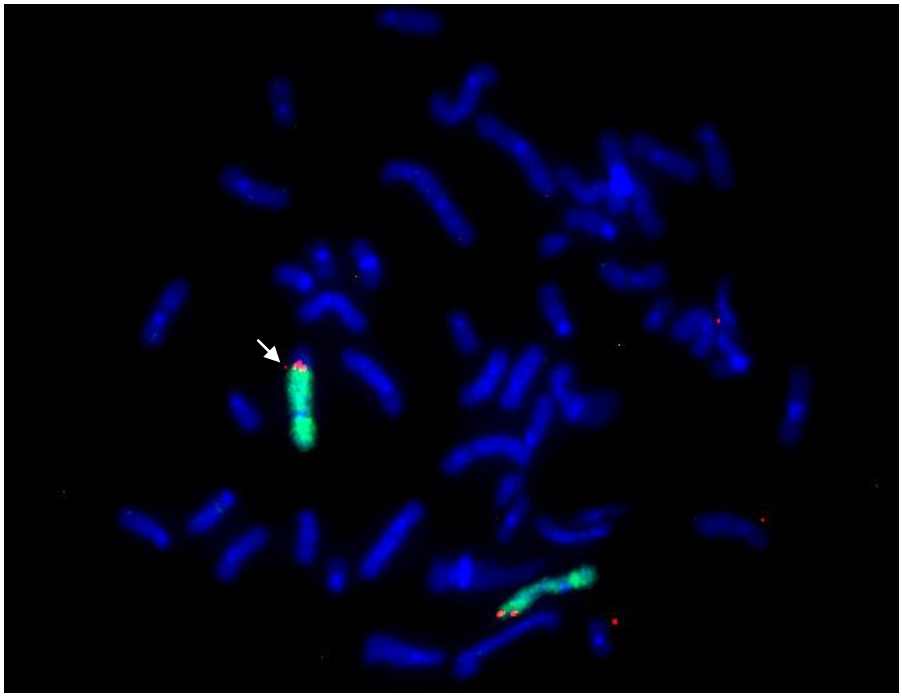


Figure 5.1: An example of dual colour FISH experiment performed on metaphase obtained from GDM-1 cell line using WCP7 (visible in green) and PAC H_DJ1121A15 for *HLXB9* (visible in red).The white arrow indicates the derivative chromosome 7; der (7) that clearly shows an additional material of DNA that delivered from other chromosome. The red signals for the *HLXB9* gene show no abnormalities within 7q36 region.

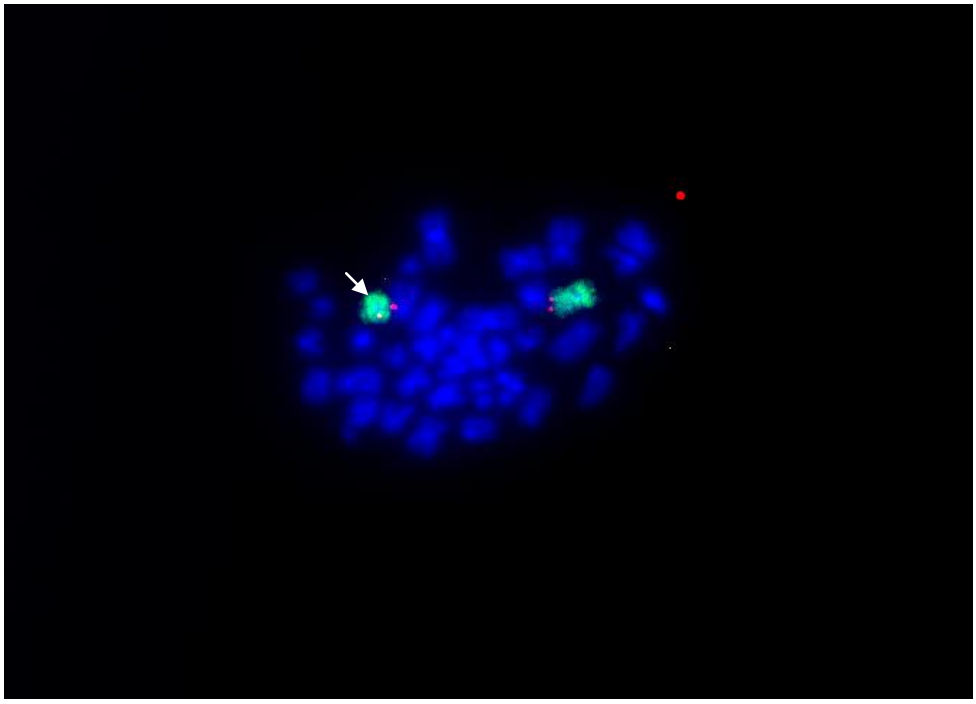


Figure 5.2: An example of a dual FISH experiment performed on metaphase obtained from patient 94-20 using WCP7 (visible in green) and PAC H_DJ1121A15 for *HLXB9* (visible in red).The white arrow indicates the del (7) that shows a deletion of 7q material and present of *HLXB9* gene red signals on chromosome 7 and del(7q).

