IDENTIFICATION OF THE GENOMIC BREAKPOINTS OF A NOVEL CHROMOSOME TRANSLOCATION IN MYELODYSPLASTIC SYNDROME

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Dissertation for a Master's Degree in Oncology

Porto, 2011

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Dissertation for applying to a **Master's Degree in Oncology** – Specialization in Molecular Oncology submitted to the Institute of Biomedical Sciences Abel Salazar – University of Porto

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"***Nothing in life is to be feared, it is only to be understood.***" — Marie Curie

AKNOWLEDGEMENTS

First of all, I would like to express my gratitude to my supervisor, Professor Manuel Teixeira for guiding me through the process of completing this thesis: for his supervision, time and advice from the very early stage of this research. Thank you for the diligence in the reading and revision of this thesis.

To Professor Carlos Lopes, as Director of the Oncology Master's Programme, and to all other lecturers who participated in this course and shared with us their knowledge and experience in Oncology.

I also want to thank my co-supervisor Nuno Cerveira for teaching me and spending countless hours of his time with me. Thank you for the endless patience and crucial contribution to this work.

A very special thanks goes to my other co-supervisor, Cecília Correia. Above all and the most needed, she was the one who provided me unflinching knowledge, encouragement and support in various ways. Thank you for the kindness, understanding, motivation and encouragement, I am grateful in every possible way. I gratefully thank to the other "Cytogenetic girls": Susana Lisboa, Lurdes Torres and Joana Vieira for receiving me into their group. Thank you for the frequent encouragement to go further and for all constructive comments on this thesis.

To Diogo, thank you for his endless patience and for spending so much of his time taught me about BACs. I am grateful for the wise suggestions and technical support. To Joana Santos, for all the attempts she made with the Panhandle PCR technique. To all the colleagues who have worked at the Genetics department of the Portuguese Oncology Institute – Porto during the year and a half I stood there, thank you for the friendly work environment.

I appreciate the constant encouragement of all my friends and family that kept me on track and working forward toward finishing the thesis, especially from Nisia and Francisca. Thanks to Gui, who surprisingly appeared so many years after and had the patient and the diligence to read and correct every word in this thesis.

To Nuno, my best friend, my rock. Thank you with all my heart for all the strength and for always being there, through both the highs and the lows.

Lastly, and most importantly, to my parents. My father, who showed me the joy of intellectual pursuit ever since I was a child, and my mother, who showed me the art of working around obstacles, and the importance of just getting it done. They bore me, raised me, supported me, taught me, and loved me. To them I dedicate this thesis.

This study was funded by grants from "Associação Portuguesa Contra a Leucemia" (2006-30.2.AP/MJ) and the "Comissão de Fomento da Investigação em Cuidados de Saúde" (P.I. 26/2007).

RELEVANT ABBREVIATIONS

AA	Aplastic anemia
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
ATG	Anti-thymocyte globulin
ATOH7	Atonal homolog 7 (Drosophila) gene
BAC	Bacterial artificial chromosome
cDNA	Complementary deoxyribonucleic acid
CDS	Commonly deleted segments
CLL	Chronic lymphocytic leukemia
CMML	Chronic myelomonocytic leukemia
CBFβ	Core-binding factor β
DAPI	4'-6-Diamidino-2-phenylindole
Del	Delection
Der	Derivative
DNA	Deoxyribonucleic acid
DNA2	DNA replication helicase 2 homolog (yeast) gene
FAB	French-American-British
FADD	Fas-associated death domain
FPD	Familial platelet disorder
FISH	Fluorescence *in situ* hybridization
HNRNPH3	Heterogeneous nuclear ribonucleoprotein H3 (2H9) gene
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplantation

IPSS	International Prognostic Scoring System
ISCN	International System for Human Cytogenetic Nomenclature
MDS	Myelodysplastic syndrome
MLL	Myeloid/lymphoid or mixed-lineage leukemia gene
NHL	Non-Hodgkin lymphomas
p	Chromosome short arm
PBLD	Phenazine biosynthesis-like protein domain containing gene
q	Chromosome long arm
qRT-PCR	Quantitative real-time polymerase chain reaction
RT-PCR	Reverse transcription polymerase chain reaction
RA	Refractory anemia
RAEB-1	Refractory anemia with excess blasts-1
RAEB-2	Refractory anemia with excess blasts-2
RARS	Refractory anemia with ring sideroblasts
RBC	Red blood cell
RCMD	Refractory cytopenia with multilineage dysplasia
RCMD-RS	Refractory cytopenia with multilineage dysplasia and ringed sideroblasts
RHD	Runt homology domain
RT-PCR	Reverse transcription polymerase chain reaction
RUFY2	RUN and FYVE domain containing 2 gene
RUNX1	Runt-related transcription factor 1 gene
t-MDS	Therapy or treatment related MDS
TAD	Transcription activation domain
TNF-α	Tumor necrosis factor α
WHO	World Health Organization
WPSS	World Prognostic Scoring System

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SUMMARY

The myelodysplastic syndromes (MDS) are a group of clonal hematopoietic stem cell diseases of which around 50 % show clonal cytogenetic abnormalities, including balanced translocations (of these, 30-50% present with *de novo* MDS and ~95% with t-MDS).Although MDS has been recognized as an important disease for more than 50 years, its molecular pathogenesis and the molecular basis for its development and progression to AML remain unclear.

Many of the recurring chromosomal abnormalities in MDS lead either to the loss of genetic material, with absence or reduction in the level of one or more critical gene products (haploinsufficiency), or to the formation of fusion genes whose tumorigenic action may be due to overexpression of a gene in one of the breakpoints or the creation of a hybrid gene through the fusion of two genes, one in each breakpoint. To date, more than 600 gene fusions created by an acquired chromosome change are known, and about 30% of human cancer cases contain a fusion gene. There is overwhelming evidence that these gene rearrangements represent important and early steps in the initiation of carcinogenesis. First, they are usually closely correlated with specific tumor phenotypes; and second, it has been shown, mainly in hematological malignancies, that successful treatment is paralleled by a decrease or eradication of the disease-associated chimera. This information has become an increasingly important tool in the management of cancer patients, mainly in hematological malignancies, helping to establish a correct diagnosis, select the appropriate treatment and predict the outcome.

Balanced chromosome aberrations involving 21q22 are commonly observed in hematopoietic disorders, mostly involving the Runt-related transcription factor 1 gene (*RUNX1*). The *RUNX1* gene is located in 21q22.3, codes for a transcription factor involved in cell lineage differentiation during development and is crucial for the establishment of definitive hematopoiesis and generation of hematopoietic stem cells in the embryo. Regarding translocations involving the *RUNX1* gene, some have been shown to be frequent while others have only been reported in a few or single cases. At present, 55 partner chromosome bands have been described in translocations with *RUNX1* gene, but the partner gene has only been identified at the molecular level in 21 cases. The main question raised by the detection of a novel translocation involving the *RUNX1* gene is to know

whether it results in a fusion with a gene located in the translocation partner chromosome, or a truncation of the *RUNX1* gene.

In this study, we describe a novel chromosomal translocation, t(10;21)(q21;q22), involving the *RUNX1* gene, in an adult patient with myelodysplastic syndrome. Commercial available FISH probes showed disruption of *RUNX1* gene and translocation of the telomeric 5' region to chromosome 10. A chromosome walking process using several locus-specific BAC clones targeting the 10q21~22 chromosomal region was performed and allowed us to restrict the der(10) breakpoint region. Five genes have been mapped on this genomic region and chosen as targets for further analyses: *RUFY2*, *ATOH7*, *HNRNPH3*, *PBLD* and *DNA2*. Despite repeated efforts, any attempt to identify a specific fusion gene transcript by both RT-PCR and panhandle PCR failed, since we could only detect *RUNX1* wild type sequences. The *RUNX1* exon expression ratio average, measured by quantitative RT-PCR, showed differences that supports both the hypothesis that we are in the presence of either a truncated *RUNX1* or a *RUNX1* fusion gene.

As far as we know, this is the first case of a confirmed translocation t(10;21)(q21;q22), involving *RUNX1* gene, in a patient with MDS. Although the clinical significance and molecular mechanism of t(10;21) are still unclear, this chromosomal abnormality seems to be a rare translocation. Interestingly, in our case the chromosomal translocation does not seem to lead to the fusion of *RUNX1* to a known candidate gene, and consequently could not be detected at the transcript level. The results suggest that the *RUNX1* gene is probably altered by the formation of a truncated *RUNX1* protein, retaining an intact RHD domain. These abnormal *RUNX1* gene products may compete with wild-type *RUNX1* and act as dominant constitutive repressor inhibiting the function of the normal *RUNX1* gene. Given the crucial role of the *RUNX1* protein in hematopoiesis it is warranted that futures studies will try to understand its role in normal hematopoiesis, thus providing more data on the leukemogenesis induced by abnormal *RUNX1* proteins.

RESUMO

As síndromes mielodisplásicas (SMD) são um grupo de doenças clonais das células progenitoras hematopoiéticas, cujas anomalias cromossómicas, incluindo translocações, são observadas em ~50% dos casos, ocorrendo em 30-50% dos doentes com SMD primária e em 95% dos casos de SMD secundária.

Embora as SMD tenham sido reconhecidas há mais de 50 anos como patologias de grande importância no espectro das neoplasias hematológicas, a patogénese e bases moleculares que levam ao seu desenvolvimento e progressão para LMA permanecem por elucidar. Muitas das alterações cromossómicas recorrentes nestas patologias, conduzem a perdas de material genético com redução ou ausência da expressão de um ou mais genes críticos (haploinsuficiência). A formação de genes de fusão cuja acção tumorigénica pode ser devida à sobre-expressão de um gene localizado nos pontos de quebra, ou à criação de um gene híbrido através da fusão de dois genes, um em cada ponto de quebra, são também resultado de alterações cromossómicas recorrentes. Até à data são conhecidos mais de 600 genes de fusão, tendo sido identificados em cerca de 30% das neoplasias existentes. Vários factores permitem confirmar que estes rearranjos cromossómicos representam passos importantes da iniciação da carcinogénese; estão estreitamente correlacionados com fenótipos tumorais específicos e, principalmente em neoplasias hematológicas, o sucesso do tratamento é acompanhado por uma diminuição ou erradicação do clone anormal. Esta informação tornou-se uma ferramenta essencial na caracterização destas doenças, fornecendo dados importantes que permitem identificar categorias clínicas e biológicas específicas, estratificar os doentes em categorias de prognóstico, avaliar a eficácia do tratamento, prever a probabilidade de progressão e seleccionar doentes que possam beneficiar de terapias inovadoras.

As alterações da região cromossómica 21q22 são frequentemente observadas em neoplasias hematológicas, nomeadamente em SMD, sendo que a maioria envolve o gene *RUNX1* ("Runt-related transcription factor 1 gene"). O gene *RUNX1* está localizado em 21q22.3, codifica factores de transcrição envolvidos na diferenciação das diferentes linhagens celulares e é essencial para o estabelecimento de uma hematopoiese eficaz. Actualmente são conhecidas translocações do gene RUNX1 com 55 bandas cromossómicas distintas, sendo que apenas em 21 casos o parceiro de fusão foi caracterizado ao nível molecular.

Algumas destas translocações são frequentes, enquanto outras foram identificadas em poucos ou mesmo casos isolados. A principal questão perante a identificação de uma nova translocação envolvendo o gene *RUNX1* é saber se esta resulta na fusão com um gene localizado no cromossoma parceiro, ou num gene *RUNX1* truncado.

Neste estudo é descrita uma nova translocação cromossómica, t(10;21)(q21;q22), envolvendo o gene *RUNX1*, num caso de SMD. Análises de FISH com sondas específicas para a região 21q22.3 demonstraram alterações do gene *RUNX1*, nomeadamente a translocação da região telomérica 5' para o cromossoma 10. Através de um processo de mapeamento cromossómico com sondas específicas originadas a partir de BACs, foi possível restringir a região de quebra no cromossoma 10 a 5 possíveis genes parceiros, que foram posteriormente alvos de análise: *RUFY2*, *ATOH7*, *HNRNPH3*, *PBLD* e *DNA2*. Através das técnicas de RT-PCR e panhandle PCR não foi possível identificar nenhum transcrito de fusão, tendo sido detectadas apenas sequências "wild type" do gene *RUNX1*. Os resultados da expressão média dos diferentes exões do gene *RUNX1* obtidos por PCR quantitativo em tempo real, permitiram suportar ambas as hipóteses: a presença de um gene *RUNX1* truncado ou de um gene de fusão.

Este é o primeiro caso confirmado de uma translocação t(10;21)(q21;q22), envolvendo o gene *RUNX1* em SMD. Embora o significado clínico e o mecanismo molecular da t(10;21) sejam ainda desconhecidos, esta alteração cromossómica pode ser considerada rara. Curiosamente, no caso descrito, a translocação não parece levar à fusão do gene *RUNX1* com um gene parceiro, uma vez que foi impossível detectar qualquer transcrito de fusão. Os resultados sugerem assim que a translocação t(10;21) poderá dar origem a um gene *RUNX1* truncado com retenção do domínio RHD. Este produto anormal provavelmente compete com o gene "wild type", actuando como regulador dominante negativo e inibindo assim as funções do gene *RUNX1* normal. Dado o papel crucial da proteína *RUNX1* na hematopoiese, serão necessários estudos futuros para esclarecer o seu papel em todo o processo hematopoiético, proporcionando desta forma informações importantes sobre a patogénese das neoplasias hematológicas.

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INTRODUCTION

1. MYELODYSPLASTIC SYNDROMES

1.1. History – a century full of challenges

As reviewd in 2009 by Steensma and Bennet, the myelodysplastic syndromes (MDS) have probably existed as long as the human lifespan was stretched beyond the reproductive age, however, the tools required for its detection — accurate hemocytometers, biological stains capable of highlighting intracellular detail, and microscopes with reduced spherical aberration — only came into widespread use at the far end of the 19th century (Steensma and Bennet, 2009).

"Preleukemia" as a unique concept originated during the begining of the 20th century with the recognition by Von Leube (Von Leube, 1900) and Parkes-Weber (Parkes-Weber, 1904) that acute myeloid leukemia (AML) may be preceded by a cytopenic state associated with dysplasia of one or more hemopoietic cell lineages (Layton and Mufti, 1986). In 1953, Block decided to incorporate in the group of "preleukemias", patients with cytopenias and dysplasia of one or more hematopoietic lineages in the bone marrow, and increased risk of leukemic evolution (Block *et al.*, 1953). Three years later, in 1956, Bjorkman established definitely a clear link between the term "preleukemia" and a subgroup of patients with refractory anemia with marrow sideroblastosis, a proportion of whom developed acute leukemia (Bjorkman, 1956).

Subsequent studies, particularly those of Saarni (Saarni and Linman, 1973) and Linman (Linman and Bagby, 1976; Linman and Bagby, 1978), revealed the extent to which these diverse disorders shared common features, in particular the presence of bi- or tri-lineage cytopenias and dyshaemopoiesis despite normal or increased marrow cellularity, and enabled criteria discriminating between cases at high or low risk of evolution to leukemia to be defined (Layton and Mufti, 1986).

The term "myelodysplastic syndrome" itself emerged in the 1970s amidst controversies surrounding the various presentations of the disorder; subsequent attempts have repeatedly been made to further specify a more precise classification (Greenberg *et al.*, 2000).

In 1982, in an effort to resolve these problems and to establish diagnostic criteria which would facilitate communication between workers, the French-American-British (FAB) cooperative group proposed criteria for the morphological classification of the myelodysplastic syndromes (Bennett, 1982). The history of MDS has been reviewd in depth by Layton and Mufty (Layton and Mufti, 1986)

Since the first FAB classification system in 1982, other classification systems with various minimal criteria have been proposed (Culligan and Jacobs, 1992; Gardais, 2000; Greenberg *et al.,* 2000) and nearly 20,000 publications have characterized various features of MDS (List *et al.,* 2004). However, even in the early 21st century, issues related to MDS terminology continue to be center of discussions for its classification and minimal diagnostic criteria (Steensma and Tefferi, 2003).

1.2. Epidemiology – a disease of the elderly

The true incidence of the myelodysplastic syndromes is not known because these disorders were defined only relatively recently (Hamblin, 2009). The 9th edition of the International Classification of Diseases (ICD-9), published in 1977, did not recognize them as distinct nosological entities, and therefore they tended to get lost under a variety of headings (Hamblin, 2009). An early estimate of the incidence of MDS in the United States (U.S.) by James Linman and Grover Bagby (Linman and Bagby, 1978) suggested approximately 1500 new cases of "preleukemia" per year, but their definition was limited to cases with fewer than 5% blasts in the marrow, and this figure is clearly a marked underestimate (Hamblin, 2009). MDS only became a reportable disease for the United States cancer registries after 2001 and results from The National Cancer Institute (NCI) Surveillance, Epidemiology and End Results (SEER) Program suggest that more than 10,268 new US MDS cases occur every year, which is more than six times the original estimate from the late 1970s (Hamblin, 2009).

In the United Kingdom (UK), MDS incidence rates published in 1992 (Cartwright, 1992) and based on 1806 cases from 26 countries, estimated an incidence of 3.6/100,000 among those less than 80 years old.

In the beginning of 2010, the crude incidence of MDS was around 4–5 per 100 000 individuals yearly (Jadersten and Hellstrom-Lindberg, 2009), rising to 30

per 100 000 in the 70-90 year age group (Radlund *et al.*, 1995; Germing *et al.*, 2004; Ma *et al.*, 2007). In fact, MDS is one of the most common hematopoietic malignancies in people above 80 years (Figure 1). However, its true incidence might be underestimated because of confusion with acquired aplastic anemia or AML (Corey *et al.*, 2007). Males are more commonly affected than females (4.5 versus 2.3 per 100 000) (Ma *et al.*, 2007), although there is evidence that this is not so for refractory anemia with ring sideroblasts (RARS) (Germing *et al.*, 2004).

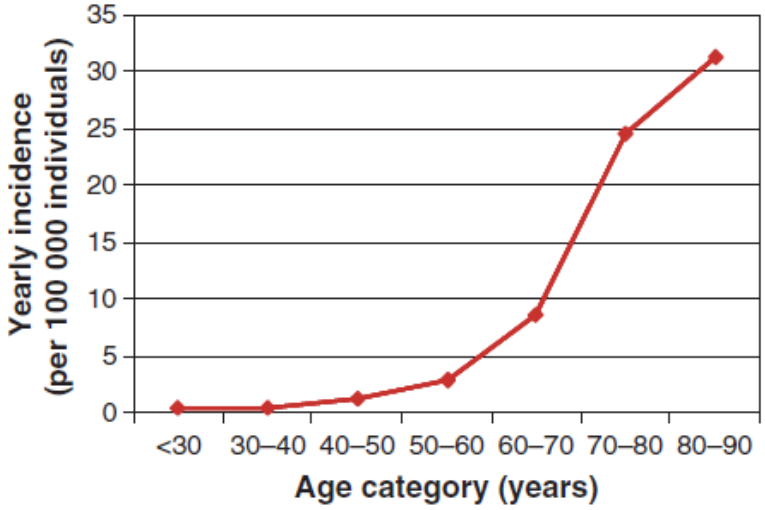

Figure 1: The incidence of MDS increases with age (Strom *et al.,* 2008).

Attention has been drawn to the fact that MDS appears to occur at a younger age in countries other than those in Europe and North America (Kuendgen, 2007). Reports from Zimbabwe, Turkey, India, Korea, China, Thailand, and Japan, suggested that in these countries, MDS is not mostly confined to the elderly (Lee, 2003; Chatterjee, 2004; Chen, 2005). Whether these differences in MDS epidemiological patterns between countries are due to genetic or environmental factors (or both) remains to be determined (Hamblin, 2009).

In relation to other hematological malignancies (Cartwright, 1997), MDS is by far the most common hematological malignancy in the very old (>age 80) subjects, being three times as common as AML, twice as common as chronic lymphocytic leukemia (CLL) and myeloma, and more common than all non-Hodgkin lymphomas (NHL) put together (Hamblin, 2009).

In 1991, an opinion poll was conducted among a group of hematologists recognized internationally for their interest in MDS about whether or not the

incidence of MDS was changing over time (Reizenstein and Dabrowski, 1991). According to the findings of Aul *et al.* (Aul *et al.,* 1998b) and Germing (Germing *et al.,* 2004), the increase in MDS reported by Reizenstein and Dabrowski (Reizenstein and Dabrowski, 1991) doesn't reflect the reality of the disease. It reflects improved case ascertainment as a consequence of improved geriatric medical care (Aul *et al.,* 1998b). Nevertheless, an aging population will produce an increase in the absolute number of patients diagnosed as will more interventional investigation of older patients (Aul *et al.,* 1998a).

MDS is much rarer in children than in adults, accounting for less than 5% of hematopoietic neoplasia in childhood (Niemeyer, 2007). Population-based data suggest an annual incidence of 1-2/100 000 (Hasle *et al.*, 1999; Passmore *et al.*, 2003). There are significant differences in presentation, underlying cytogenetic abnormalities, and classification between MDS in children and adults, and unlike the adult disease, risk inversely correlates with age and is therefore highest among the youngest individuals (Hasle, 2002). The therapeutic aim in children with MDS is a cure, and therapeutic efforts concentrate on hematopoietic stem cell transplantation (HSCT) rather than on novel therapeutics (Niemeyer, 2007).

1.3. Etiology and risk factors

Several studies have assessed risk factors associated with development of *de novo* and treatment-related MDS (t-MDS). However, while the association between chemotherapy and radiation therapy with t-MDS is well established, risk factors for *de novo* MDS, which account for the large majority of cases, have not been fully elucidated (Strom *et al.,* 2008).

1.3.1. Cytotoxic drugs and radiation

The extensive use of chemotherapy and/or radiotherapy has improved cancer survival, but these treatments have had the unintended side effect of inducing t-MDS (Mayer, 1996).

The majority of secondary MDS or t-MDS cases arise following chemotherapy for other cancers, particularly with alkylating agents or topoisomerase inhibitors, and following radiation therapy (Aul *et al.,* 1992). It is estimated that prior

exposure to chemotherapy increases the risk of developing MDS at least 100-fold (Pedersen-Bjergaard *et al.,* 2002b). Patients previously treated with alkylating agents tend to have deletions of regions on the long arm of chromosomes 5 or 7, whereas patients treated with topoisomerase II inhibitors such as doxorubicin, etoposide and teniposide tend to have translocations involving the "myeloid/lymphoid or mixed-lineage leukemia" (MLL) gene located at 11q23 (Corey *et al.,* 2007). The typical latency period for t-MDS is 5 to 10 years, and the risk appears to be dose dependent (Smith *et al.*, 2003). This category of MDS holds an overall worse prognosis than the non-treatment-related, or primary MDS, counterparts. This prognostic distinction is closely linked to the unfavorable chromosomal abnormalities characteristic of treatment-related or secondary MDS (Aul C, 2002). These chromosomal abnormalities (predominantely delections involving chromosomes 5, 7, 17, 12 and 3) (Pedersen-Bjergaard *et al.,* 2002a) are similar to those in poor-risk *de novo* MDS.

Patients treated with radiotherapy for several types of cancers (non-Hodgkin's lymphoma, Ewing's sarcoma, breast and uterine cancer) have a two- to threefold increased risk of t-AML (Boice, 1996)

1.3.2. Ionizing radiation

MDS cases are reported in cohorts of individuals exposed to radiation, for treatment of diseases such as ankylosing spondylitis (Brown and Doll, 1965), or following exposure to the A-bomb in Hiroshima and Nagasaki (Matsuo *et al.,* 1988). Some of these cases ocurred up to 40 years after exposure and thus the precise association between the development of MDS and exposure to radiation is impossible to quantify (Bowen, 2007).

Non-ionizing radiation exposure (as from magnetic fields) does not seem to play a significant role in MDS development (West *et al.*, 1995).

1.3.3. Benzene

Solvents, including benzene, are the class of chemicals probably most often reported as linked with MDS. Benzene is the most well-known leukemogenic chemical and has been the subject of multiple epidemiological studies, the first

as early as 1897 (Le Noire, 1897). Chromosomal aberrations in lymphocytes have been found to be present up to 30 years after high benzene exposure. These cytogenetic abnormalities were similar to those found in patients with t-MDS treated with alkylating agents (Park and Koeffler, 1996).

Some studies (Nisse *et al.*, 1995; Rigolin *et al.*, 1998; Strom *et al.*, 2005) but not all (West *et al.*, 1995; Albin *et al.*, 2003) have reported associations between pesticides, herbicides, or fertilizers, and MDS risk. These associations seem to be stronger among men, possibly due to the limited number of exposed women (Strom *et al.,* 2008).

1.3.4. Other potential causative factors

Cigarette smoking represents the greatest source of non-occupational benzene exposure, with smokers exposed to a 10-fold increase in benzene inhalation as compared to nonsmokers (Brownson *et al.,* 1993). An association between cigarette smoking and MDS is well established and was found in most studies, with many reporting a statistically significant increase in risk (Ido *et al.*, 1996; Pasqualetti *et al.*, 1997; Bjork *et al.*, 2000; Strom *et al.*, 2005; Bjork *et al.*, 2009). Risk seems to be related to intensity and duration of smoking (Pasqualetti *et al.*, 1997; Bjork *et al.*, 2000; Strom *et al.*, 2005), and the effect of smoking on MDS risk may persist up to 15 years after smoking cessation (Bjork *et al.,* 2000; Strom *et al.,* 2005). Furthermore, it has been demonstrated that smoking is associated with chromosomal aberrations such as deletions in −5 and −7 (Bjork *et al.,* 2000).

Other factor with an increased odds ratio for MDS that have emerged from case-control studies include alcohol excess (including a possible dose effect) (Ido *et al.,* 1996). However, each of this studies is limited by insufficient number of cases and controls to identify significant odds ratios with high statistical power (Bowen, 2007).

1.4. Clinical and morphological diagnosis

Myelodysplastic syndromes are a heterogenous group of clonal bone marrow disorders characterized by the presence of dysplastic maturation of

hematopoietic cells coupled with one or more peripheral cytopenias and a propensity to progress to an acute leukemia (Vardiman, 2003a) in approximately 30% of cases (Mufti *et al.,* 2008). In keeping with the broad spectrum of disorders covered by the term MDS, the clinical presentation of patients varies widely (Greenberg *et al.,* 2000), with the majority having macrocytic anemia with or without additional cytopenias present at the time of diagnosis (Scott and Deeg, 2010). Other symptoms are a direct result of cytopenias and cell function abnormalities (Scott, 2007). The differential diagnosis includes other causes of macrocytic anemia, such as vitamin B12 and folate deficiencies, alcohol consumption, and thyroid disorders. The initial laboratory workup includes blood cell counts, serum ferritin levels, total iron binding capacity, serum iron levels, reticulocyte counts, levels of vitamin B12, red blood cell (RBC) folate, and thyroid stimulating hormone. Persistent unexplained cytopenias warrant additional investigation via bone marrow aspiration and biopsy, including cytogenetic testing and iron stains (Scott and Deeg, 2010).

1.5. Classification

As refered in section 1.1, during the past 20 years several MDS classification and prognostic scoring systems have been proposed and gained acceptance.

The French-American-British (FAB) (Bennett, 1982) diagnostic scheme has largely been supplanted by the World Health Organization (WHO) criteria (Table 1) (Vardiman, 2003b). The WHO classification has proved helpful for prognostication (Malcovati *et al.,* 2005) and in therapy selection (Howe *et al.,* 2004). The FAB scheme distinguish MDS from AML on the basis of a blast count of 30%, whereas the WHO uses a threshold of 20% (Corey *et al.,* 2007).

Table 1: WHO diagnostic classification of myelodysplastic syndromes (Vardiman *et al.*, 2002).

Disease	Blood findings	Bone marrow findings
Refractory anemia (RA)	Anemia No or rare blasts	Erythroid dysplasia only <5% blasts <15% ringed sideroblasts
Refractory anemia with ringed sideroblasts (RARS)	Anemia No blasts	Erythroid dysplasia only ≥15% ringed sideroblasts <5% blasts
Refractory cytopenia with multilineage dysplasia (RCMD)	Cytopenias (bicytopenia or pancytopenia) No or rare blasts No Auer rods <1 × 10^9/L monocytes	Dysplasia in ≥10% of cells in 2 or more myeloid cell lines <5% blasts in marrow No Auer rods <15% ringed sideroblasts
Refractory cytopenia with multilineage dysplasia and ringed sideroblasts (RCMD-RS)	Cytopenias (bicytopenia or pancytopenia) No or rare blasts No Auer rods <1 × 10^9/L monocytes	Dysplasia in ≥10% of cells in 2 or more myeloid cell lines ≥15% ringed sideroblasts <5% blasts No Auer rods
Refractory anemia with excess blasts-1 (RAEB-1)	Cytopenias <5% blasts No Auer rods <1 × 10^9/L monocytes	Unilineage or multilineage dysplasia 5% to 9% blasts No Auer rods
Refractory anemia with excess blasts-2 (RAEB-2)	Cytopenias 5% to 19% blasts Auer rods ± <1 × 10^9/L monocytes	Unilineage or multilineage dysplasia 10% to 19% blasts Auer rods ±
Myelodysplastic syndrome, unclassified (MDS-U)	Cytopenias No or rare blasts No Auer rods	Unilineage dysplasia in granulocytes or megakaryocytes <5% blasts No Auer rods
MDS associated with isolated del(5q)	Anemia <5% blasts Platelets normal or increased	Normal to increased megakaryocytes with hypolobated nuclei <5% blasts No Auer rods Isolated del(5q)

In 1997 an alternative International Prognostic Scoring System (IPSS) based on the presence of cytopenia(s), cytogenetic abnormalities and blasts was proposed (Table 2) (Greenberg *et al.,* 1997). The IPSS divides patients into four risk groups – low, intermediate-1 (int-1), intermediate-2 (int-2), and high – and reliably discriminates between these groups in regard to expected survival and AML progression (Figure 2) (Scott and Deeg, 2010).

Table 2: International Prognostic Scoring System (IPSS) classification criteria (Scott and Deeg, 2010).

	Score value[a]				
Prognostic variable	0	0.5	1.0	1.5	2.0
BM blasts (%)	<5	5-10	—	11-20	21-30
Karyotype[b]	Good	Intermediate	Poor		
Cytopenias	0/1	2/3			

[a]Scores for risk groups are as follows: low, 0; INT-1, 0.5-1.0; INT-2, 1.5-2.0; high, ≥2.5.

[b]Karyotype was as follows: good: normal, –Y, islolated del(5q), isolated del(20q); poor: complex (≥3 abnormalities), chromosome 7 abnormalities; and intermediate: other abnormalities.

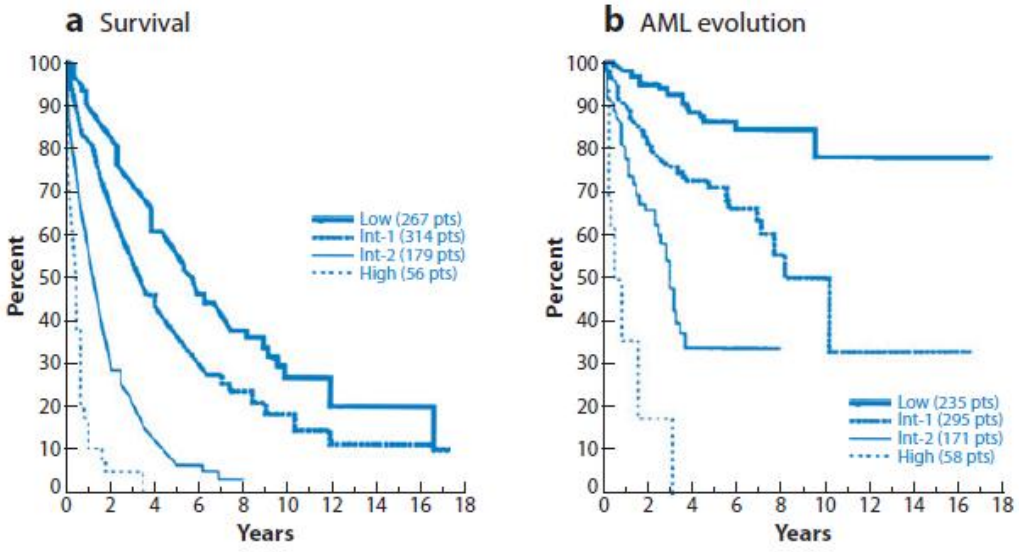

Figure 2: Expected survival and AML progression based on the IPSS classification. **(a)** Survival by IPSS category. **(b)** Risk of AML evolution by IPSS category (Greenberg *et al.,* 1997).

In addition, the association of both transfusion burden and high ferritin levels with inferior survival, and a modification of the IPSS by Malcovati and co-workers (Malcovati *et al.,* 2007), the so-called WPSS (World Prognostic Scoring System), have surfaced as an important components of our understanding of the natural history of MDS (Table 3) (Mufti *et al.,* 2008). Recently, a flow-cytometric scoring system (FCSS) has been validated, and may have prognostic information, particularly in patients who are otherwise thought to be low risk (Scott *et al.,* 2008; van de Loosdrecht *et al.,* 2008).

As research continues to advance our knowledge of the etiology and the pathogenesis of MDS, refinements in classification are definitly necessary.

Table 3: World Prognostic Scoring System (WPSS) classification criteria (Scott and Deeg, 2010).

Variable	0	1	2	3
WHO= category[a]	RA, RARS, 5q–	RCMD, RCMD-RS	RAEB-1	RAEB-2
Karyotype[b]	Good	Intermediate	Poor	—
Transfusion requirement[c]	No	Regular	—	—

[a]Abbreviations: MDS, myelodysplastic syndrome; RA, refractory anemia; RARS, refractory anemia with ringed sideroblasts; 5q–, myelodysplastic syndrome with isolated del(5q) and marrow blasts <5%; RCMD-RS, refractory cytopenia with multilineage dysplasia and ringed sideroblasts; RAEB-1, refractory anemia with excess of blasts-1; RAEB-2, refractory anemia with excess of blasts-2.

[b]Karyotype was as follows: good: normal, –Y, islolated del(5q), isolated del(20q); poor: complex (≥3 abnormalities), chromosome 7 abnormalities; and intermediate: other abnormalities.

[c]Red blood cell transfusion dependency was defined as having at least one red blood cell transfusion every 8 weeks over a period of 4 months.

2. PATHOGENESIS

Normal hematopoiesis is organized as a hierarchy sustained by a small population of primitive multipotent hematopoietic stem cells (HSCs) (Figure 3) (Wang and Dick, 2005). These are also known as SCID repopulating cells (SCRs), because they can reconstitute hematopoiesis in non-obese diabetic severe combined immunodeficient (NOD/SCID) mice. HSCs give rise to lineage-restricted progenitors capable of extensive proliferation and differentiation but with reduced or no self-renewal capacity (long-term colony-initiating cells (LTC-ICs) and colony forming cells (CFCs)), which in turn produce functionally mature non-proliferating blood cells. When these processes become deregulated through the acquisition of transforming mutations, the result is often leukemia (Corey *et al.,* 2007).

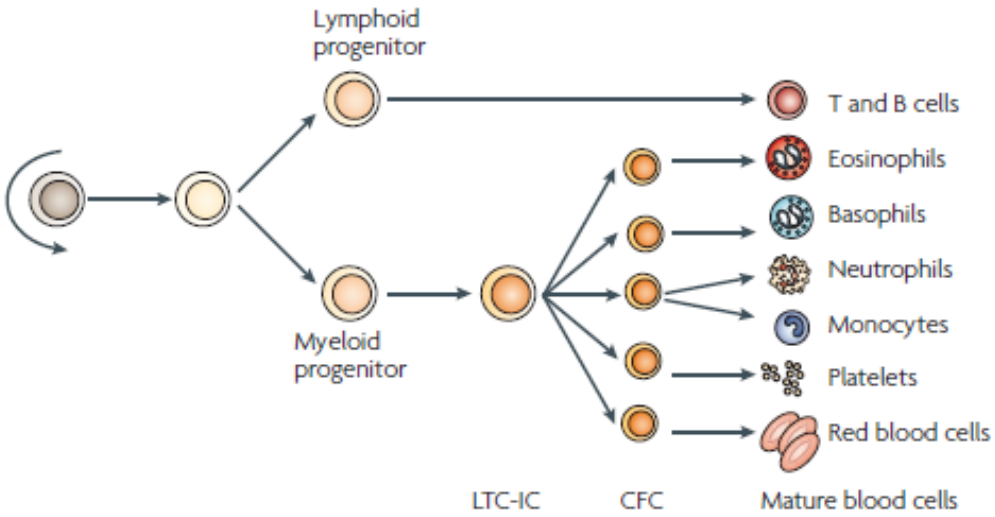

Figure 3: The hierarchical organization of hematopoiesis (Corey *et al.,* 2007).