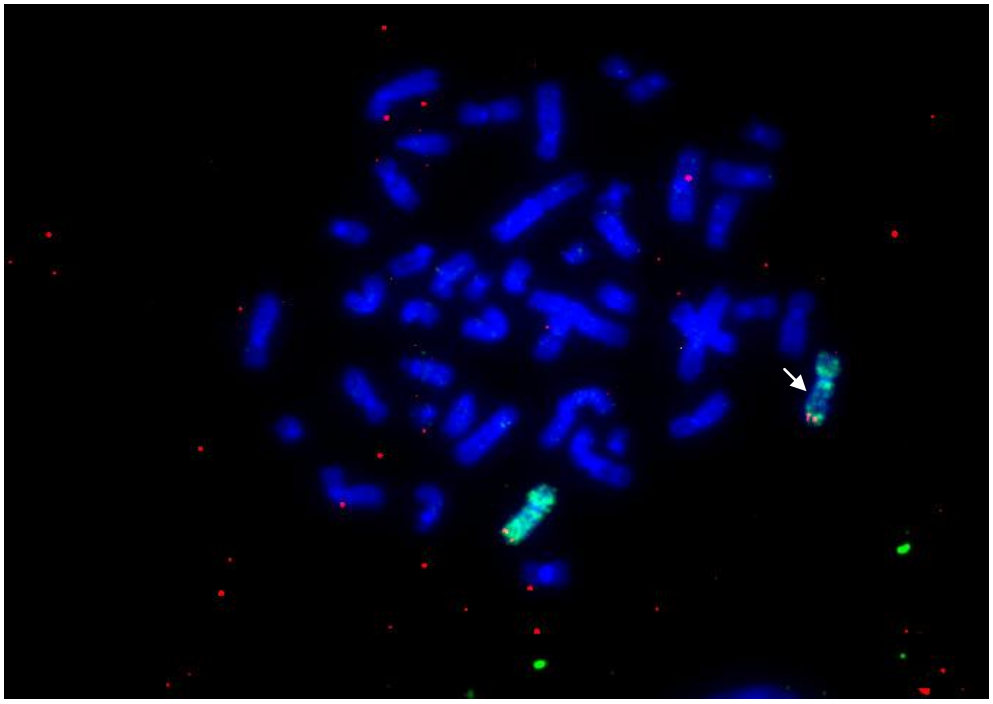


Figure 5.3: An example of a dual FISH experiment performed on metaphase obtained from patient 94-2086 using WCP7 (visible in green) and PAC H_DJ1121A15 for *HLXB9* (visible in red). The white arrow indicates the del(7) that shows a deletion of 7q material, but does not involve the *HLXB9* gene at 7q36 region. The red signal for *HLXB9* gene is present

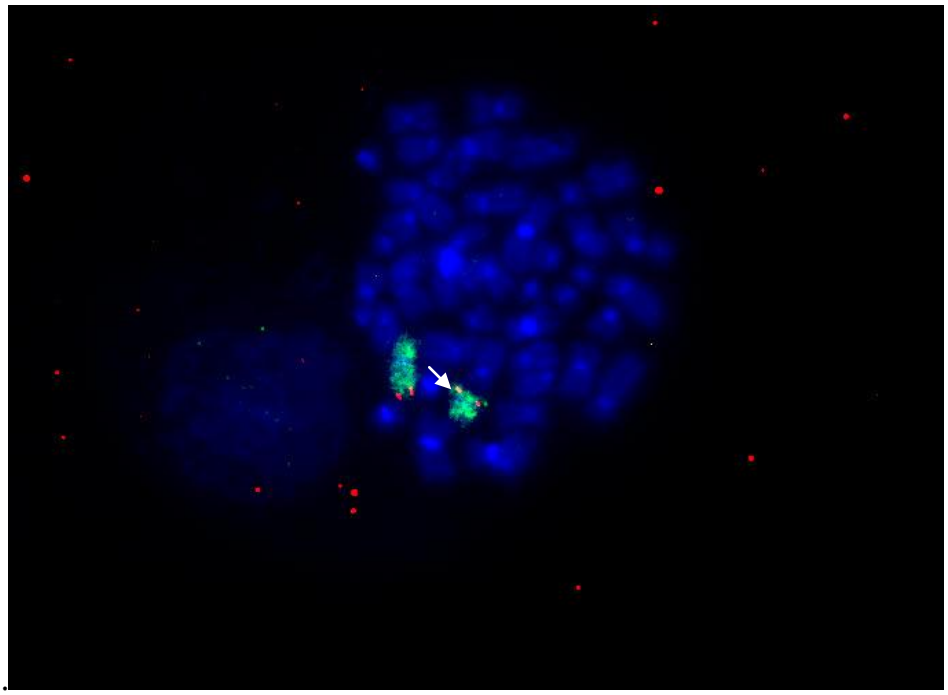


Figure 5.4 : An example of a dual FISH experiment performed on metaphase obtained from patient C using WCP7 (visible in green) and PAC H_DJ1121A15 for *HLXB9* (visible in red).The white arrow indicates the del(7) that shows a deletion of 7q material. Red signals for *HLXB9* are present on both normal chromosome 7 and del(7q).