The natural history of MDS is highly variable. This likely reflects the myriad of cytogenetic, genetic and epigenetic alterations that are associated with MDS. It has generally been suggested that MDS arises from an hematopoietic stem cell that has suffered irreversible DNA damage. Further events result in dominance of this damaged clone (Figure 4). Immunologic responses may occur that promote progenitor survival and eventual clonal dominance (Corey *et al.,* 2007).

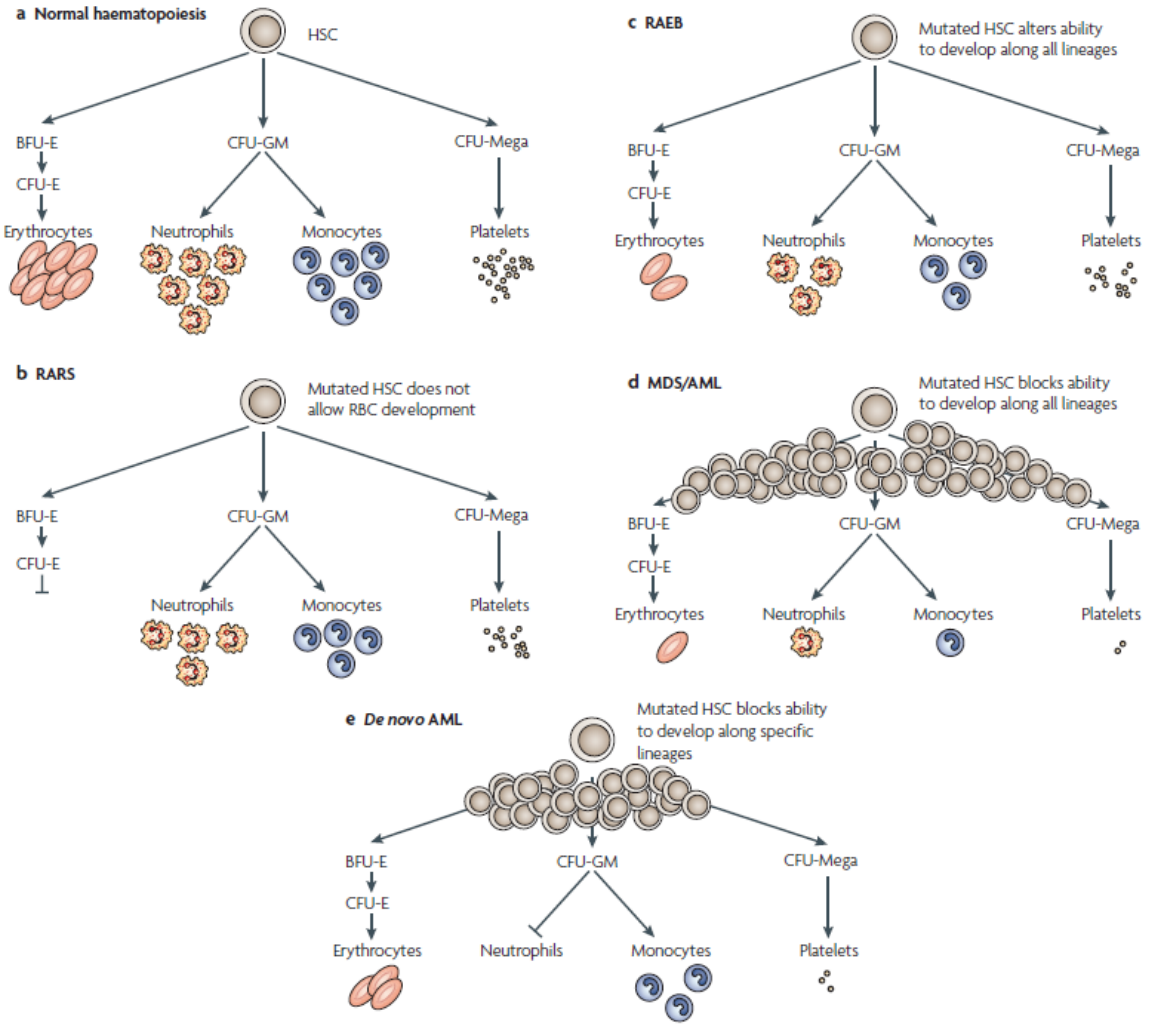

Figure 4: MDS constitute a complex range of stem-cell diseases. The MDS cell clone can suppress normal hematopoiesis **(a)** directly or indirectly through stroma. Stem-cell defects can result in single-lineage deficiency (refractory anemia and ringed sideroblasts (RARS; **b)**) or multiple-lineage deficiencies (refractory anemia with excess blasts (RAEB; **c)**). MDS stem-cell diseases **(d)** might seem like de novo AML, however, the two are distinguishable. For example **(e)**, cytopenias in de novo AML can be more restricted owing to a failure in differentiation (Corey *et al.,* 2007).

2.1. Clonal stem cell disorder

Considered to be a clonal disorder of an early hematopoietic progenitor or stem cell, clonality of MDS has been demonstrated with fluorescence *in situ* hybridization (FISH) analysis in patients with known cytogenetic aberrations, generally demonstrating clonal involvement of the hematopoietic stem cells, all myeloid lineages and less often also B and NK cells (Jaju *et al.*, 2000; Nilsson *et*

al., 2002). In addition, several studies have shown a nonrandom X-inactivation pattern in all MDS categories, including patients with RARS (Abrahamson *et al.,* 1991; Delforge, 2003).

However, abnormalities seen so frequently in MDS may be acquired during disease progression, rather than reflecting the initial inciting clonal event (Nilsson *et al.*, 2002; Delforge, 2003). Whether a primary or secondary event, genomic instability, as evidenced by karyotypic changes common in MDS, is thought to play an important role in disease pathogenesis (Nivatpumin, 2007).

2.2. Increased apoptosis in the bone marrow

First described by Wyllie and colleagues (Wyllie *et al.,* 1980), apoptosis is an energy-dependent process characterized morphologically by cytoplasmic and nuclear condensation, fragmentation of nuclei into "apoptotic bodies", preservation of plasma membrane integrity and phagocytosis of cellular debris by macrophages in the absence of an inflammatory response (Walker *et al.*, 1988; Greenberg, 1998). This death mechanism is crucial in maintaining a precise number of cells in a given organism.

Increased apoptosis in the hematopoietic progenitors resulting in peripheral cytopenias is a hallmark of MDS (Parker and Mufti, 2004), and has been shown by morphology, immunohistochemistry, flow cytometry and the molecular detection of activated apoptosis-related proteins (Parker *et al.,* 2000). Both the extrinsic and the intrinsic pathways of apoptosis have been shown to be involved (Jadersten and Hellstrom-Lindberg, 2009).

The death receptors (Fas, TNF-α and TRAIL) and the Fas-associated death domain (FADD) can be over-expressed in MDS (Zang *et al.*, 2001; Claessens *et al.*, 2002; Sawanobori *et al.*, 2003). However, blocking the extrinsic pathways has generated conflicting results *in vitro* (Claessens *et al.*, 2002; Claessens *et al.*, 2005), and clinical studies using tumor necrosis factor α (TNF-α) inhibitors failed to demonstrate any significant activity (Raza, 2000).

The pro-apoptotic members of the Bcl-2 family are up-regulated in low-risk disease leading to increased apoptotic signaling (Parker *et al.*, 1998; Boudard *et al.*, 2002). Defects in the mitochondrial function may also be present: the ringed sideroblasts observed in RARS are in fact iron-overloaded mitochondria, with the

iron bound to aberrant mitochondrial ferritin (Cazzola *et al.,* 2003; Tehranchi *et al.,* 2005). In patients with RARS, there is a constitutive leakage of cytochrome *c* from the mitochondria leading to subsequent caspase activation and increased apoptosis (Tehranchi *et al.,* 2003). Furthermore, mutations of the mitochondrial DNA are present in as much as half of the patients with MDS (Wulfert *et al.,* 2008).

The cause of abnormal apoptosis in MDS is unknown. Further insight into the intrinsic and extrinsic factors that affect apoptosis is a central area of research and holds significant promise for the development of clinical therapies (Bowen, 2007).

2.3. Epigenetic modifications

While genetic alterations are critical in the pathogenesis of MDS, epigenetic changes also contribute significantly to the disease phenotype (Nivatpumin, 2007).

The most studied epigenetic alterations in cancer are promoter hypermethylation and histone deacetylation, although several other ways of epigenetic modulation of gene expression exist (Jones and Baylin, 2002; Esteller, 2008). Hypermethylation of the tumor suppressor $p15^{INK4B}$ gene promoter has been observed in 30-50% of MDS cases and has been shown to correlate with the percentage of bone marrow blasts (Quesnel *et al.*, 1998). Further evidence of the importance of this event in MDS pathogenesis derives from the observation that the degree of methylation correlates with the risk of evolution to AML and clinical prognosis (Quesnel *et al.,* 1998). Other genes frequently methylated and silenced in myeloid malignancies include *E-Cadherin*, *RARβ*, and *SOCS-1* (Esteller and Herman, 2002; Herman and Baylin, 2003).

The clinical activity of the DNA methyltransferase inhibitors 5-azacitidine (Silverman, 2004) and 2'-deoxy-5-azacitidine (Wijermans *et al.*, 1997; Wijermans *et al.*, 2000) in MDS suggests that the methylation status of a subset of genes is likely to contribute significantly to the biological and clinical behavior of MDS. However, attempts to correlate the clinical activity of these agents with reversal of p15 methylation have demonstrated that methylation reversal may not be

required for clinical response (Daskalakis *et al*., 2002; Lubbert, 2003; Issa *et al*., 2004).

Transcriptional silencing of methylated genes is mediated at least in part through the establishment of repressive chromatin conformation through the recruitment of histone deacetylases (Cameron *et al.,* 1999). This has led to the strategy of combined DNA methyltransferase and histone deacetylase inhibition for the treatment of MDS (Bowen, 2007).

2.4. Immune dysregulation

There is growing evidence that immune deregulation plays a role in MDS pathophysiology (Bowen, 2007). The relationship between MDS and autoimmunity stimulated the investigation about the role of the immune system in MDS. The incidence of autoimmune disorders appears to be increased in patients with MDS (Saif *et al.,* 2002). Autologous cytotoxic T lymphocytes have been observed to exert inhibitory effects on MDS myelopoiesis in vitro. Moreover, the features of MDS may overlap with aplastic anemia (AA) and large granular lymphocyte (LGL) disease, two diseases thought to be related to autoreactive T lymphocytes (Barrett *et al.,* 2000; Kanchan and Loughran, 2003).

Deeg *et al.* treated fourteen transfusion-requiring MDS patients with the combination of anti-thymocyte globulin (ATG) and the soluble TNF receptor protein etanercept (Deeg *et al.,* 2004). Forty-six percent of the patients responded, with five patients achieving periods of red blood cell and platelet independence that exceeded 2 years. These impressive results lend further evidence to the premise that immunomodulation may be effective in selecting patients with MDS (Bowen, 2007).

As stated by Bowen, fundamental questions remain unanswered about the precise mechanisms underlying autoimmunity in MDS (Bowen, 2007). The hypothesis that T lymphocytes attack specific antigens on MDS clonal progenitors remains unproven. Likewise, it is unclear why some patients respond to immunosuppression and others do not.

3. CYTOGENETIC DIAGNOSIS OF MYELODYSPLASTIC SYNDROMES

The cytogenetic evaluation of bone marrow samples from patients with a myelodysplastic syndrome has become an intrinsic part of clinical care. Cytogenetic analysis not only confirms the diagnosis but is invaluable in defining the prognosis, as well as the risk for progression to AML (Olney, 2007). On a more fundamental level, we can say that cytogenetic analysis has been essential in establishing the clonality of these syndromes, as well as providing hints about the numerous biologic mechanisms implicated in their pathophysiology.

The value of cytogenetic analysis in predicting survival and risk of leukemic transformation during a patient's clinical course has been well established (Morel *et al.*, 1993; Toyama *et al.*, 1993b; Jotterand and Parlier, 1996; Sole *et al.*, 2005; Haase *et al.*, 2007). At the time of diagnosis, recurring chromosomal abnormalities are found in ~50% of patients with primary MDS and in 95% of patients with therapy-related MDS (t-MDS) (Vallespi *et al.,* 1998). Clonal chromosome abnormalities can be detected in bone marrow cells of 25% of patients with RA, 10% of patients with RARS, 50% of patients with RCMD, 50-70% of patients with RAEB-1,2, and all of the patients with MDS with isolated del(5q) (Olney, 2009b).

Most recurring cytogenetic abnormalities found in MDS are unbalanced, most commonly the result of the loss of a whole chromosome or a delection of part of a chromosome, but translocations (balanced and unbalanced) and more complex derivative (rearranged) chromosomes can also be found (Figures 5). The most common cytogenetic abnormalities encountered in MDS are del(5q), −7, and +8, which have been incorporated into the more robust prognostic scoring systems of MDS (Tables 2 and 3).

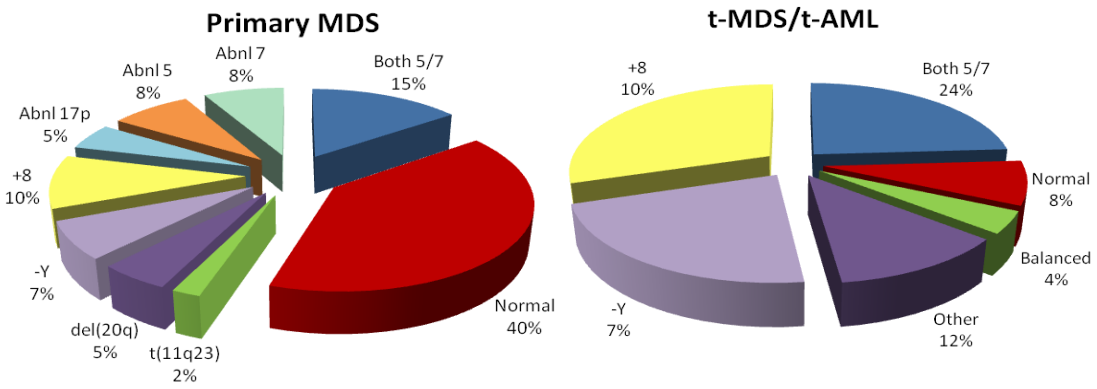

Figure 5: Recurring chromosomal abnormalities in MDS (Olney, 2007).

The frequency of cytogenetic abnormalities increases with the severity of disease, as does the risk of leukemic transformation (Olney, 2009b). Cytogenetic evolution of the karyotype includes the appearance of an abnormal clone where only normal cells had been seen previously, or progression from the presence of a single clone (often with a simple karyotype) to multiple related, or occasionally unrelated, abnormal clones. The abnormal clones may evolve acquiring additional abnormalities with disease progression, and typically resolve with remission of disease following treatment. In published series, most MDS patients die of bone marrow failure, close to half progress to acute leukemia, and a few die of unrelated intercurrent illness (Olney, 2009a).

The natural history of MDS is generally characterized by one of three clinical scenarios: (1) a gradual worsening of pancytopenia where the marrow blast count is found to be increasing (the karyotype typically remains stable); (2) a relatively stable clinical course followed by an abrupt change with a clear leukemic transformation, typically with a change in the karyotype with the gain of secondary clones, and complex karyotypes; or (3) a stable course over many years without significant change in the marrow blast counts when reevaluated, and a stable karyotype (Hamblin and Oscier, 1987). Few series with sequential cytogenetic studies have been published, and most series are small with short follow-up periods (Horiike *et al.*, 1988; Geddes *et al.*, 1990; de Souza Fernandez *et al.*, 2000).

3.1. Cytogenetic analysis

Cytogenetic analysis should be requested for any patient with a suspected or confirmed MDS. The identification of recurring chromosomal abnormalities can aid in the diagnosis of the disorder, represent independent predictors of response to therapy and outcome and can be used as a biological marker to monitor the response to therapy or to detect residual disease in follow-up specimens. In the future, cytogenetic results may be used to select risk-adapted therapies (Olney, 2009b).

The International System for Human Cytogenetic Nomenclature (ISCN) has served as the central reference for describing the human chromosome complement since 1960 (Denver Conference 1960), and is used to describe

abnormalities in a consistent manner (Mitelman, 1995). Using this system, abnormalities can be described using abbreviated terms for the rearrangement, followed by a numerical description of the chromosome(s), chromosome arm(s), and bands involved (ISCN 2009).

3.2. Cytogenetic findings in MDS

3.2.1. Normal karyotype

A normal karyotype is found in 30-60% of patients with MDS (Catenacci and Schiller, 2005). This group of patients is almost certainly genetically heterogeneous, where technical factors precluded the detection of chromosomally abnormal cells or where leukemogenic alterations occurred at the molecular level and were not detectable with standard cytogenetic methods. Nonetheless, despite this heterogeneity, these cases are a standard reference for comparison of outcomes. The International MDS Risk Assessment Workshop found that patients with a normal karyotype fall within the favorable risk group. The median survival for these patients is 3.8 years, and the time to progression to AML of 25% of this cohort was 5.6 years (Greenberg *et al.,* 1997).

3.2.2. Loss of chromosome Y

The clinical and biological significance of the loss of the Y chromosome, –Y, is unknown. Loss of the Y chromosome has not only been observed in a number of malignant diseases, but has also been reported to be a phenomenon associated with aging (Pierre and Hoagland, 1972). The UKCCG undertook a comprehensive analysis of this abnormality in both normal and neoplastic bone marrows (UKCCG, 1992). A –Y could be identified in 7.7% of patients without evidence of a malignant hematologic disease, and in 10.7% of patients with MDS; thus, the presence of a –Y alone was not reliable in documenting a malignant process. Analysis of a large series of 215 male patients found that patients with a hematological disease had a significantly higher percentage of cells with a –Y (52% vs. 37%; p = 0.036) (Wiktor *et al.,* 2000). In this series, the presence of –Y in

>75% of metaphase cells accurately predicted a malignant hematological disease. While loss of a Y chromosome may not be diagnostic of MDS, once the disease is identified by clinical and pathologic means, the International MDS Risk Analysis Workshop found that −Y as the sole cytogenetic abnormality conferred a favorable outcome (Greenberg *et al.,* 1997).

3.2.3. Loss of chromosome 5 or del(5q)

In MDS or AML arising de novo, loss of a whole chromosome 5, or a deletion of its long arm, −5/del(5q), is observed in 10% to 20% of patients, whereas it is identified in 40% of patients with t-MDS/t-AML (Vallespi *et al.,* 1998). A significant occupational exposure to potential carcinogens is present in many patients with AML or MDS de novo and either −5/del(5q) or a −7/del(7q) (discussed below), suggesting that abnormalities of chromosome 5 or 7 may be a marker of mutagen-induced hematological malignant diseases (West *et al.,* 2000).

In primary MDS, abnormalities of chromosome 5 are observed in the 5q− syndrome or, more commonly, in RAEB-1, 2, as part of a complex karyotype. Clinically, the patients with del(5q) coupled with other cytogenetic abnormalities have a poor prognosis, with early progression to leukemia, resistance to treatment, and short survival (Olney, 2009b). Abnormalities of 5q are associated with previous exposure to standard and high-dose alkylating agent therapy, including those used in immunosuppressive regimens (Larson *et al*., 1996; Aul *et al*., 1998a; McCarthy *et al*., 1998; Pedersen-Bjergaard *et al*., 2000).

MDS with an isolated del(5q) (5q− syndrome) represents a distinct clinical syndrome characterized by a del(5q) as the sole karyotypic abnormality (Boultwood *et al.,* 1994; Van den Berghe and Michaux, 1997). Unlike the male predominance in MDS in general, the 5q− syndrome has an overrepresentation of females (2:1) (Olney, 2009b). The initial laboratory findings are usually a macrocytic anemia with a normal or elevated platelet count. The diagnosis is usually RA (in two-thirds), or RAEB (in one-third); some investigators exclude patients with excess blasts from the definition of 5q− syndrome. On bone marrow examination, abnormalities in the megakaryocytic lineage (particularly micromegakaryocytes) are prominent. These patients have a favorable outcome, in fact the best of any MDS subgroup, with low rates of leukemic transformation, and a relatively long survival of several years duration (Boultwood *et al.,* 1994;

Greenberg *et al.,* 1997). The loss of a single copy of the *RPS14* gene may be involved in the pathogenesis of this syndrome (Ebert *et al.,* 2008).

3.2.4. Loss of chromosome 7 or del(7q)

A −7/del(7q) is observed as the sole abnormality in ~5% of adult patients with de novo MDS (Toyama *et al.*, 1993a; Sole *et al.*, 2000), but in ~50% of children with de novo MDS (Kardos *et al.,* 2003) and in ~55% of patients with t-MDS (Vallespi *et al.,* 1998). It can occur in three clinical settings: (1) de novo MDS and AML, (2) myeloid leukemia associated with constitutional predisposition, and (3) t-MDS/t-AML. The similar clinical and biological features of the myeloid disorders associated with −7/del(7q) suggest that the same gene(s) is altered in each of these contexts. The IPSS considers the −7/del(7q) to be a poor prognostic cytogenetic finding (Table 2) (Greenberg *et al.,* 1997). It is characterized by a preponderance of males (~4:1), hepatosplenomegaly, leukocytosis, thrombocytopenia, and a poor prognosis. Juvenile myelomonocytic leukemia, is a myelodysplastic/myeloproliferative (MDS/MPD) disease in the WHO classification, and shares many features with this entity; −7 is observed either at diagnosis or as a new cytogenetic finding associated with disease acceleration (Luna-Fineman *et al.,* 1995). As with −5/del(5q), occupational or environmental exposure to mutagens including chemotherapy, radiotherapy, benzene exposure, and smoking, as well as severe AA (regularly treated with immunosuppressive agents alone) have been associated with −7/del(7q) (Bjork *et al.,* 2000).

3.2.5. Trisomy of chromosome 8

The incidence of a gain of chromosome 8 in MDS is ~10% (Olney, 2009b). This abnormality is observed in all MDS subgroups, varying in frequency with age, gender, and prior treatment with cytotoxic agents or radiation (Greenberg *et al.*, 1997; Vallespi *et al.*, 1998; List *et al.*, 2006). The prognostic significance of the gain of chromosome 8 in MDS patients is not fully characterized. This abnormality is often associated with other recurring abnormalities known to have prognostic significance, for example, −5/del(5q) or −7/del(7q), and may also be seen isolated as sole abnormality unrelated to the

primary clone in up to 5% of cases (Greenberg *et al.,* 2000). The presence of cryptic abnormalities at other sites within the genome in association with +8 has also been described in some cases using molecular methods (Paulsson *et al.,* 2006b), which may explain the variability in the clinical course reported in patients with trisomy 8. The International MDS Risk Analysis Workshop ranked this abnormality in the intermediate-risk group (Greenberg *et al.,* 1997), and this ranking remains unchanged with the newly proposed time-dependent score of the WPSS (Malcovati *et al.,* 2007). In univariate analysis, one large confirmatory study found that +8 as a sole abnormality had a worse behavior than expected for an intermediate IPSS risk group, which was also the case in a large retrospective study (Sole *et al*., 2000; Haase *et al*., 2007). This latter study found that the prognosis improved with one additional abnormality, but worsened with more than one additional abnormality.

3.2.6. The 17p− syndrome

Loss of the short arm of chromosome 17 (17p−) has been reported in up to 10% of patients with MDS. This loss can result from various abnormalities, including simple deletions, unbalanced translocations, dicentric rearrangements (particularly with chromosome 5), or less often, −17 or isochromosome formation (Johansson *et al.,* 1993). The dic(5;17)(q11.1-13;p11.1-13) is a frequent recurring rearrangement (Lai *et al.,* 1995; Wang *et al.,* 1997), and approximately one-third of these patients have t-MDS (Merlat *et al.,* 1999). Morphologically, the 17p− syndrome is associated with a characteristic form of dysgranulopoiesis combining pseudo-Pelger-Hüet hypolobulation and the presence of small granules in granulocytes. Clinically, the disease is aggressive with resistance to treatment and short survival. The *TP53* (p53) gene, an important tumor suppressor gene that functions in the cellular response to DNA damage, is located at 17p13.1. In these cases, one allele of *TP53* is typically lost as a result of the abnormality of 17p; an inactivating mutation in the second allele on the remaining, morphologically normal chromosome 17 occurs in ~70% of cases (Lai *et al.,* 1995; Wang *et al.,* 1997).

3.2.7. del(20q)

A deletion of the long arm of chromosome 20, del(20q), is a common recurring abnormality in malignant myeloid disorders, and is seen in approximately 5% of MDS and 7% of t-MDS cases (Vallespi *et al.,* 1998). Clinical features characterizing MDS patients with isolated del(20q) include low-risk disease (usually RA), low rate of progression to AML, and prolonged survival (median of 45 months vs. 28 months for other MDS patients) (Wattel *et al.,* 1993). Morphologically, the presence of a del(20q) is associated with prominent dysplasia in the erythroid and megakaryocytic lineages (Kurtin *et al.,* 1996). The International MDS Risk Analysis Workshop noted that patients with a del(20q) observed in association with a complex karyotype identified a poor-risk group with a median survival for the entire poor-risk group of 9.6 months, whereas the prognosis for patients with an isolated del(20q) was favorable (Greenberg *et al.,* 1997).

3.2.8. Complex karyotypes

Complex karyotypes are variably defined, but generally involve the presence of ≥3 chromosomal abnormalities within one cell clone (ISCN 2009). Between 10 and 20% of all patients with primary MDS have complex karyotypes (Silver *et al.,* 2004). The majority of cases with complex karyotypes have unbalanced chromosomal abnormalities leading to the loss of genetic material, and abnormalities involving chromosomes 5, 7, or both are identified in most cases (Greenberg *et al.,* 1997). Complex karyotypes are observed in ~20% of patients with primary MDS and in as many as 90% of patients with t-MDS, and carry a poor prognosis (Malcovati *et al.,* 2007).

3.2.9. Recurring translocations

The first specific translocation identified in human neoplasia was t(9;22)(q34;q11) resulting in the Philadelphia chromosome (Ph) (Rowley, 1973). After that, recurrent balanced rearrangements, most often translocations, have been detected in almost every tumor type (Mitelman, 2006). Many of these

balanced abnormalities are — with remarkable specificity — associated with distinct tumor types and clinical features (Harrison and Foroni, 2002; Mrozek *et al.*, 2004; Mitelman *et al.*, 2005), as well as with characteristic global gene expression profiles (Andersson *et al.,* 2005). This information has become an increasingly important tool in the management of cancer patients, helping to establish a correct diagnosis, select the appropriate treatment and predict outcome (Mitelman *et al.,* 2007).

To date, more than 600 gene fusions created by an acquired chromosome change are known, and about 30% of human cancer cases contain a fusion gene (Mitelman, 2010). Most balanced structural rearrangements characterized at the molecular level have been found to exert their action through one of two alternative mechanisms. Either deregulation occurs, usually resulting in the overexpression of a seemingly normal gene in one of the breakpoints, or the creation of a hybrid, chimeric gene occurs through the fusion of parts of two genes, one in each breakpoint (Mitelman *et al.,* 2007). There is overwhelming evidence that these gene rearrangements represent important and early steps in the initiation of carcinogenesis. First, they are usually closely correlated with specific tumor phenotypes (Mrozek *et al.*, 2004; Mitelman *et al.*, 2005). Second, it has been shown, mainly in hematological malignancies, that successful treatment is paralleled by a decrease or eradication of the disease-associated chimera (Oehler and Radich, 2006). Analysis of gene partners in balanced translocations can be very informative, however, unlike AML, recurrent chromosomal translocations in MDS are rare (Look, 2005; Mitani, 2009). Among the first chromosomal translocations described in MDS patients is t(3;21)(q26;q22), involving the *MDS1-EVI1* locus on chromosome 3q (Poppe *et al.,* 2006; Vardiman, 2010). The *MDS1-EVI1* gene locus is unusual in that it encodes two related proteins with partially opposing functions. Alternative splicing of transcripts results in either expression of the full length *MDS1-EVI1* gene product or just the EVI1 protein alone (Bordereaux *et al.,* 1990). *EVI1* can repress the function of *GATA1* and thus alter normal hematopoietic differentiation. It can also down-regulate activity of the *MDS1-EVI1* fusion protein (Soderholm *et al.,* 1997). The *MDS1-EVI1* locus is one of the most common translocation partners in myeloid malignancies and presence of a 3q translocation is typically associated with a poor prognosis (Russell *et al.,* 1994). Although translocations involving this locus on 3q are typically associated with AML, they are frequent enough in MDS that

they can be used as evidence to support a diagnosis of MDS in patients with equivocal evidence of dysplasia (Bejar and Ebert, 2010).

Translocations between chromosomes 6p and 9q are also considered evidence of MDS in patients with unexplained anemia and little evidence of marrow dysplasia (Tefferi and Vardiman, 2008). The genes involved in this translocation are *DEK* (on 6p21) and *NUP214* on 9q34. *DEK* encodes a DNA binding protein and can inhibit histone acyltransferase activity and modulate transcript splicing. *NUP214* encodes a member of the nuclear pore complex. The *DEK/NUP214* fusion protein up-regulates protein synthesis in myeloid cells, but how it might contribute to leukemogenesis or dysplasia is not yet clear (Bejar and Ebert, 2010). In AML, where this translocation is more common, it is a marker of poor prognosis (Oancea *et al.,* 2010). Other genes that are involved in rarer translocations include *RUNX1*, *TEL*, *MEL1*, *NUP98*, and *IER3* (Raza-Egilmez *et al.*, 1998; Xinh *et al.*, 2003; Lahortiga *et al.*, 2004; Steensma *et al.*, 2009).

The t(11;16)(q23;p13.3) occurs primarily in t-MDS, but rare cases have presented as t-AML. The t(11;16) is unique among MLL translocations in that most patients have t-MDS. The MLL gene on chromosome 11 is fused with the CREBBP (CREB binding protein) gene on chromosome 16 (Rowley *et al.,* 1997).

The t(5;12)(q32;p13) is observed in ~1% of patients with CMML. The translocation creates a fusion gene, and the encoded fusion protein contains the 5' portion of TEL gene on chromosome 12 (also known as ETV6) and the 3' portion of PDGFRB, encoding the beta chain of the platelet-derived growth factor receptor (PDGFRB) on chromosome 5 (Golub *et al.,* 1994).

The majority of translocations in MDS occur in the setting of a more widely disturbed cytogenetic profile, making it unclear if these represent disease modifying changes or incidental rearrangements in a highly unstable genome (Bejar and Ebert, 2010). The prognostic implications of these rare translocations are not considered independently in common clinical prognostic scoring systems such as the IPSS. These lesions are lumped into the intermediate-risk group when they occur in isolation or with another single abnormality besides -7/7q-.

4. GENETIC PATHWAYS AND MOLECULAR MODELS FOR CHROMOSOME ABNORMALITIES IN MDS

Although MDS has been recognized as an important disease for more than 50 years, its molecular pathogenesis and the molecular basis for its development and progression to AML remain unclear. Despite all the chromosomal abnormalities that have been detected in MDS, most of the genes involved have not yet been identified, and it is not known with certainty whether these genetic aberrations are initial events leading to the development of MDS or are secondary events (Tormo *et al.,* 2010). As described earlier, many of the recurring chromosomal abnormalities in MDS lead to the loss of genetic material. The genetic consequences may be a reduction in the level of one or more critical gene products (haploinsufficiency), or complete loss of function (Olney, 2009b). The latter model, widely known as the "Knudson two-hit model", predicts that loss of function of both alleles of the target gene would occur: in one instance, through a detectable chromosomal loss or deletion, and in the other, as a result of a subtle inactivating mutation or another mechanism, such as transcriptional silencing (Knudson, 1971). A clinical example that may illustrate this principle is t-MDS/t-AML. The relatively long latency period between the time of exposure and t-MDS/t-AML with abnormalities of chromosomes 5 or 7 (~5 years) is compatible with a two-step mechanism, in which two mutations of a target gene must occur in a hematopoietic stem/progenitor cell. These patients may have two normal alleles initially, one of which is mutated as a result of therapy. Subsequent stochastic loss of the other allele in a bone marrow stem cell would then contribute to leukemogenesis (Nakao *et al.,* 2004). Alternatively, because t-AML develops in only 5% to 10% of patients who are treated for a primary tumor, these individuals may have inherited a predisposing mutant allele; subsequent mutagenic exposure may then induce the second mutation, eventually giving rise to leukemia when additional cooperating mutations arise. In these cases, characterization of the predisposing mutations will be important in identifying individuals who are at risk of developing t-AML, which might aid in the selection of the appropriate therapy for the primary malignant disease (Olney, 2009a).

In an alternative model, loss of only a single copy of a gene may result in a reduction in the level of one or more critical gene products (haploinsufficiency). There is growing evidence that a number of leukemia-related genes are haploinsufficient, for example, *TP53*, *SPI1/PU.1*, and *RUNX1* (Venkatachalam *et*

al., 1998). In humans, haploinsufficiency of the *RUNX1* gene results in a familial platelet disorder with a predisposition to AML (Michaud *et al.,* 2002). Support for the existence of this mechanism was provided by the subsequent identification of point mutations in the *RUNX1* gene in sporadic cases of MDS and AML (Nakao *et al.,* 2004).

To manifest clinically, an MDS clone must pass several biologic milestones (Figure 6) (Chao *et al.,* 2008). In addition, each step in the process can be achieved by alterations in one of several genes, providing a multitude of molecular routes to disease development (Bejar *et al.,* 2011). Thus, an important aspect of leukemia biology is the elucidation of the spectrum of chromosomal abnormalities and molecular mutations that cooperate in the pathways leading to leukemogenesis, which may lead to the identification of therapeutic targets and facilitate the development of targeted therapies (Pedersen-Bjergaard *et al.,* 2002a). Gilliland and colleagues have described an emerging paradigm in AML, namely, the cooperation between constitutively activated tyrosine kinase molecules, such as *FLT3*, and aberrant transcription factor fusion proteins (Kelly and Gilliland, 2002). In this model, the activated tyrosine kinase (Class I mutation) confers a proliferative and/or anti-apoptotic activity, whereas the fusion protein (Class II mutation) impairs normal differentiation pathways, but has a limited effect on cellular proliferation (Pedersen-Bjergaard *et al.,* 2002a). In other words, Class I mutations (*NRAS* or *JAK2* mutations) drive proliferation and Class II mutations (transcription factors like *RUNX1*) impair differentiation (Virtaneva *et al.,* 2001).

Although our understanding of the association of chromosomal abnormalities with gene mutations in MDS is incomplete, several patterns of cooperating mutations have emerged, suggesting that there are multiple genetic pathways leading to MDS (Olney, 2009a). A variety of experimental evidence suggests that the recurring chromosomal abnormalities in MDS and AML are likely to be the initiating event. With respect to the recurring translocations, the rearrangement seems to occur in a hematopoietic progenitor cell or, in some cases, in a committed myeloid progenitor cell. Leukemogenesis may entail a linear process in which the initiating mutation leads to a specific pattern of stepwise events: In the first stage, multiple abnormal events have been linked to intrinsic increases in apoptotic response and the presence of inflammation that converts myeloid progenitors into abnormal progenitors in the bone marrow. The apoptotic response might be modulated by many distinct environmental insults,

such as altered T-cell activation, inflammatory microenvironment, stromal cell defects, abnormal ribosomal processing, and turnover of progenitors. In the second stage, myeloid progenitor clones with abnormalities in early-regulatory genes (such as *NRas* and *RUNX1* mutations) would lead to acquired survival benefit for some myeloid progenitor cells. A greater survival and proliferative advantage and/or loss of natural killer (NK) immunosurveillance effector mechanisms would allow these abnormal cells to escape and undergo expansion. In the third stage, DNA repair abnormalities (error-prone DNA repair, loss of genomic stability, proliferative expansion) would convert MDS into AML (Figure 6) (Tormo *et al.,* 2010).