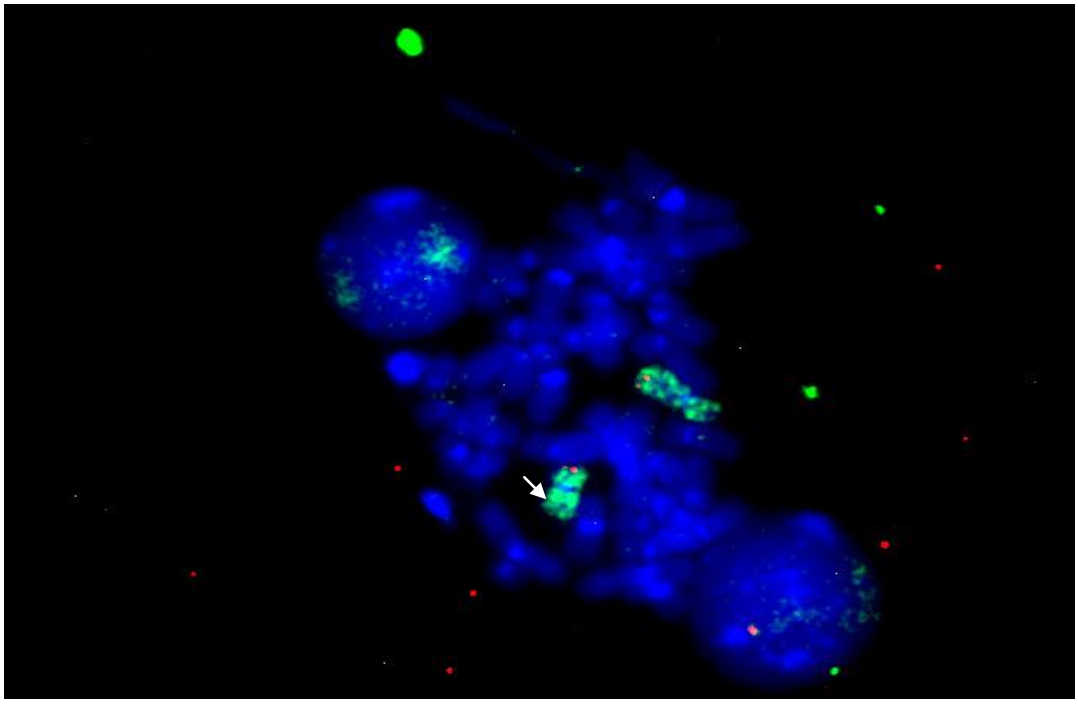
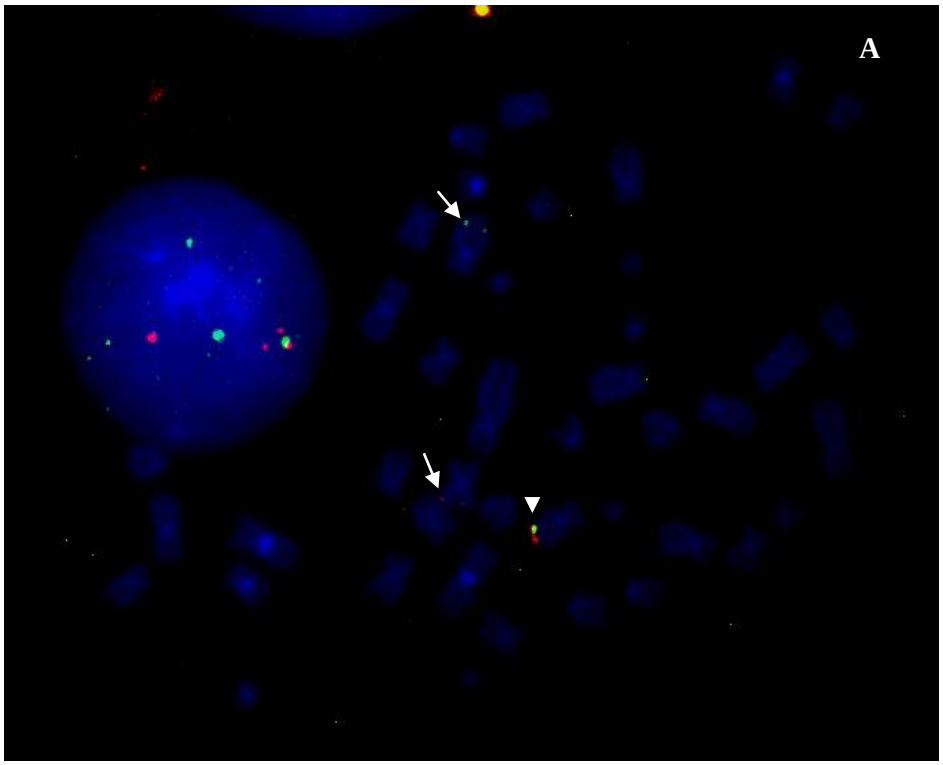