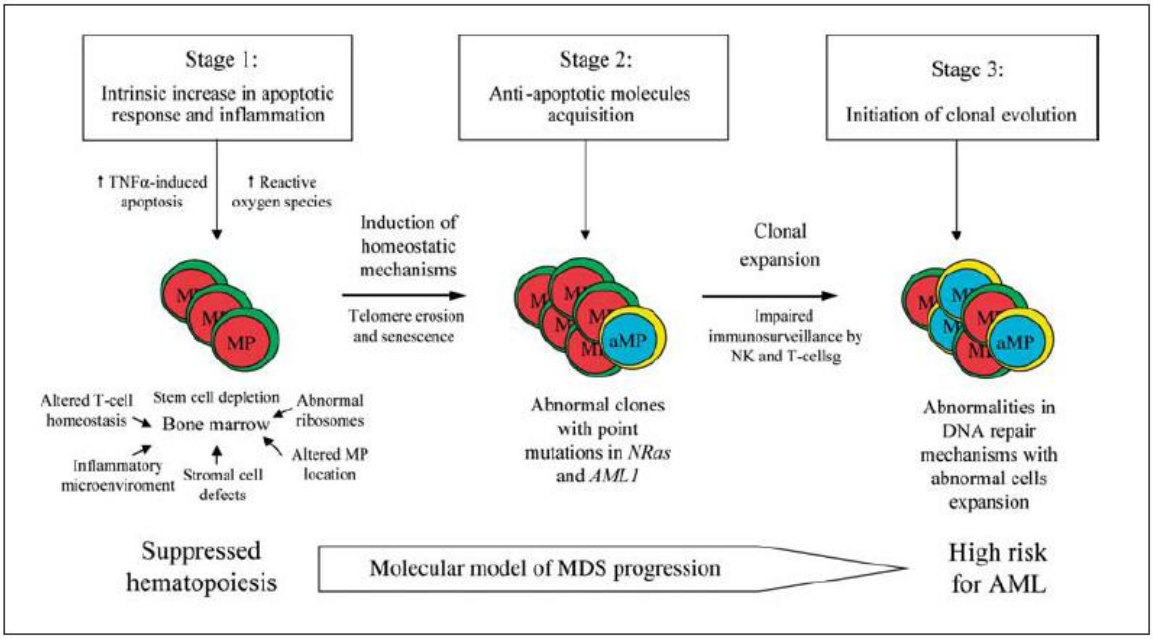

Figure 6: Molecular model of MDS progression (Tormo *et al.* 2010).

OBJECTIVES

The aim of this study was to characterize the novel translocation t(10;21)(q21;q22) in a case of MDS, including:

- To perform FISH analysis with available commercial probes targeting the regions of interest.

- To map specific genomic regions with Bacterial Artificial Chromosome (BAC) generated probes used in a FISH-based positional cloning strategy, in order to define the breakpoints.

- To uncover the genes involved in the translocation with a combination of molecular genetic methodologies, including Qualitative Reverse-Transcription Polymerase Chain-Reaction (RT-PCR), cDNA Panhandle Polymerase Chain-Reaction (cDNA panhandle PCR) and Quantitative Real-Time Polymerase Chain-Reaction (qRT-PCR).

MATERIAL AND METHODS

1. PATIENT CLINICAL HISTORY

A patient from the Portuguese Oncology Institute – Porto (IPO-Porto) with cytogenetic evidence of a novel chromosome translocation was studied in order to characterize an unknown rearrangement at the molecular level.

A 65-year-old male, with previous history of diabetes mellitus (DM), insulin-treated but poorly controlled, presented with fatigue, lack of energy, shortness of breath and dyspnea. The peripheral blood values were: hemoglobin 6.5 g/dl, hematocrit 0.211 L/L, MCV 114.7 fL, no leukocytosis and thrombocytopenia. Giving these findings, the patient was diagnosed with MDS, AR variant, with an IPSS of 1 and transfusion support dependency every two weeks.

Approximately 1 year after diagnosis, due to worsening of the hematologic conditions, the patient was transferred to IPO-Porto. The peripheral blood values were: hemoglobin 5.9 g/dl, hematocrit 0.176 L/L, MCV 87.6 fL and myeloid lineage dysplasia. Bone marrow biopsy revealed a hypercellular marrow with marked dysplasia of myeloid lineage, 1.15% of blasts and karyotype abnormalities (see below), suggesting disease progression. Due to progressive worsening of the values of anemia with increased transfusion requirements, a reevaluation of the bone marrow showed eosinophilia (27% eosinophils), absence of lymphoid precursors and erythroid series. Cytogenetic changes detected previously remained.

Alo-BMT was performed 5 months later and the reassessment of the bone marrow 1 month after the transplant showed minimal residual disease less than 1%. The patient remained hospitalized for 4 months due to digestive graft versus host disease (GVHD) and returned 1 month after discharge due to worsening of general condition. The patient died after progression to septic shock with multi-organ failure.

2. CYTOGENETIC STUDIES

2.1. Conventional cytogenetic analysis

The diagnostic bone marrow sample was cultured with two different methods: 24 hours unstimulated culture and synchronized cell culture with methotrexate (MTX), both in RPMI 1640 medium with GlutaMAX-I (Invitrogen, London, UK) supplemented with 20% foetal bovine serum (Invitrogen, London, UK).

Both cultures were processed according to standard methods and chromosome preparations were stained with trypsin-Leishman. Karyotypes were described according to the International System for Human Cytogenetic Nomenclature (ISCN, 2009).

2.2. Molecular cytogenetic analysis

Fluorescence *in situ* hybridization (FISH) analysis for *RUNX1* gene rearrangement was performed using Vysis LSI AML1/ETO Dual Color, Dual Fusion Translocation Probe (Abbott Molecular/Vysis, Des Plaines, IL, USA) and AML Breakapart (Cytocell, Cambridge, UK). Together with these two probes, CEP 10 Spectrum Orange (Abbott Molecular/Vysis) probe was used in order to confirm the co-localization of the *RUNX1* signal. Sample processing and hybridization were performed according to the manufacturer's instructions.

Bacterial artificial chromosome (BAC) analysis targeting the 10q21~22 region was performed with clones RP11-318J23, RP11-296K5, RP11-315J10, RP11-367H22, RP11-910N24, RP11-867F19, RP11-979F3, RP11-338J10, RP11-260C17, RP11-135E4, RP11-1133N9, RP11-960P24, RP11-152M14, RP11-960F15, RP11-104F15, RP11-155B22, CTD-3205J21 and CTD-2233C19.

The clones were selected according to their physical and genetic mapping data on chromosome 10 as reported by the UCSC Human Genome Browser, and obtained from the BACPAC Resources Center, California, USA.

All clones were grown in selective media. DNA was extracted using the Plasmid DNA Purification Kit (Machereynagel GmbH KG, Duren, Germany) and

amplified using the GenomiPhi V2 DNA amplification kit (WGA kit, GE, Healthcare, UK) according to the manufacturer's instructions. After a 5-min pretreatment at 90ºC, BAC DNA was labeled with SpectrumGreen or SpectrumRed (Abbott Molecular/Vysis) conjugated nucleotides in nick translation reactions using the same protocol as described for comparative genomic hybridization (CGH) (Ribeiro *et al.,* 2006, Vieira *et al.,* 2010). About 700 ng of each labeled BAC probe was then mixed with 30 µg unlabeled Cot-1 DNA (Life Technologies, Rockville, MD), ethanol precipitated, dried, and dissolved in hybridization buffer (Abbott Molecular/Vysis). Sample processing and hybridization were performed as described previously (Ribeiro *et al.*, 2006; Ribeiro *et al.*, 2007). Slides were counterstained with 4,6-diamidino-2-phenylindole (DAPI) in an antifade solution (Vector Laboratories, Burlingame, CA). Single-color fluorescent images corresponding to DAPI, SpectrumGreen and SpectrumOrange were sequentially captured with a Cohu 4900 CCD camera, with the use of an automated filter wheel coupled to a Zeiss Axioplan fluorescence microscope (Zeiss, Oberkochen, Germany) and a CytoVision system version 3.9 (Applied Imaging, Santa Clara, CA, USA). Adequate mapping and probe specificity of all BAC clones was confirmed by hybridization onto normal human metaphases.

3. MOLECULAR STUDIES

3.1. RNA extraction and cDNA synthesis

Cell pellets from bone marrow nucleated cells were obtained in two moments (at diagnosis and 3 months after diagnosis), and kept at -80ºC until RNA and/or DNA extraction was performed.

Total RNA was extracted from the bone marrow samples using 1 ml of Tripure isolation reagent (Roche Diagnostics, Indianapolis, USA) and quantified in a NanoDrop ND-100 spectrophotometer (NanoDrop Technologies, Wilmington, USA).

For cDNA synthesis, 1 µg of total RNA was subjected to reverse transcription with random hexamers using the Superscript III First-Strand Synthesis System for RT-PCR (Invitrogen), according to the manufacturer's instructions. The final cDNA was diluted with 30 µl of H_2O.

3.2. Qualitative Reverse-Transcription Polymerase Chain-Reaction (RT-PCR)

Five genes located on chromosome 10: 69,990,386 – 70,231,879 were completely analysed by RT-PCR. Forward primers for *RUNX1* (exons 1, 3, 4, 5, 6 and 7) were derived from the published sequence of *RUNX1* mRNA with GenBank accession no. NM_001754. Reverse primers for *RUFY2* (exons 2, 3, 5, 8, 10, 12, 14, 16 and 18), *ATOH7* (exon 1), *HNRNPH3* (exons 2, 3, 5, 7 and 9), *PBLD* (exons 1, 3, 4, 6, 7 and 9) and *DNA2* (exons 1, 3, 4, 6, 8, 10, 12, 14, 15, 17, 19 and 21) were derived from their published sequences with GenBank accession numbers NM_017987, NM_145178, NM_012207, NM_022129 and NM_001080449, respectively. Sequences of the primers used are listed in Table 4. Primers were designed with Primer Express 2.0 (Applied BioSystems, Foster City, USA) and purchased from Metabion (Metabion, Martinsried, Germany).

PCR reactions were performed in a 50 μl reaction volume containing 2 μl of synthesized cDNA, 5 μl of 10x GeneAmp PCR buffer II (100 mM Tris-HCl pH 8.3, 500 mM KCl) (Applied Biosystems, Foster City, USA), 5 μl of 25 mM MgCl2, 0.4 μl dNTP mix (25 mM each dNTP) (Applied Biosystems), 0.4 mM of each primer (Metabion), and 1 unit of AmpliTaq Gold DNA Polymerase (Applied Biosystems). Reaction tubes were kept on ice at all times to prevent non-specific amplification and incubated for 5 min. at 94ºC, followed by 35 cycles of 30 sec. at 95ºC, 1 min. at 63ºC, and 1.5 min. at 72ºC, followed by a final elongation of 10 min. at 72ºC on a GeneAmp PCR System 9700 (Applied BioSystems). Amplified products were analysed on a 2% agarose gel (SeaKem LE Agarose, Rockland, USA) and the results were visualised in an image analyser ImageMaster VDS (Amersham Biosciences, Little Chalfont, UK).

Table 4: Sequence of the primers used for RNA analyses.

Primer	*Target*	*Sequence*
RUNX1-E1S	*RUNX1* exon 1	5'-ATGGCTTCAGACAGCATATTTGAG-3'
RUNX1-E3S-O	*RUNX1* exon 3	5'-CAACTTCCTCTGCTCCGTGC-3'
RUNX1-E3S-In	*RUNX1* exon 3	5'-GACCCTGCCCATCGCTTT-3'
RUNX1-E4S-O	*RUNX1* exon 4	5'-TCACTGTGATGGCTGGCAAT-3'
RUNX1-E4S-In	*RUNX1* exon 4	5'-GCCATGAAGAACCAGGTTGC-3'
RUNX1-E5S-O	*RUNX1* exon 5	5'-ACCGCAAGTCGCCACCTA-3'
RUNX1-E5S-In	*RUNX1* exon 5	5'-AATCACAGTGGATGGGCCC-3'
RUNX1-E6S-O	*RUNX1* exon 6	5'-AGATGATCAGACCAAGCCCG-3'
RUNX1-E6S-In	*RUNX1* exon 6	5'-TCCCTGAACCACTCCACTGC-3'
RUNX1-E7S-O	*RUNX1* exon 7	5'-CCCACCGTGGTCCTACGAT-3'
RUNX1-E7S-In	*RUNX1* exon 7	5'-ATTGCCTCTCCTTCTGTGCAC-3'
RUFY2-E2AS	*RUFY2* exon 2	5'-AGACCGTGTTTCAGGCAATGT-3'
RUFY2-E3AS	*RUFY2* exon 3	5'-GACCAGGTAGATCCCGGACAC-3'
RUFY2-E5AS	*RUFY2* exon 5	5'-ACTTGTGAGTCTAAATCCTCTCCCTT-3'
RUFY2-E8AS	*RUFY2* exon 8	5'-GTATTTGACTTTTCTAATGAATCAACTCTTG-3'
RUFY2-E10AS	*RUFY2* exon 10	5'-TTCATTGTACATTTCATCTAGCCCC-3'
RUFY2-E12AS	*RUFY2* exon 12	5'-ATGGCTGCAGTAATTTTATTGGTTT-3'
RUFY2-E14AS	*RUFY2* exon 14	5'-TGCCTCCATTCCTTCTCAATCT-3'
RUFY2-E16AS	*RUFY2* exon 16	5'-TCTTTTATGTCTTCAATTTTAAGTTTTGATTC-3'
RUFY2-E18AS	*RUFY2* exon 18	5'-TGAGCAGTGCATGACAGGAATC-3'
ATOH7-E1AS1	*ATOH7* exon 1	5'-TTTATCCTGGCCCACTGG-3'
ATOH7-E1AS2	*ATOH7* exon 1	5'-GCCATGATGTAGCTCAGGGC-3'
HNRNPH3-E2AS	*HNRNPH3* exon 2	5'-CCTTGTGTTTCCCCAGAGCAT-3'
HNRNPH3-E3AS	*HNRNPH3* exon 3	5'-GGTCTATCATATGGTCCCGGTC-3'
HNRNPH3-E5AS	*HNRNPH3* exon 5	5'-ACCTGAACTTGCATCACCAGCT-3'
HNRNPH3-E7AS	*HNRNPH3* exon 7	5'-CCGCCTCCAGGAGTAGAATTC-3'
HNRNPH3-E9AS	*HNRNPH3* exon 9	5'-GCCTTGCCCATAGTAACCTCC-3'
PBLD-E1AS	*PBLD* exon 1	5'-GGCAAACAGCAGCAGGATTC-3'
PBLD-E3AS-In	*PBLD* exon 3	5'-CCATCTCAGTCCAAAGCAGGA-3'
PBLD-E3AS-O	*PBLD* exon 3	5'-CAGAAGCCAGGGTGGCAT-3'

PBLD-E4AS-In	*PBLD* exon 4	5'-AGTGACAAACGTGAGCGTGC-3'
PBLD-E4AS-O	*PBLD* exon 4	5'-AAGTCCAGGACGATGCCATC-3'
PBLD-E6AS-In	*PBLD* exon 6	5'-ACAGATGTCCTGGACCAGTGTG-3'
PBLD-E6AS-O	*PBLD* exon 6	5'-ACGAGGAGCTTTTGGGTATCTG-3'
PBLD-E7AS-In	*PBLD* exon 7	5'-GAATAAGCCCTTTCACCTTCCC-3'
PBLD-E7AS-O	*PBLD* exon 7	5'-CCCACCAGGCTCTCCTTTAAG-3'
PBLD-E9AS-In	*PBLD* exon 9	5'-CGAAGGGAAATTCCCAGCTC-3'
PBLD-E9AS-O	*PBLD* exon 9	5'-GCACCTCCTCTAATGTCAACCCT-3'
DNA2-E1AS-In	*DNA2* exon 1	5'-CACCTGAGCAGCAGGGCT-3'
DNA2-E1AS-O	*DNA2* exon 1	5'-CAGTTCGTTCAGCTGCTCCAT-3'
DNA2-E3AS	*DNA2* exon 3	5'-AGTGTCAGATGTGCAGTCTCCCT-3'
DNA2-E4AS	*DNA2* exon 4	5'-GAAACACCTCATGGAGAACCG-3'
DNA2-E6AS	*DNA2* exon 6	5'-TCACGACTTCAATGTTACATGTTGA-3'
DNA2-E8AS	*DNA2* exon 8	5'-GCTAATACGGTGAAACAATGAGAATG-3'
DNA2-E10AS	*DNA2* exon 10	5'-AGGTTTCCAATGCAACTGCC-3'
DNA2-E12AS	*DNA2* exon 12	5'-TTGCATCATGTGGAAGAACAGAA-3'
DNA2-E14AS	*DNA2* exon 14	5'-AAAAGAATATTGTCAACAGCAGC-3'
DNA2-E15AS	*DNA2* exon 15	5'-GGTCCCCCACTAACACAAATCTC-3'
DNA2-E17AS	*DNA2* exon 17	5'-AGGATTGTTGGGTTCAAATACTCC-3'
DNA2-E19AS	*DNA2* exon 19	5'-TTTAATTGCTGCCTGTACGGTG-3'
DNA2-E21AS	*DNA2* exon 21	5'-TTATGTTTGGCTCTGGTTATAGCAAC-3'

3.3. cDNA Panhandle Polymerase Chain-Reaction (cDNA Panhandle PCR)

A modified cDNA panhandle PCR approach, previously described by Megonigal *et al.* (2000), was used to detect the unknown *RUNX1* fusion partner. Briefly, as shown in Figure 7, first-strand cDNA was synthesized using *RUNX1*-specific random hexamer oligonucleotides at the 3' ends (Table 5) primers RH1-RH3). The random hexamers anneal to and prime from many complementary sequences in total RNA and produces first strand cDNAs of different sizes.

The initial PCR required 2.5 U Taq/Tgo DNA polymerase mix (Roche Molecular Biochemicals, Mannheim, Germany), 350 µM 1x Expand Long template buffer I, 12 pmols of primer 1 (Table 2, primers 1A–1C), and H2O, to a final volume of 50 µL. The mixture was preheated to 80ºC for 5 min., and then 2 µl of cDNA was added to the respective tubes. Primer 1 extension was achieved by denaturing at 94ºC for 1 min. followed by one cycle at 94ºC for 10 sec. and 68ºC for 7 min. After reheating to 80ºC for 5 min., 12.5 pmols of RUNX1 primer 2 (Table 2, primers 2A–2C) were added and further cycling performed at 94ºC for 1 min., 94ºC for 10 sec., and 68ºC for 7 min. (10 cycles), 94ºC for 10 sec. and at 68ºC for 7 min. with extend segment at 20 sec. for every cycle (20 cycles), and final extension at 68ºC for 7 min.

The second round nested PCR contained 2.5 U Taq/Tgo DNA polymerase mix (Roche), 350 µM 1x Expand Long template buffer I, RUNX1 primers 3 and 4 (12.5 pmols each) (Table 5) primers 3A–3C, 4A–4C), and H_2O. The mixture was preheated to 80ºC for 5 min., and then 1 µl from initial round of PCR was added, followed by thermal cycling at 94ºC for 1 min.; 94ºC for 10 sec. and 68ºC for 7 min. (10 cycles); 94ºC for 10 sec. and at 68ºC for 7 min. with extend segment at 20 sec. for every cycle (20 cycles); and final extension at 68ºC for 7 min.

The cDNA panhandle PCR products of various sizes were subcloned into a Topo TA cloning vector (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions and sequenced.

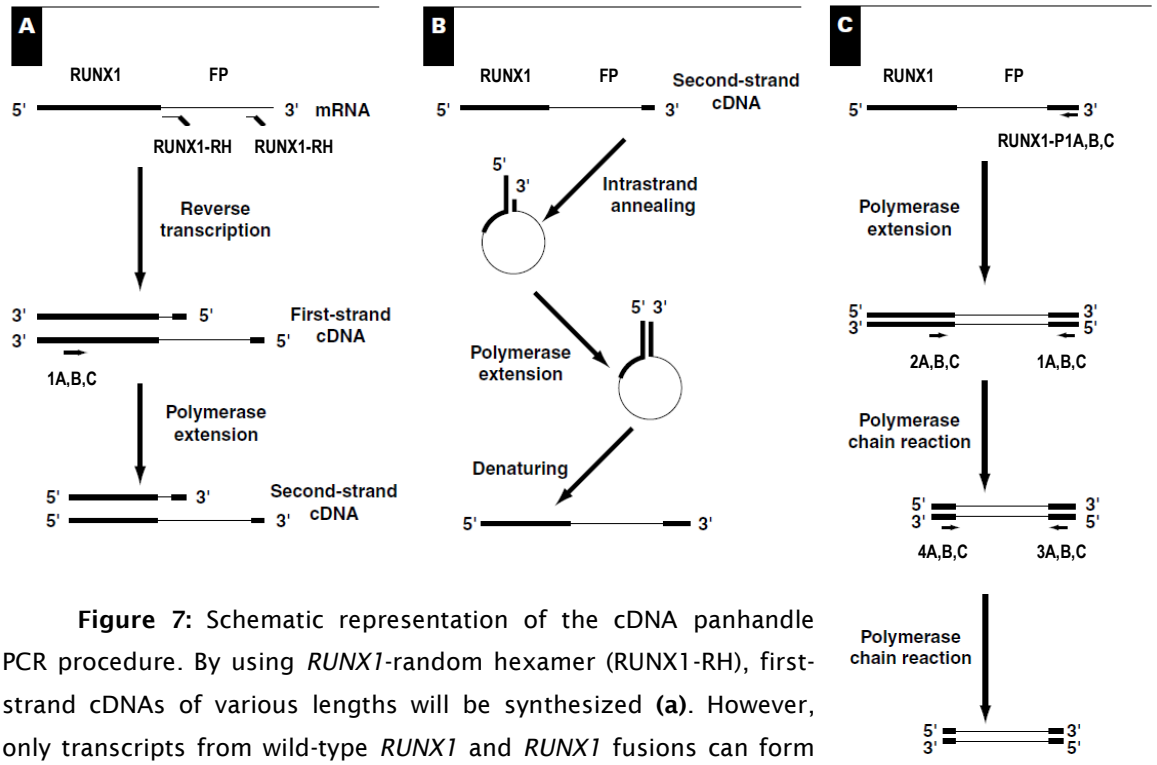

Figure 7: Schematic representation of the cDNA panhandle PCR procedure. By using *RUNX1*-random hexamer (RUNX1-RH), first-strand cDNAs of various lengths will be synthesized **(a)**. However, only transcripts from wild-type *RUNX1* and *RUNX1* fusions can form the panhandle structure **(b)**. Extension of the 3' region of the panhandle structure creates a new *RUNX1* primer 1 binding site, which ensures the successful amplification of *RUNX1* or *RUNX1* break region by the PCR technique **(b)**. Thick lines, *RUNX1* gene; thin lines, *RUNX1* unknown fusion partner gene; FP, fusion partner; 1A~4C, *RUNX1* primer 1A~*RUNX1* primer 4C (adapted from Fu *et al.* 2007).

Table 5: Sequence of the primers used for cDNA Panhandle PCR analyses.

Primer	***Target***	***Sequence***
RH1	*RUNX1* exon 3	5'-CGACCGCAGCATGGTGGAGGTGCTGGCCGACCNNNNNN-3'
RH2	*RUNX1* exon 4	5'-CTACCGCAGCCATGAAGAACCAGGTTGCAAGANNNNNN-3'
RH3	*RUNX1* exon 6	5'-CGCACAGCCATGAGGGTCAGCCCACACCACCCNNNNNN-3'
1A	*RUNX1* exon 2-3	5'-TCCTTCTAGAGACGTCCACGATGC-3'
1B	*RUNX1* exon 4	5'-GTGGCCCTAGGGGATGTTCCAG-3'
1C	*RUNX1* exon 6	5'-AGCCCGGGAGCTTGTCCTTTTC-3'
2A	*RUNX1* exon 3	5'-CAACTTCCTCTGCTCCGTGCTG-3'
2B	*RUNX1* exon 5	5'-TGTCTTCACAAACCCACCGCAA-3'
2C	*RUNX1* exon 6	5'-CTCGTGCCTCCCTGAACCACTC-3'
3A	*RUNX1* exon 3	5'-TGAGCCCAGGCAAGATGAGCGA-3'

3B	*RUNX1* exon 5	5'-ACTCTGGTCACTGTGATGGCTGGCAA-3'
3C	*RUNX1* exon 6	5'-CGAGCGGCTCAGTGAACTGGAG-3'
4A	*RUNX1* exon 3	5'-CCTACGCACTGGCGCTGCAACA-3'
4B	*RUNX1* exon 5	5'-GTCGCCACCTACCACAGAGCCAT-3'
4C	*RUNX1* exon 6	5'-CACTGCCTTTAACCCTCAGCCT-3'

3.4. Sequencing analysis

Sequence analysis was directly performed on the cDNA panhandle PCR products by use of the BigDye Terminator Cycle Sequencing Chemistry (Applied Biosystems) on an automated sequencer ABI Prism 310 Genetic Analyser (Applied BioSystems) according to the manufacturer's instructions. When multiple bands were observed, gel band extraction and purification was performed with the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare), again according to the manufacturer's instructions.

3.5. RUNX1 exon-level expression quantification by Quantitative Real-Time Polymerase Chain-Reaction (qRT-PCR)

We have evaluated the mRNA expression of *RUNX1* exons 2, 5 and 7 by qRT-PCR on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Primers and probes for *RUNX1* were derived from the published mRNA sequence of *RUNX1* (GenBank accession no. NM_001754), designed with Primer Express 2.0 (Applied BioSystems) and purchased from Metabion (Metabion). Primers and probes for the *ABL1* gene (GenBank accession no. NM_005157), used as endogenous control, were previously described and approved for qRT-PCR based diagnosis and minimal residual disease detection in leukemic patients, due to be similarly expressed in diagnostic samples as well as in normal samples (Gabert *et al.* 2003).

Beyond our case, we studied 3 cases of diagnostic bone marrow samples with a *RUNX1-RUNX1T1* rearrangement and, as a control group, 3 cases with a *BCR-ABL1* rearrangement. All primers and probes were designed outside of *RUNX1* most probable breakpoint regions in exons 2-3, exons 5-6 and exons 6-7. To determine the relative expression levels of the target exons in each sample, the relative amount of the target exon was calibrated to the relative amount of the internal reference gene and expressed in terms of ratios between the target and the reference that were then multiplied by 100 for easier tabulation (target exon/ABL1 x 100). PCR reactions were performed in a 25 μl volume containing 5 μl of synthesized cDNA, 12.5 μl of TaqMan universal PCR master mix (Applied BioSystems), 0.3 μM of each primer and 0.2 μM of each probe. PCR was performed in separate wells for each primer/probe set and each sample was run in triplicate. PCR parameters were as follows: 50 °C for 2 min., 95 ºC for 10 min., followed by 50 cycles at 95 °C for 15 sec. and 60 °C for 1 min.

Each plate included non-template controls and serial dilutions of a strongly expressing sample (*RUNX1*) to construct the standard curve. Relative expression levels of the target transcripts were calculated using the comparative CT method (Schmittgen *et al.*, 2008).

RESULTS

1. CYTOGENETIC STUDIES

1.1. Conventional cytogenetic analysis

Identification of chromosome translocation t(10;21)(q22;q22) in the patient's bone marrow cells.

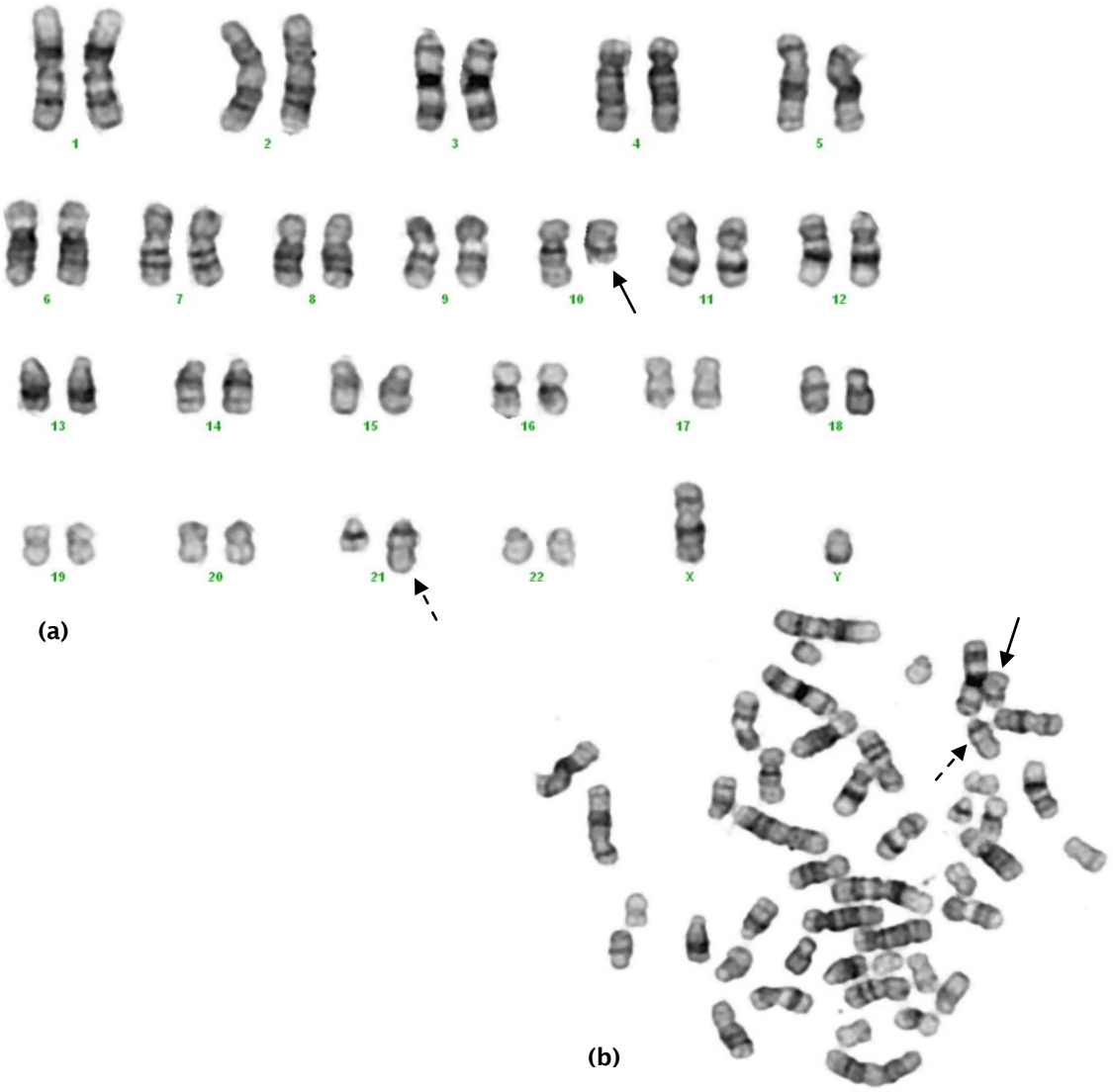

Figure 8: Conventional cytogenetic analysis. **(a)** A representative G-banded karyotype of the patient's bone marrow (BM) cells, described as 46,XY,t(10;21)(q22;q22)[20], showing the derivative chromosome 10 [der(10)] and derivative chromosome 21 [der(21)]. **(b)** Metaphase plate from the same cell. Solid arrows: der(10); dashed arrows: der(21).

Of 20 metaphases analyzed by GTL-banding, all were clonally abnormal and had 46 chromosomes with an apparently reciprocal translocation involving the long arm of chromosome 10 and the long arm of chromosome 21. These cells had the karyotype: 46,XY,t(10;21)(q22;q22)[20] (Figure 8a,b).

The 21q22 breakpoint suggested a possible involvement of the *RUNX1* locus. The partial karyogram and schematic diagrams of normal and derivative chromosome 21 is shown in Figure 9.

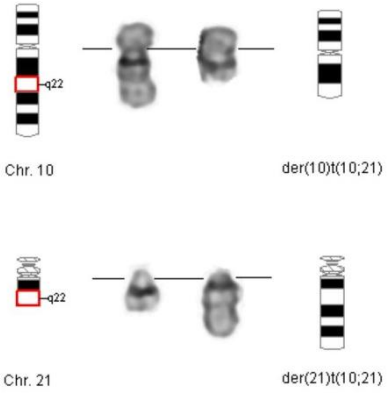

Figure 9: Partial karyogram and schematic diagrams of normal (left) and derivative (right) chromosomes 10 and 21 from metaphases with the 46,XY,t(10;21)(q22;q22)[20] karyotype. Red squares indicate the most probable breakpoint regions, presumably affecting the *RUNX1* gene on 21q22.

1.2. Molecular cytogenetic analysis

Confirmation of involvement of the RUNX1 gene in the translocation t(10;21)(q22;q22).

Since translocations involving chromosomal band 21q22 are frequently identified in leukemia patients and most of them are associated with a rearrangement of the *RUNX1* gene, FISH analyses using Vysis LSI AML1/ETO Dual-color, Dual-fusion Translocation Probe (Abbott Molecular/Vysis) and AML1 Breakapart probe (Cytocell) was performed.

Interphase FISH analysis using the AML1/ETO translocation probe showed that one of the *RUNX1* signals (green) was split and metaphase FISH analysis showed one normal *RUNX1* signal (green) on the intact chromosome 21 and two

small green signals, one probably on the derivative chromosome 21 and other probably on the derivative chromosome 10 (Figure 10a).

FISH with the breakapart probe for *RUNX1* also showed an abnormal pattern: one co-localized green and red signal on the normal chromosome 21 that represents an intact *RUNX1,* and one green signal that could be localized either on the derivative chromosome 10 or on the derivative chromosome 21. This doubt comes from the similar size of the two derivative chromosomes, as can be seen in Figure 9. There is also loss of the red signal, which means that there is a deletion, which may include part of 3' *RUNX1*, around the breakpoint region (Figure 10b).

To confirm the localization of the isolated green signal, a centromeric probe for chromosome 10 was used simultaneously with *RUNX1* breakapart probe. As can be seen in Figure 10c, there is one co-localized green and red signal that corresponds to an intact *RUNX1*, as well as one green signal corresponding to 5' *RUNX1* present in the same chromosome as the red signal from the centromeric probe for chromosome 10. This confirms that the translocation between chromosomes 10 and 21 moves the 5' *RUNX1* region from the latter to the former.

Because different commercial probes for the same gene have different designs and can provide different information, the LSI ETV6(TEL)/RUNX1(AML1) ES Dual Color Translocation Probe (Abbott Molecular/Vysis) was also tested. The approximately 500 kb *RUNX1* probe spans the entire gene, and in a normal nucleus the expected pattern would be the two red (*RUNX1*), and two green (*ETV6*) signal pattern. As can be seen in Figure 10d, there is a normal signal pattern, which is somewhat contradictory to the results obtained with previous probes.