Figure 5.5: An examples of a dual colour FISH experiment performed on metaphase obtained from patient K using WCP7 (visible in green) and PAC H_DJ1121A15 for *HLXB9* (visible in red). The white arrow indicates the del(7) that shows a deletion of 7q material Red signals for *HLXB9* are present on both normal chromosome 7 and del(7q).



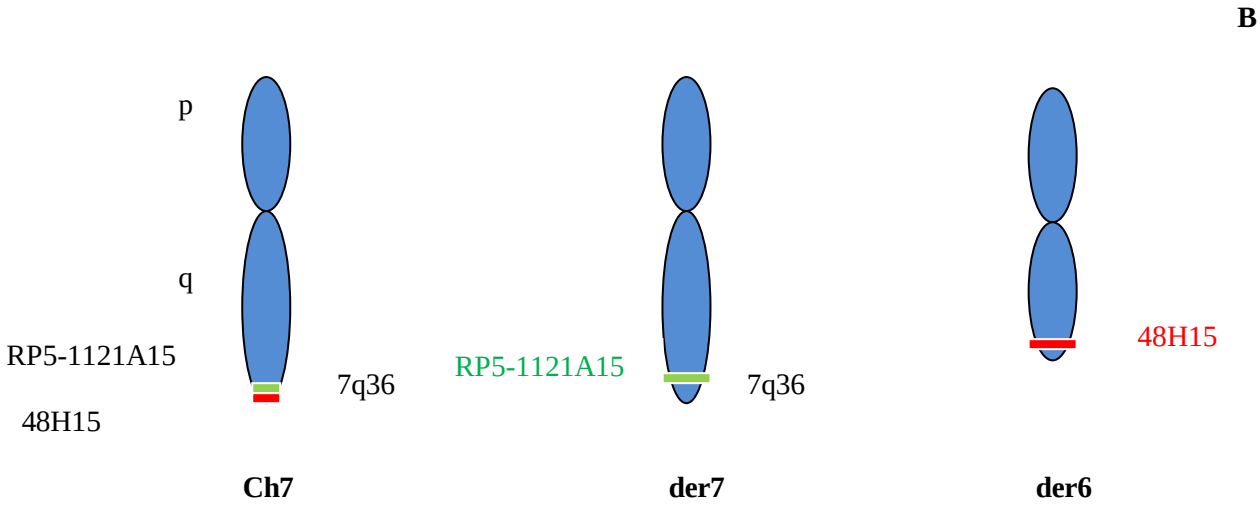
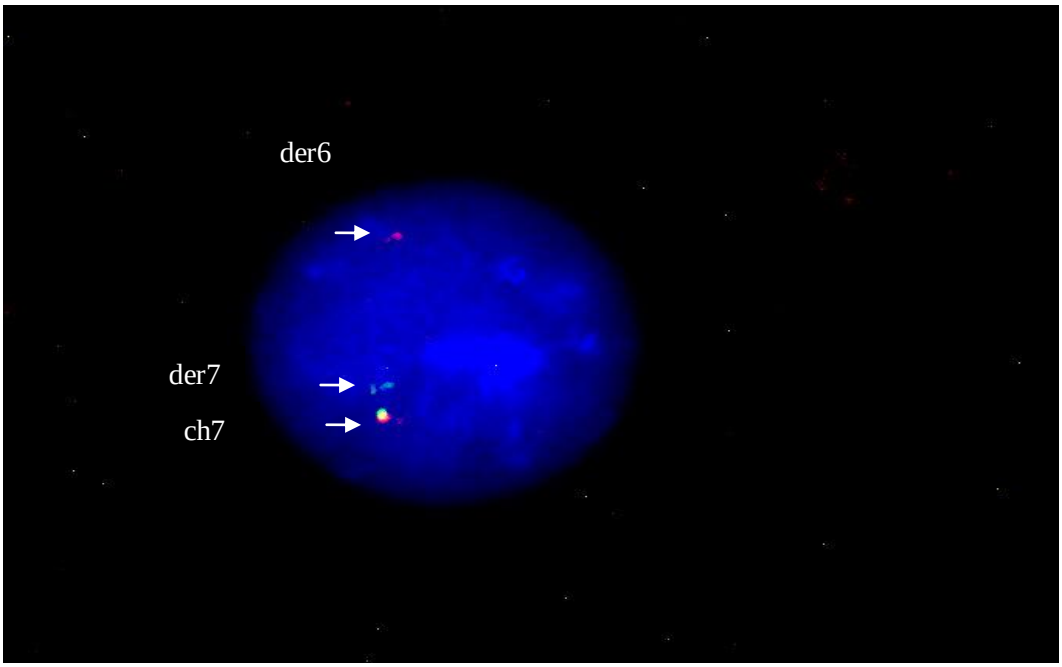