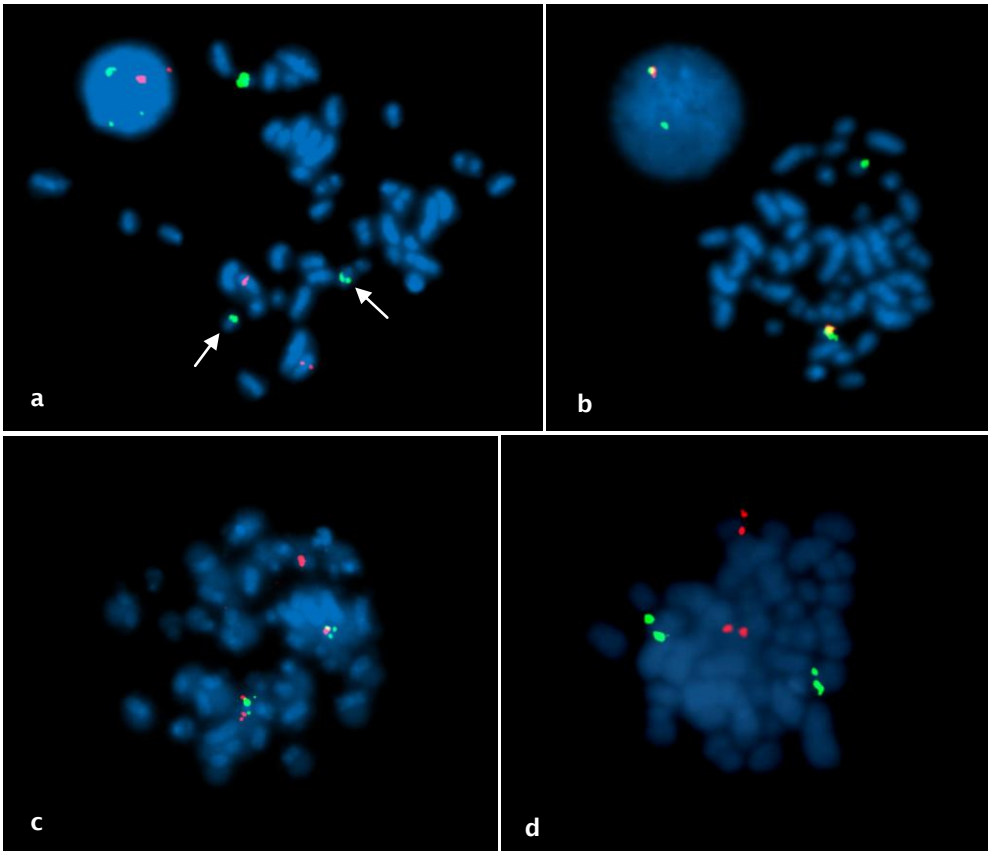

Figure 10: Metaphase and interphase FISH. **(a)** FISH using LSI AML1/ETO dual-color, dual-fusion translocation probe (Abbott Molecular/Vysis) showed two normal red (*RUNX1T1*) and one normal green (*RUNX1*) signals on normal chromosomes 8 and 21, respectively, as well as two more green signals, one probably on der(21) and the other probably on der(10) (solid arrows). **(b)** FISH using AML Breakapart probe (Cytocell). Co-localized green and red signals on the normal chromosome 21 indicate an intact *RUNX1*. The isolated green signal can be localized either on the derivative chromosome 10 or on the derivative chromosome 21 (with similar sizes). **(c)** FISH using AML Breakapart probe (Cytocell) together with LSI CEP 10 Spectrum Red (Abbott Molecular/Vysis). One co-localized green and red signals indicate an intact *RUNX1* on the normal chromosome 21 and the other represents the green signal of 5' *RUNX1* together with the red signal from the centromeric probe for chromosome 10. Single red signal indicates the normal chromosome 10. **(d)** LSI ETV6(TEL)/(RUNX1)AML1 ES Dual Color Translocation Probe (Abbott Molecular/Vysis) hybridized to a metaphase showing the two red and two green normal signal pattern.

Representative diagrams of AML1 Breakapart probe (Cytocell), LSI AML1/ETO Dual-color, Dual-fusion Translocation Probe (Abbott Molecular/Vysis) and LSI ETV6(TEL)/RUNX1(AML1) ES Dual Color Translocation Probe (Abbott Molecular/Vysis) can be seen in Figure 11.

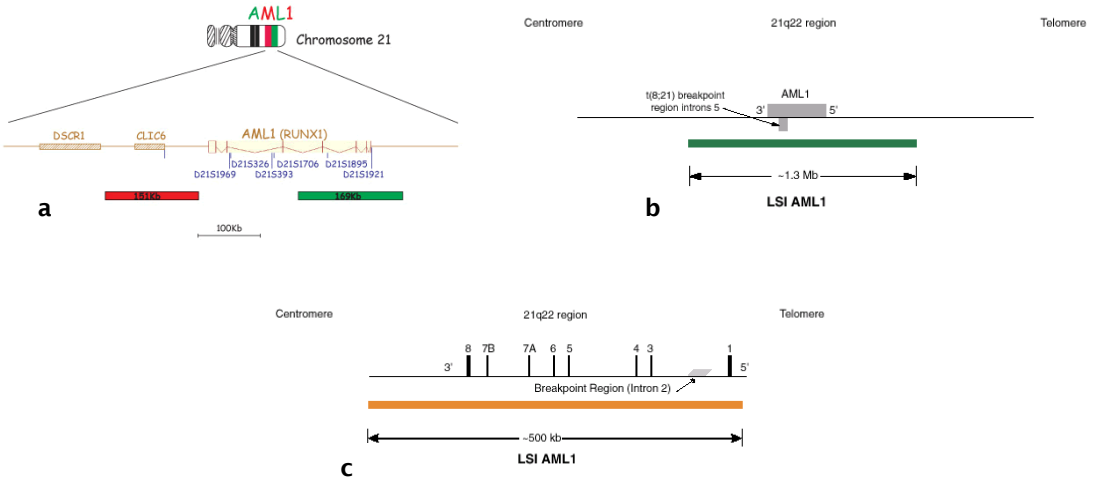

Figure 11: Representative diagrams of commercially available probes. **(a)** AML1 Breakapart probe design (Cytocell). **(b)** LSI AML1/ETO Dual-color, Dual-fusion Translocation Probe (Abbott Molecular/Vysis); only the AML1/RUNX1 locus is shown. **(c)** LSI ETV6(TEL)/(RUNX1)AML1 ES Dual Color Translocation Probe (Abbott Molecular/Vysis); only the AML1/RUNX1 locus is shown.

Characterization of the der(10) chromosomal breakpoint region (chromosome walking with BAC-FISH).

Bacterial artificial chromosome (BAC) analysis targeting the 10q21~22 region was performed with clones described in Chapter III. Since the region on der(10) chromosome thought to harbor the breakpoint comprises more than one hundred genes, BAC clones were selected, combined in pairs according to the distance between each element of the pair, and labeled with different colors (Spectrum Green or Spectrum Red) in order to obtain a "breakapart probe". A chromosome walking procedure using several locus-specific BAC clones was then followed.

The first clone pair, consisting of BACs RP11-155B22 and RP11-910N24, suggested that the breakpoint was located between these loci, as the RP11-155B22 probe, mapping to chromosome 10q21.3, yielded one hybridization

signal on the derivative chromosome 21, whilst RP11-910N24, mapping to chromosome 10q22.2, yielded one hybridization signal on the derivative chromosome 10 (Figure 12a).

Following the chromosome walking process, successive attempts were made with BACs mapped between BACs RP11-155B22 and RP11-910N24 (Figure 12a,b,c,d,e). In Figure 12e one can see the signal of probe RP11-338J10 present on both derivative chromosomes 10 and 21.

Two BACs inside the region covered by RP11-338J10 were selected (CTD-3205J21 and CTD-2233C19). Due to sample limitation, no metaphases were available to perform metaphase FISH. Hybridization results with BAC CTD-3205J21 were normal (data not shown), and interphase FISH image from BAC CTD-2233C19 can be seen in Figure 12f. A split green signal was found with the latter BAC, defining the breakpoint on der(10) to the sub-band 10q21.3 (70035644–70123355). This result allowed refinement of the karyotype description, changing the breakpoint region on der(10) from 10q22 to 10q21. The correct karyotype description became: 46,XY,t(10;21)(q21;q22)[20].

In the region covered by the BAC CTD-2233C19 there are twelve genes, the majority of which has already been characterized according to biological function, but none of them has ever been associated with fusion genes in hematological malignancies.

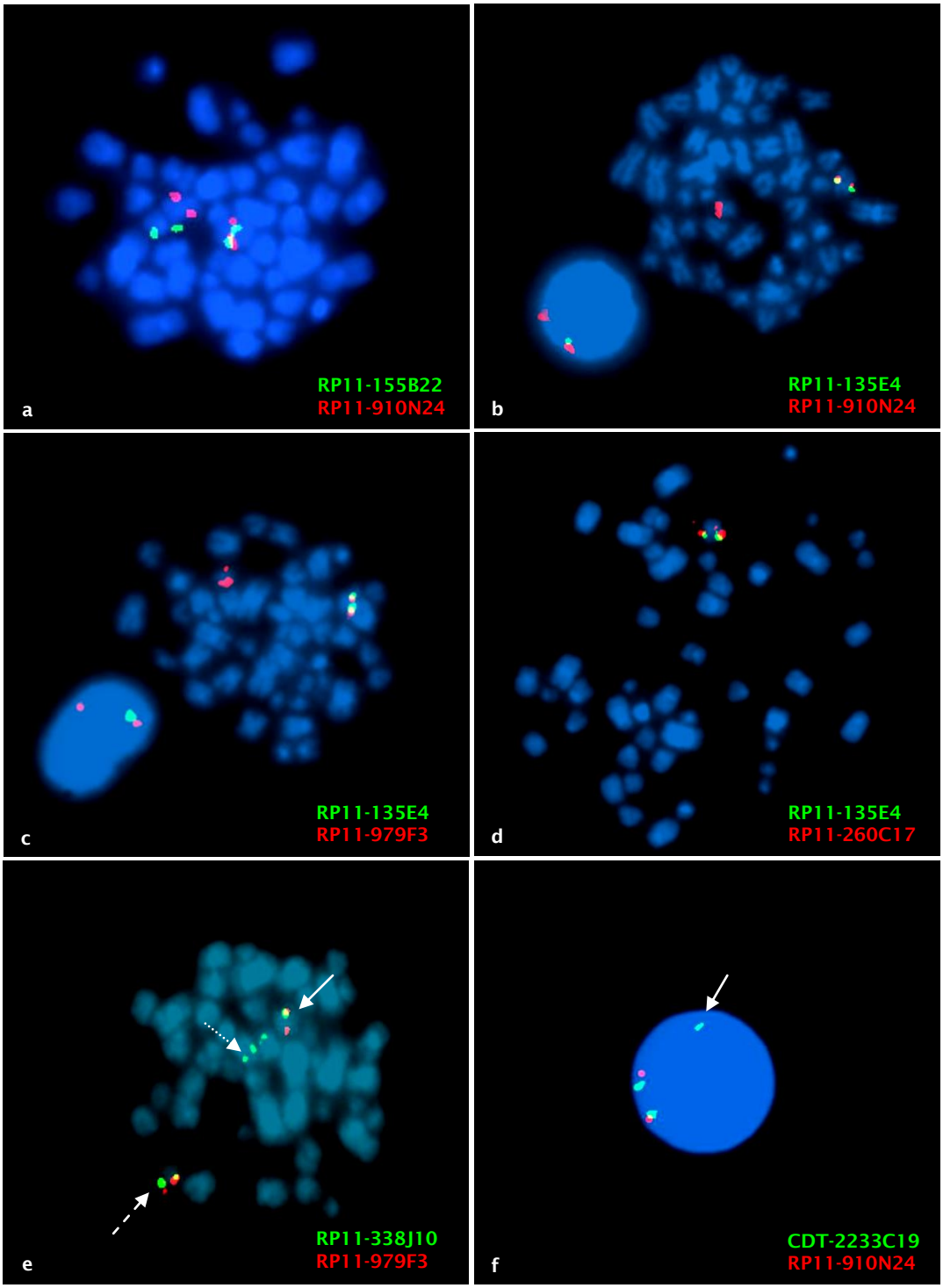

Figure 12: Metaphase and interphase FISH. **(a)** FISH on metaphasic chromosomes with RP11-155B22 labeled with Spectrum Green (telomeric) and RP11-910N24 labeled with Spectrum Red (centromeric) probes. Co-localized green and red signals indicate a normal chromosome 10. RP11-155B22 probe, mapping to chromosome 10q21.3, yielded one hybridization signal located on derivative chromosome 21, whilst RP11-910N24, mapping to chromosome 10q22.2, yielded one hybridization signal located on derivative chromosome 10, thus indicating a breakpoint between these loci. **(b)** Hybridization with BACs RP11-135E4 labeled with Spectrum Green (telomeric) and RP11-910N24 labeled with Spectrum Red (centromeric). Co-localized green and red signals indicate

a normal chromosome 10. BAC RP11-910N24, mapping to chromosome 10q22.2, yielded one hybridization signal located on derivative chromosome 10, whilst the region that comprises BAC RP11-135E4 is deleted. **(c)** Hybridization with BACs RP11-135E4 labeled with Spectrum Green (telomeric) and RP11-979F3 labeled with Spectrum Red (centromeric). Co-localized green and red signals indicate a normal chromosome 10. RP11-970F3 region is present on the derivative chromosome 10, whilst deletion of RP11-135E4 is confirmed. **(d)** Hybridization with BACs RP11-135E4 labeled on Spectrum Green (telomeric) and RP11-260C17 labeled on Spectrum Red (centromeric). Co-localized green and red signals indicate a normal chromosome 10. Both RP11-135E4 and RP11-260C17 probes were deleted, which indicates that the breakpoint region should be upstream these regions and downstream BAC RP11-979F3 region. **(e)** Hybridization with BACs RP11-338J10 labeled with Spectrum Green (telomeric) and RP11-979F3 labeled with Spectrum Red (centromeric). Two co-localized green and red signals are present, one representing a normal chromosome 10 (solid arrow) and the other the derivative 10 (dashed arrow). Localization of probe RP11-338J10 was also detected on derivative chromosome 21 (pointed arrow). **(f)** Hybridization in interphase nucleus with BACs CTD-2233C19 labeled with Spectrum Green (telomeric) and RP11-910N24 labeled with Spectrum Red (centromeric). Two co-localized green and red signals are present, one corresponding to a normal chromosome 10 and the other to the derivative 10. One green split signal (isolated signal) is visible (solid arrow), resulting from the split and localization of probe CTD-2233C19 also on derivative chromosome 21.

2. MOLECULAR STUDIES

2.1. Qualitative Reverse-Transcription Polymerase Chain-Reaction (RT-PCR)

Owing to the lack of quality of metaphases and suitable material for more BAC-FISH analyses, we decided that the better way to identify the fusion partner in the der(10) would be analysis by RT-PCR of the candidate genes located at the CTD-2233C19 locus and surrounding region. According to their closest localization to CTD-2233C19 locus, and after an *in silico* analysis, five existing genes in this region were selected: *RUFY2, ATOH7, HNRNPH3, PBLD* and *DNA2.*

However, any attempt to demonstrate the presence of specific fusion gene transcripts using RT-PCR failed.

2.2. cDNA Panhandle Polymerase Chain-Reaction (cDNA Panhandle PCR)

A cDNA panhandle PCR approach, which is usually used for cloning the fusion partners of *MLL* or *NUP98* (Megonigal *et al.*, 2000; Taketani *et al.*, 2002), was used in order to detect a hybrid transcript.

cDNA generated from total RNA resulted in panhandle products of various sizes, and recombination PCR yielded several subclones that were selected for further sequencing. However, BLAST analysis of the sequenced panhandle products revealed only wild type *RUNX1* fragments, and no gene fused with *RUNX1*.

2.3. RUNX1 exon-level expression quantification by Quantitative Real-Time Polymerase Chain-Reaction (qRT-PCR)

To determine the breakpoint localization in the *RUNX1* gene we designed specific primer pairs and probes for the junction region of *RUNX1* exons 2 and 3, 5 and 6, and 6 and 7. The results of quantitative RT-PCR for *RUNX1* exon-level

expression revealed that the individual exon expression values between our case, cases with the *RUNX1-RUNX1T1* rearrangement, and normal controls did not show significant differences. However, the ratio average between all the exon-level expression evaluates for each case, from the normal controls and from the cases with the *RUNX1-RUNX1T1* rearrangement show great differences: in the normal controls the ratio exon-level expression average was 0.99 and in the cases with the *RUNX1-RUNX1T1* rearrangement was 2.41. In our case, the ratio was 2.54, which is closer to the value observed in the cases with the *RUNX1-RUNX1T1* rearrangement.

DISCUSSION

It is now well established that cancers, including leukemia, develop through the accumulation of genetic and epigenetic alterations that act in concert to confer malignant phenotypes (Niebuhr *et al*. 2008). However, many of the genes and signal pathways targeted in this process have not yet been identified.

Translocations and their corresponding gene fusions have an important role in the initial steps of tumorigenesis (Mitelman *et al*. 2007), usually being usually closely correlated with specific tumor phenotypes. In this way, the identification of balanced translocations and the resulting fusion genes is of great importance, since the involved breakpoints can point to the location of cancer-relevant genes. These translocations, which are mainly balanced, have been found to exert their action through one of two alternative mechanisms. Either deregulation of gene expression occurs, usually resulting in overexpression of a seemingly normal gene in one of the breakpoints, or a hybrid, chimeric gene, is formed through the fusion of parts of two genes, one in each breakpoint (Mitelman *et al.,* 2007).

The role of RUNX1 in leukemogenesis

Balanced chromosome aberrations involving 21q22 are commonly observed in hematopoietic disorders, mostly involving the *RUNX1* gene. The *RUNX1* gene (aliases *AML1, CBFA2* and *PEBP2aB*) is located in chromosome 21q22.3 and belongs to a family of three evolutionarily conserved genes (*RUNX1, RUNX2* and *RUNX3*) that code for transcription factors involved in cell lineage differentiation during development (De Braekeleer *et al.,* 2011). This gene is crucial for the establishment of definitive hematopoiesis and the generation of hematopoietic stem cells in the embryo (Okuda *et al*., 1998; North *et al*., 2002), as well as for differentiation of myeloid progenitor cells to granulocytes (Tanaka *et al.,* 1995).

Members of this family share a 128 amino acid region of high sequence homology, called “Runt domain”, first identified in the Drosophila runt gene (Golling *et al*. 1996). In addition to this “Runt homology domain” (RHD), there is a 5 amino acid sequence, VWRPY, that is 100% conserved at the C-terminal end

(Levanon and Groner, 2004). The Runt domain is responsible for heterodimerization of the RUNX1 protein with the core-binding factor β (*CBFβ* or *PEBP2β*) originating a transcription factor and for DNA binding (Ito, 2008).

The *RUNX1* gene spans 260 kb and consists of 12 exons with two distinct promoters, each followed by the initiation codon ATG. The protein is composed of 453 amino acids and contains several regions of major importance: the RHDs coded by exons 3–5 and located in the N-terminal part (amino acids 50–177), the transcription activation domain (TAD; amino acids 243–371) coded by part of exon 7 and exon 8, the transcription inhibition domain (amino acids 372–411) and the VWRPY sequence (Tanaka *et al.*, 1996; Zeng *et al.*, 1997; Yamaguchi *et al.*, 2004).