Figure 5.6: (**A**) An example of a dual colour FISH experiment performed on metaphase obtained from GDM-1cell line using PAC RP5-1121A15 for *HLXB9* at 7q36 (visible in green) and PAC 48H15 for a region distal to *HLXB9* (visible in red). The arrowhead shows normal chromosome 7 that has a fused signal in red and green. A green signal for *HLXB9*

gene is visible on the der(7) and a split red signal for PAC 48H15 is visible on der(6). The white arrows indicate the derivative chromosome 6 and derivative chromosome 7, der(6) and der(7) respectively, that shows just one signal on each chromosome in red or green which suggests the telomeric region on ch.7 is translocated on the der(6). No evidence for the *HLXB9* gene split signal was detected by FISH. **(B)** An ideogram illustrates the chromosomal translocation t (6:7) in GDM-1 cell line and the breakpoint is just between these 2 probes.



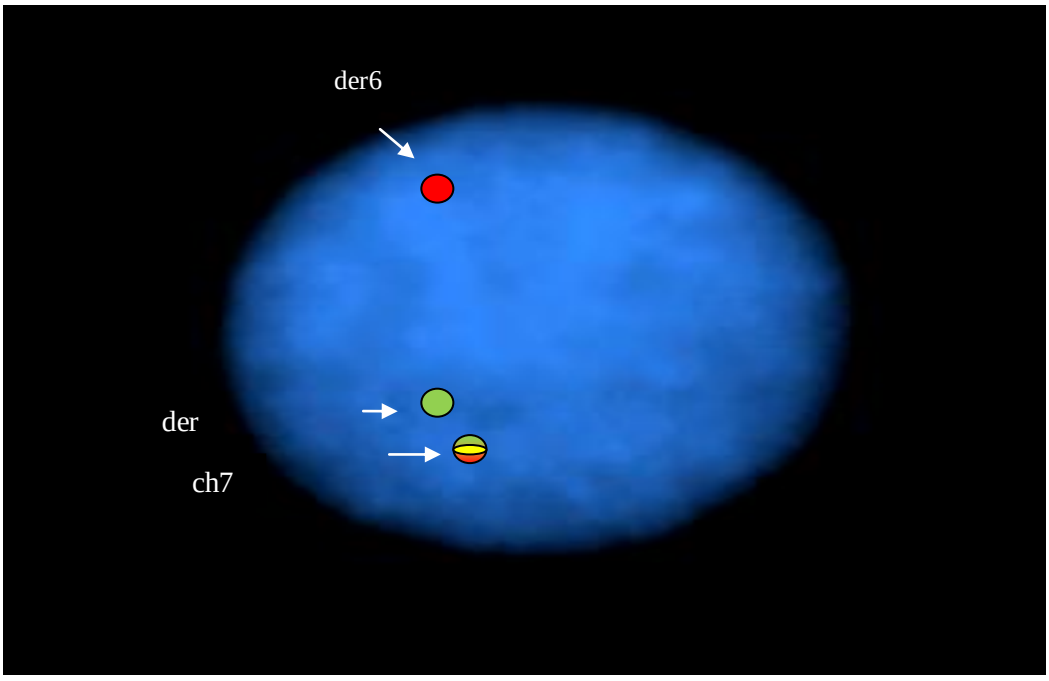


Figure 5.7: (**A**) An example of interphase FISH experiment on GDM-1 cell line nucleus that shows one fused signal (red and green) for normal chromosome 7 and two individual signals (one in red and one in green), which confirm a chromosomal translocations and the breakpoint is just between these two probes. (**B**) An Ideogram illustrates the chromosomal translocation.

5.5 Discussion

Several studies have reported an involvement of *HLXB9* in some leukaemia cases (Beverloo et al., 2001; Tosi et al., 2003 and Von Bergh et al., 2006). In infant AML patients, the *HLXB9* gene has been identified as a partner gene for the *ETV6* gene in t(7;12) (Beverloo et al., 2001; Tosi et al., 2003). The translocation results in a fusion transcript of *HLXB9-ETV6* in 50% of t(7;12) patients and over-expression of the *HLXB9* in the all of the t(7;12) patients reported to date (Tosi et al., 2003; Von Bergh et al., 2006).

In the present study, the FISH localization of the *HLXB9* gene has been investigated in AML cell line (GDM-1) and 4 AML patients. FISH investigation using a specific PAC clone (H_J 1121A15) containing *HLXB9* on AML cell line and patients indicated no involvement of the *HLXB9* gene in any breakages or rearrangements. Furthermore, the breakpoint was proximal of the *HLXB9* gene in all four AML patients that have been analysed in this study. In contrast, the breakpoint was confirmed to be distal to *HLXB9* in the AML cell line (GDM-1).

Previous studies of the t(7;12) cases have shown that the 7q36 breakpoint is usually proximal to *HLXB9.* This implies that, in these cases, the *HLXB9* gene is translocated; hence, localized on the der(12) (Tosi et al., 2000 and 2003; Von Bergh et al., 2006).

Tosi and collaborators (Tosi et al., 2000 and 2003) have investigated 7q36 breakpoint in details in 6 leukaemia patients with t(7;12) and rearrangement of the *ETV6* gene using a series of PAC clones and cosmids to cover 7q22 to 7q36 region (from centromere to telomere) by FISH. The breakpoint was identified at 7q36 by G-banding analysis previously in the all patients. FISH using clone PAC H_DJ1121A15 containing *HLXB9* showed a split signal in normal chromosome (7), der (7) and der (12) in 3 patients, which confirmed the breakpoint was within the same PAC clone at 7q36. Further FISH investigation was carried out using 2 cosmids derived from this PAC on these patients. The cosmids signals were detected in der

(12) in 2 patients, which confirmed the breakpoints at 7q36 was proximal to the the cosmids probes. On the other patients, the cosmids signal was not detected, which suggested the breakpoint was distal of the cosmids probe in these patients. This data support the heterogeneity of the breakpoints in patients.

Tosi's and her group's study (Tosi et al., 2003) that was on fluorescence in situ hybridization (FISH) and Southern Blotting analysis reported an interesting case of t(7;12) (q22;p13) that also included a breakpoint at 7q36 genomic region. This case showed there is no involvement of *ETV6* gene rearrangement in t(7;12). However, this finding suggests that there is another mechanism of t(7;12), which does not include a significant role for the *ETV6* gene. *HLXB9* expression was not investigated in this case. However, if *HLXB9* is proved to be over-expressed in this case, one could speculate that the breakpoint close to the *HLXB9* gene region could be the triggering factor for causing leukaemogenesises.

In some t(7;12) patients, over-expression of *HLXB9* has been reported to associate with nuclear changed position. The *HLXB9* gene is normally located toward the nuclear periphery. Interestingly, it has been reported to be towards a more nuclear interior position in t(7;12) (Ballabio et al., 2009). It seems gene position affecting mechanism could be an another possible mechanism that results in over-expression of the *HLXB9* gene in some AML positive t(7;12)patients.

5.5.1 Conclusions

The *HLXB9* gene has been investigated in this study in AML cell line and 4 patients. The *HLXB9* gene was not lost or disrupted either in the AML cell line or in the patients that have been analysed in this study. However, from the FISH patterns it was possible to deduce that the breakpoint in relation to *HLXB9* was different in the cell line compared to all del(7q) patients.

5.5.2 Future work

To date, there is no supporting data of *HLXB9* gene breakage or rearrangement in leukaemia (particularly AML patients). However, the over-expression of the *HLXB9* has been established in AML patients by several groups (Beverloo et al., 2001; Tosi et al., 2003; Von Bergh et al., 2006).

The level of *HLXB9* expression must be investigated in more leukaemia patients, as some data showed support of the expression of *HLXB9* in very limited numbers of ALL patients and AML patients negative t(7;12) (Ballabio et al., 2009; Von Bergh et al., 2006). The study of the *HLXB9* gene in more leukaemia patients might enable the identification of a sub-group of leukaemia patients that share the expression of *HLXB9.*

Following from the data analysed in this chapter, it would be interesting to see whether the expression of *HLXB9* is observed in patients with interstitial del(7q) that do not show loss of *HLXB9.* This would prove that the disruption of 7q36 in a region proximal to *HLXB9* is sufficient to promote its expression.

CHAPTER 6

GENERAL DISCUSSION

6. General discussion

6.1 Understanding chromosomal abnormalities using conventional and molecular cytogenetics

In present study, both conventional cytogenetic (through G-banding analysis) and molecular cytogenetic (through chromosome painting, 24 colour painting and single locus probe FISH) were performed on leukaemia and lymphoma cell lines, as well as on patients materials. The scope of this study was to understand, at the chromosomal level, a possible cause of activation of the homeobox gene *HLXB9* resulting in the expression. All three cell lines analysed in this study are characterized by the expression of *HLXB9*. This was proven by reverse transcription (RT)-PCR experiments carried out in our lab (personal communication with Dr. Sabrina Tosi). It is known that *HLXB9* resides on chromosome 7, therefore a detailed characterization was carried out to investigate this chromosome in particular, and in the context of the whole karyotype in these cell lines. In the case of patient material, chromosome painting was carried out in a single colour manner specifically for chromosome 7.