RUNX1 acts as a key regulator of hematopoiesis, activating or repressing transcription, depending on the context in which it binds DNA. This context is determined by both the cell type and the combination of different transcription factors, coactivators, corepressors and binding sites within a particular target gene regulatory region (Canon and Banerjee, 2003; Stein *et al.,* 2004). Some genes regulated by *RUNX1* included: growth factors (*GM-CSF*, *MPO* and *IL-3*), surface receptors (*TCRA, TCRB, M-CSF* receptor and *FLT3*), signaling molecules (*CDKN1A, BLK* and *BCL2*) and transcription activators (*STAT3* and *MYB*) (Ito, 2004; De Braekeleer *et al.*, 2011). In addition, *RUNX1* functions as an organizing protein that facilitates assembly of transcriptional activation or repression complexes. By recruitment of non-DNA binding proteins such as p300/CBP and histone acetyltransferase (HAT), it contributes to the activation of transcription of target genes (De Braekeleer *et al.,* 2011). Upon recruitment of non-DNA binding repressors such as mSin3A, Groucho/TLE and histone deacetylase (HDAC), it represses transcription of target genes (Speck and Gilliland, 2002; Kurokawa and Hirai, 2003; Zelent *et al.*, 2004).

The *RUNX1* gene presents a challenge to the simple binary classification of cancer genes as oncogene or tumor suppressor gene: an oncogenic role was revealed by retroviral insertional mutagenesis showing that *RUNX* genes are frequent targets for proviral insertion in murine leukemia virus (*MLV*) induced T cell tumors in CD2-MYC transgenic mice (Wotton *et al.,* 2002), by the finding of *RUNX1* amplification in some cases of childhood acute lymphoblastic leukemia (ALL), being possible that increased dosage of *RUNX1* in cases of trisomy 21 is also causally related to Down's syndrome-related acute megakaryoblastic leukemia (Roumier *et al.,* 2003), and by the involvement of *RUNX1* in oncogenic

fusion genes. On the other hand, the *RUNX1* gene can also act as tumor suppressor, as it can be disrupted by translocation events, affected by hemizygous deletion, and silenced by hypermethylation (Nimmo and Woollard, 2008).

A number of experimental results support the hypothesis that *RUNX1* translocations or mutations are initiating events in hematopoietic malignancies, establishing a preleukemic clone, but requiring additional, secondary events for leukemia development (Niebuhr *et al.,* 2008). Supporting evidence comes from patient studies, including data showing the prolonged and variable latency of ALL induction in identical twin pairs, which share the initiating clone carrying the t(12;21) from birth (Ford *et al.,* 1998; Wiemels *et al.,* 1999), and studies linking *RUNX1* mutations to hereditary familial platelet disorder (FPD) - AML syndrome (Song *et al.,* 1999). Strikingly, the *RUNX1/CBFβ* fusion proteins seems to be strong determinants of the leukemia phenotype (Niebuhr *et al.,* 2008). Indeed, several studies have clearly demonstrated that the t(8;21) and inv(16) are present in an HSC/P compartment (Schwieger *et al*., 2002; McHale *et al*., 2003; Morrow *et al*., 2004), thus supporting the importance of the fusion protein itself in determining the leukemia phenotype. The case for the t(12;21) associated with pre B-cell ALL is less clear. Contradictory evidence as to whether the translocation occurs in the HSC or in cells committed to the B-cell lineage have been reported (Schwieger *et al.,* 2002). The inability to detect the translocation in earlier cells or cells of different lineages may reflect the strong expansion pressure that the *ETV6-RUNX1* fusion protein has on the B-cell compartment, coupled with the fact that its expression is incompatible with differentiation along specific lineages (Niebuhr *et al.,* 2008).

Fusion genes involving *RUNX1* can be classified into five categories according to their structure and, as a consequence, to their function: in-frame fusion genes retaining the RHD but not the TAD of *RUNX1* [ex. t(8;21)(q22;q22), *RUNX1-RUNX1T1* (Miyoshi *et al.,* 1993)]; truncated fusion genes retaining part of the RHD but not the TAD of *RUNX1* [ex. t(1;21)(q21;q22), *RUNX1-ZNF687* (Nguyen *et al.,* 2006)]; truncated fusion genes retaining the RHD but not the TAD of *RUNX1* [ex. t(1;21)(p36;q22), *RUNX1-PRDM16* (Hromas *et al.,* 2000)]; fusion genes not retaining the RHD of *RUNX1* [(ex. inv(21)(q21q22), *USP16-RUNX1* (Gelsi-Boyer *et al.,* 2008)]; and in-frame fusion genes retaining the RHD and the TAD of *RUNX1* [(ex. t(12;21)(p13;q22), *ETV6-RUNX1* (Golub *et al.,* 1995)]. In addition, the same translocation can lead to different fusion products acting through different

mechanisms. Indeed, alternate splicing transcripts have been reported in many *RUNX1* fusion genes, leading to both in-frame and out-of-frame fusion products (De Braekeleer *et al.,* 2011).

Regarding translocations involving the *RUNX1* gene, some have been shown to be frequent while others have only been reported in a few or single cases. At present, 55 partner chromosome bands have been described in translocations with *RUNX1* gene, but the partner gene has only been identified at the molecular level in 21different rearrangements (De Braekeleer *et al.,* 2011). The most frequent translocations involving *RUNX1* are: t(1;21)(p36;q22), resulting in the *RUNX1–PRDM16* fusion gene (Hromas *et al.,* 2000); t(3;21)(q26;q22), where three genes (*RPL22L1, MECOM* and *EVI1*) have been shown to fuse with *RUNX1* (Nucifora *et al.*, 1994; Nucifora, 1997); t(8;21)(q22;q22), involving the *RUNX1T1* gene and found in 30% of the patients with AML-M2 subtype (Miyoshi *et al.*, 1993); t(12;21)(p13;q22), being the most common structural chromosomal abnormality in pediatric acute lymphoblastic leukemia, fusing the 5´ region of *ETV6* with almost the entire coding region of *RUNX1* (Romana *et al.,* 1995); and t(16;21)(q24;q22) that results in the formation of a chimeric gene involving the *RUNX1* and *CBFA2T3* genes (De Braekeleer *et al.,* 2008).

In the rare 33 translocations described, the *RUNX1* gene was shown to be disrupted, presumably leading to the fusion of *RUNX1* with an as yet unidentified gene partner. Whether these translocations involve an already known partner gene, a different gene, or don't even lead to a fusion gene, remains to be determined (De Braekeleer *et al.,* 2011). In this way, the main question raised by the detection of a novel translocation involving the *RUNX1* gene is to know whether it results in a fusion with a gene located in the translocation partner chromosome, which can lead, or not, to truncation of the RUNX1 gene. The t(1;21)(p35–36.1;q22) translocation, described in a 68 year-old male with AML by Nguyen *et al.,* leads to the formation of a *RUNX1–YTHDF2* (YTH domain family member 2) transcript that includes the RHD, but not the TAD of *RUNX1*(Nguyen *et al.,* 2006) and the fusion protein has a truncated *RUNX1* protein without translating *YTHDF2*. Likewise, the t(4;21)(q28;q22) translocation fuses the 5´ region of the *RUNX1* gene (breakpoint in intron 6) to a 27 codon sequence of a novel gene named *FGA7* (fused gene 7 to *RUNX1*) (Mikhail *et al.,* 2004) and this rearrangement leads to a stop codon in the chimeric gene, resulting in a truncated RUNX1.

On the other hand, truncation of the *RUNX1* has been described by other mechanisms, meaning point mutations (Roumier *et al.,* 2003). Point mutations of the *RUNX1* gene have been detected in about 10–20% patients classified as MDS/AML and have been identified at a substantially higher frequency among therapy-related MDS/AML cases (Harada *et al.,* 2004; Steensma *et al.,* 2005; Zharlyganova *et al.,* 2008). The *RUNX1* mutations are distributed throughout of the full length of the protein, and they can be roughly divided into four types according to the structures of the functional domains: (a) truncated mutations in N-terminal region; (b) inframe mutations in N-terminal runt domain; (c) short truncated mutations in C-terminal region; and (d) chimera-like mutations which are frameshift mutations in C-terminal region resulting in the formation of bigger proteins than wild-type *RUNX1* (Harada and Harada, 2009). Usually, frameshift mutations, mainly the ones of the C-terminal, result in truncation of the authentic protein followed by a relatively short additional stretch of amino acid residues originating from the wrong reading frame or from intronic sequences (Harada and Harada, 2009).

The target gene(s) of the t(10;21)(q21;q22) in myeloid neoplasia

In this study, we describe a novel chromosomal translocation t(10;21)(q21;q22) involving the *RUNX1* gene, in an adult patient with MDS. An identical translocation to the one we present was previously reported in one patient with MDS (Rashaan *et al.,* 2007), but the translocation was only described according to the karyotype analysis and no molecular genetics investigation of this chromosomal rearrangement was described.

At the time of diagnosis, the patient presented with what seemed to be a reciprocal translocation involving the long arms of chromosomes 10 and 21. By karyotype analysis is not possible to conclude whether the rearrangement creates a fusion gene and, if it does, which is the partner gene. Conventional cytogenetic and FISH techniques are widely used for detection of cytogenetic abnormalities that can play an important role as diagnostic markers of malignant hematological diseases, and, in some instances, are independent predictors of disease progression and survival (Karauzum *et al*. 2005). However, karyotyping relies on G-band quality and resolution. In general, bone marrow samples give poorer quality chromosomes, and, allied to the fact that the number of alternating light

and dark bands detectable with G-banding varies with the quality and level of chromosomal contraction in each metaphase cell (range of 350–550 bands, with one band representing approximately 5–10 × 10^6 base pairs (bp) of DNA, containing hundreds of genes), cytogenetic analysis is not considered a high-resolution technique.

In our patient, the karyotype was initially described as 46,XY,t(10;21)(q22;q22) and FISH analysis using the Vysis LSI AML1/ETO Dual Color, Dual Fusion Translocation Probe (Abbott Molecular/Vysis) and AML Breakapart probe (Cytocell) showed disruption of the *RUNX1* gene and its localization to another chromosome, the derivative chromosome 10. FISH analysis lead us to several conclusions: (1) the telomeric 5' region of the *RUNX1* gene was translocated to chromosome 10; (2) a large region in chromosome 21, 3' upstream of *RUNX1* gene was deleted; and (3) the comparison of the results achieved with the three commercial FISH probes, which are complementary, show that no *RUNX1* sequences remain on chromosome 21. Commercially available single-copy sequence probes are very limited and expensive, but are valuable for research purposes and are an important tool in the identification of new molecular disease biomarkers. In our case, they allowed us to confirm the disruption of the *RUNX1* gene, already suspected by karyotype analysis, and to get new insights about its localization. On the other side, the use of custom locus-specific probes for interphase and metaphase FISH, namely by using BACs, allows more flexibility to characterize new genes involved in chromosome translocations. In the present case, FISH analyses performed with BAC clones located on chromosome 10q21~22 allowed us to restrict the breakpoint region. In fact, clones RP11-338J10 and CTD-2233C19, normally located in band 10q21.3, were found to be split, allowed the initial karyotype description to be revised as 46,XY,t(10;21)(q21;q22).

At least five genes map to the genomic region covered by BAC clone CTD-2233C19 but none of them have been described as a fusion partner in any chromosomal rearrangement, nor been associated with hematologic neoplasms. According to their closest localization to the region we thought to harbor the breakpoint in chromosome 10, these five genes were chosen as targets for further analyses: *RUFY2*, the second member of the *RUFY* family, contains an N-terminal RUN domain and a C-terminal FYVE domain with two coiled-coil domains in-between, and its expression seems to be relatively tissue restricted to the brain, lung, and testis (Yang *et al.,* 2002); *ATOH7*, alias *Math5*, is a *bHLH*

transcription factor required for retinal ganglion cell (RGC) and optic nerve development (Prasov *et al.,* 2010); *HNRNPH3*, a member of a subfamily of ubiquitously expressed heterogeneous nuclear ribonucleoproteins (hnRNPs), that complex with heterogeneous nuclear RNA (hnRNA) (Kim *et al.,* 2011); *PBLD*, alias *MAWDBP*, the only representative of the phenazine biosynthesis-like protein family in the human genome, that has its expression elevated in several diseases, including insulin resistance, folate deficiency and hypotension, and presumably also in carcinogenesis (Zhang *et al.,* 2007); and *DNA2*, a dual polarity exo/endonuclease, and 5' to 3' DNA helicase involved in Okazaki Fragment Processing (OFP) and Double Strand Break (DSB) Repair (Budd *et al.,* 2011).

Despite repeated efforts, any attempt to identify a specific fusion gene transcript by RT-PCR failed. RNA PCR amplification of the region encompassing the breakpoint of an unknown fusion gene can be problematic. Indeed, since the breakpoint localization is not known, several exon combinations between the two putative rearranged genes can occur, requiring appropriate selection of PCR primers that cover all possible exonic rearrangements. In our case, despite the intensive coverage of the five genes studied, no fusion transcript could be detected. Also, in this type of study, DNA analysis is not usually used due to the fact that introns can be very long, generating very large PCR products that may not be completely amplified, thus reducing the sensitivity of the analysis.

Although panhandle PCR approaches are highly effective for cloning genomic breakpoint, for instance in the identification of unknown *MLL* partner genes, a big problem remains to be solved: sometimes the genomic target sequence may be too large to amplify. In addition, if the amplicon does not contain an exonic sequence, containing only intronic sequences, this approach may not lead to the identification of the fusion partner gene (Megonigal *et al.*, 1997; Felix and Jones, 1998). As occurred with the RT-PCR approach, all the attempts to isolate and identification of the *RUNX1* partner gene by panhandle PCR were unsuccessful, since we could only detect *RUNX1* wild type sequences.

As far as we know, besides the description of Rashaan *et al.* of a t(10;21) translocation (Rashaan *et al.,* 2007), this is the first time that this translocation is described and confirmed at a molecular level, meaning the involvement of the *RUNX1* gene, in a patient with MDS. Although the clinical significance and molecular mechanism of the t(10;21) are still unclear, this chromosomal abnormality seems to be a rare translocation. The *RUNX1 is* a promiscuous gene that may be fused with different partners as a result of different chromosomal

translocations, resulting in distinct fusion genes, as discussed above. However, it appears that not all of these rearrangements result in chimeric genes, instead giving rise to a truncated *RUNX1* with an abnormal fusion (De Braekeleer *et al.,* 2011).

As we can see from the quantitative RT-PCR results, the exon expression ratio average showed differences that support the hypothesis that we are in the presence of either a *RUNX1* truncated gene or a *RUNX1* fusion gene. Most of the chimeric genes involving *RUNX1* fuse the 5´ region of the gene with the 3´ region of the partner gene (De Braekeleer *et al.,* 2011), and we have shown in our case that the 5´ part of *RUNX1* is indeed translocated to chromosome 10. Furthermore, the FISH and BAC-FISH assays disclosed the presence of deletions in the breakpoint regions of both 10 and 21 derivative chromosomes, including deletion of the 3' part of *RUNX1*. Deletions surrounding translocation breakpoints have been reported in several other leukemia-associated rearrangements, such as the t(9;22)(q34;q11) in CML (Paulsson *et al.,* 2006a). The RHD, located in the 5´ region of *RUNX1*, is retained in the majority of the fusion genes, but not the TAD situated in the 3´ part of the gene (Miyoshi *et al.,* 1993). Several translocations involving *RUNX1* can also lead to truncated fusion genes retaining the RHD but not the TAD domain of *RUNX1* (Hromas *et al.,* 2000). These truncated *RUNX1* gene products, either by loss of 3' region or by point mutations, may compete with wild-type *RUNX1* and act as dominant constitutive repressors inhibiting the function of the normal *RUNX1* in the regulation of hematopoietic differentiation (De Braekeleer *et al.,* 2011), and this is how we can relate the dominant negative effect of the fusion gene or the truncated *RUNX1* to leukemogenesis. Although each chimeric gene may have its own leukemogenic mechanism, *RUNX1* insufficiency may be a common underlying mechanism in *RUNX1* leukemias (Osato, 2004).

On the other hand, *RUNX1* mutations in the C-terminus of the gene have recently been shown to predict transformation to AML (Kuo *et al.*, 2009; Kohlmann *et al.*, 2010), and since the domains disrupted by the C-terminal mutations are critical for protein–protein interactions and for appropriate sub-nuclear targeting of *RUNX1*, these cells exhibit deregulation of *RUNX1* target genes (Vradii *et al.,* 2005). The resulting phenotype is enhanced proliferation of myeloid progenitor cells concomitant with a differentiation block, similar to that caused by chromosomal translocations that retain the RHD domain of the *RUNX1* protein, but replace the *RUNX1* C-terminus with segments from other proteins.

CONCLUSIONS

A novel chromosomal translocation t(10;21)(q21;q22) involving the *RUNX1* gene was here described by conventional cytogenetics and molecularly confirmed in an adult patient with myelodysplastic syndrome.

In agreement with already reported literature where most of the chimeric genes involving *RUNX1* fuse the 5´ region of the gene with the 3´ region of the partner gene, we have shown in our case that the 5´ part of *RUNX1* is indeed translocated to chromosome 10. Furthermore, we have demonstrated the presence of large deleted regions in the breakpoint regions of both derivative chromosomes. Part of the *RUNX1* gene, meaning the 3' region, also suffered deletion.

Since we could not find any gene fused to the 3' region of *RUNX1*, our results are compatible both with the existence of a truncated *RUNX1* gene product with an abnormal fusion that does not give rise to a chimeric gene, and with a truncated *RUNX1* without gene fusion. The truncated *RUNX1* gene product may compete with wild-type *RUNX1* and act as a dominant-negative inhibitor of the normal *RUNX1* protein, inducing malignant transformation in hematopoietic cells.

Although the clinical significance of the t(10;21) is still unclear, this chromosomal abnormality seems to be a rare translocation, being one of the rare 33 translocations described where the *RUNX1* gene was shown to be disrupted but no partner gene has yet been identified.

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