The study of human myeloid leukaemia cell lines K562 and GDM-1 revealed numerical and structural abnormalities involving chromosome 7. The chronic myeloid leukaemia cell line K562 presented tetrasomy of chromosome 7, whereas the acute myeloid leukaemia cell line GDM-1 carries a translocation t(6;7). Interestingly, a very complex karyotype has been identified in the lymphoma cell line Pfeiffer (CRL2632), but no evidence of chromosome 7 abnormalities has emerged.

The conventional cytogenetic technique G-banding was a useful first line investigation tool to screen the whole chromosomes within the cell lines. Furthermore, M-FISH technique was a powerful tool to identify the origin of translocated chromosomes in a 24-colour fashion.

In this study, FISH has enabled us to identify cryptic translocations that were misdiagnosed as deletions by only G-banding analysis (Chapter 3). The importance of M-FISH as a screen tool in detecting cryptic translocations was previously reported (Hilgenfeld et al., 2001; Mathew et al., 2001; Nordgren et al., 2001).

However, there is a limitation of M-FISH technique in detecting very small balanced translocations; for example, in this study the der(6) was not detected by M-FISH analysis in the balanced translocation t(6;7) in the GDM-1 cell line, although der(7) was clearly indicated. The der(6) was indicated by G-banding analysis in this study and by G-banding and FISH using specific locus probes previously (Nagel et al., 2005). This study confirmed the importance of using molecular cytogenetic investigations, such as M-FISH, to enable a broad screening of the gross chromosomal aberrations with a limitation of indicating very small deleted or translocated chromosomal regions. The combination of M-FISH analysis and classic banding techniques is required to obtain a very accurate karyotype.

Naumann et al. (2001) successfully established a complete karyotype of the K562 cell line using G-banding, M-FISH, FISH (using whole chromosome painting and specific locus probes) and comparative genomic hybridization (CGH). Similar chromosomal abnormalities were reported in this study with more chromosomal abnormalities that have been detected by FISH using the whole chromosome painting previously.

In the present study, the GDM-1 cell line was analysed using M-FISH technique for the first time. The analysis of M-FISH showed a very similar karyotype reported by G-banding analysis that was published previously by Nagel (Nagel el al., 2005). This finding has proven that G-banding is a valuable tool to detect the chromosomal abnormalities despite the difficulty of identifying the added genetic materials origin and the special training that is required for performing the analysis.

The GDM-1 cell line was shown previously (Nagel et al., 2005 and in this study) to carry t(6;7)(q23;q36) with breakpoint at 7q36, which makes it a good candidate to study *HLXB9* gene further, as it localizes at the same breakpoint.

M-FISH was a good method to indicate general chromosomal abnormalities. Unfortunately, the investigation of the *HLXB9* gene could not be achieved by M-FISH due to the lower resolution of the technique. In order to confirm the chromosomal abnormalities found by M-FISH, further FISH investigations using a probe specific for the *HLXB9* should be carried out.

6.2 Cryptic translocations revealed by FISH using WCP7 on AML patients and the GDM-1 cell line

The importance of the whole chromosome painting technique was proven in the classification of chromosome 7 rearrangements as deletions or translocations in the AML patients and the GDM-1 cell line. FISH using WCP7 was a useful tool to detect cryptic unbalanced translocations that was reported as (del)(7q) by G-banding analysis in 3 patients.

The powerful of the relatively simple method of whole chromosome painting in detecting cryptic translocations that are misdiagnosed by G-banding was reported previously (Pan et al., 2012; Tosi, et al., 1996 and 1997).

The balanced chromosomal translocation t(6;7) in the GDM-1 cell line was confirmed by FISH using WCP7. The der(7) with the additional genetic material was clearly shown. The translocated part of chromosome 7 was not possible to be detected on the der(6) using WCP7, as the region is very small and not detected by the whole chromosome painting technique. However, The translocated region in der(6) was reported previously by Nagel using the G-banding analysis and FISH using specific probes for the 6q23 and 7q36 regions (Nagel et al., 2005).

In this study, the whole chromosome painting showed a limitation of the identification of the breakpoint by only FISH analysis. However, the use of both G-banding and FISH analysis has enabled us to estimate the breakpoints in these cases.

In order to confirm the breakpoint further FISH investigations using specific probes along the different regions of chromosome 7 are required. Chapter 4 focused on the use of chromosome painting in order to select those patients with 7q deletions and discard those patients with unbalanced translocations that involved the loss of the telomeric region of chromosome 7 (the area of interest in this study, as *HLXB9* gene is localized at 7q36 very close to the telomere).

6.3 Chromosomal rearrangements and *HLXB9* gene expression

The role of the homeobox gene *HLXB9* in leukaemia has been investigated previously in numerous studies (Ballabio et al., 2009; Beverloo et al., 2001; Nagel et al., 2005; Tosi et al., 2003; Von Bergh et al., 2006) and two main mechanisms of pathogenesis have been described.

The translocation t(7;12) results in a fusion transcript *HLXB9-ETV6* in 50% of AML patients and over expression of *HLXB9* gene was reported in all t(7;12) patients that have been analysed (Ballabio et al., 2009; Von Bergh et al., 2006).

It has been suggested that over-expression of *HLXB9* in the AML cell line GDM-1, happens by juxtaposition with the oncogene *MYB*. In fact, the translocation t(6;7) in GDM-1 requires breakpoints at 6q23 (where *MYB* is located) and at 7q36 in a region that very close to *HLXB9* and distal to it (Nagel et al., 2005).

To date, only two partner genes for *HLXB9* have been identified. These are (i) the *ETV6* gene, that codes for a transcription factor in the t(7;12) (q36;p13) t(7;12) (q36;p13) (Beverloo et al., 2001; Tosi et al., 2003) and (ii) the proto oncogene *MYB* at 6q23 with via the t(6;7) (Nagel et

al., 2005). However, the involvement of *MYB* with *HLXB9* has been so far reported only in the GDM-1 cell line. Further investigation is required to understand the role of *MYB* in this translocation.

In this thesis, (Chapter 5) the role of the *HLXB9* gene has been investigated in AML cell line (GDM-1) and 4 AML patients by FISH using a specific PAC clone containing *HLXB9* gene. The dual FISH analysis revealed that no evidence of the *HLXB9* gene structural rearrangements in the AML cell line and patients. The translocation breakpoint was confirmed to be distal to *HLXB9* in the AML cell line (GDM-1). All the patients studied in chapter 4 were characterized by the presence of an interstitial deletion of chromosome 7.In all these cases, the deleted region was proximal to the *HLXB9* gene. This means that both proximal and distal breakpoint in the del(7q) did not involve *HLXB9*, that was retained and not deleted.

The involvement of *HLXB9* in structural rearrangements and breakage were excluded previously (Tosi et al., 2003; Nagel et al., 2005). Although the over-expression of *HLXB9* was reported in leukaemia patients and the GDM-1cell line (Ballabio et al., 2009; Beverloo et al., 2001; Nagel et al., 2005; Tosi et al., 2003; Von Bergh et al., 2006).

Recently, in some t(7;12) patients, over-expression of *HLXB9* has been reported to associate with nuclear changed position (Ballabio et al., 2009). The *HLXB9* gene is found to be located towards the nuclear periphery in the absence of translocation. Interestingly, the *HLXB9* allele on the derivative (12) is assumed to have an intermediate position towards the nuclear interior (Ballabio et.al, 2009). The alteration of gene position could be a mechanism that favours over- expression of the *HLXB9* gene in t(7;12) positive AML patients.

6.4 Future work

Currently, there is no evidence of *HLXB9* gene breaks or structural rearrangements in leukaemia patients. However, the over expression of *HLXB9* has been recognized in AML patients (Tosi et al., 2003; Von Bergh et al., 2006) and cell line (GDM-1) (Nagel et al., 2005).

The study of the *HLXB9* expression in more leukaemia patients will be a valuable as these studies would be important to establish the real incidence of *HLXB9* positive leukaemia cases. As some data established already an over expression of *HLXB9* in very limited number of ALL patients and AML patients negative for t(7;12) (Ballabio et al., 2009; Von Bergh et al., 2006). The characterization of *HLXB9* over-expression in more leukaemia patients would facilitate the identification a sub-group of leukaemia patients that shared the over-expression of *HLXB9* abnormality. This would have implications in diagnosis and prognosis and subsequently in therapy decisions.

Further investigation of *HLXB9* gene in patients will enable a better understanding of the molecular changes of gene over expression and will result in more accurate therapy targeting the gene being delivered to these patients.

A position effect mechanism involving *HLXB9* gene has been recently proposed in leukaemia patients positive for the t(7;12) (Ballabio el al., 2009). Investigations that focus on the *HLXB9* gene position in a series of leukaemia patients involving t(7;12) and t(6;7) would be valuable, as this could give an indication of the changes in the nuclear architecture in leukaemia. Moreover, this mechanism could be a sole charge mechanism in these patients.

It would be interesting to understand what are the consequences of the ectopic expression of *HLXB9* gene and what the target genes are for *HLXB9*, being this a transcription factor.

Techniques of Chromatin Immuno Precipitation (ChIP) on chip are useful tools to shed some light on *HLXB9* pathways and their alteration in cancer.

Finally, the oncogenic potential role of the *HLXB9* gene could be explored in mouse models to see whether an altered gene expression is sufficient to promote leukaemia development in animals.

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