Characterization of bone marrow mesenchymal stem cells from patients with myeloid disorders using chromosomal analysis and molecular genetic techniques

Graduate Program

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Τίτλος

Χαρακτηρισμός των αρχέγονων μεσεγχυματικών κυττάρων του μυελού των οστών σε ασθενείς με μυελικές διαταραχές χρησιμοποιώντας χρωμοσωμική ανάλυση και τεχνικές μοριακής γενετικής

Μεταπτυχιακό Πρόγραμμα

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ΕΥΧΑΡΙΣΤΙΕΣ

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ΠΕΡΙΛΗΨΗ

Στο μυελό των οστών βρίσκονται τουλάχιστον δύο τύποι αρχέγονων πολυδύναμων κυττάρων: τα αρχέγονα αιμοποιητικά κύτταρα (HSCs) και τα αρχέγονα μεσεγχυματικά κύτταρα (MSCs). Αν και παραδοσιακά οι δύο αυτοί τύποι κυττάρων θεωρούνται ξεχωριστοί ως προς την προέλευση και τη δυνατότητα διαφοροποίησης, υπάρχουν ωστόσο ορισμένες έμμεσες ενδείξεις που υποστηρίζουν ότι τα HSCs και MSCs μπορούν να έλκουν την καταγωγή τους από κοινό πρόγονο.

Στόχος της παρούσας μελέτης είναι η διερεύνηση του κατά πόσο ο παθολογικός κλώνος σε ασθενείς με οξεία μυελογενή λευχαιμία (AML), μυελοδυσπλαστικά σύνδρομα (MDS) ή μυελοϋπερπλαστικά σύνδρομα (MPD) αφορά στο προαναφερθέντα κοινό πρόγονο των HSCs και MSCs. Προκειμένου να απαντηθεί το ερώτημα αυτό εκτιμήθηκε αν οι σχετιζόμενες με την μυελική κακοήθεια μεταλλάξεις και χρωμοσωμικές ανωμαλίες που ανιχνεύονται στα αιμοποιητικά κύτταρα των ασθενών απαντώνται επίσης στα MSCs αυτών.

Μελετήθηκαν 16 ασθενείς με MDS, 12 με AML και 7 με MPD, καθώς 10 υγιή άτομα αναλόγου ηλικίας και φύλου ως μάρτυρες. Απομονώθηκε DNA και RNA από το κλάσμα των μυελικών μονοπύρηνων κυττάρων και από καλλιεργηθέντα μυελικά MSCs. Με PCR σε γενωμικό DNA ελέγχθηκε η παρουσία μεταλλάξεων στο γονίδιο FLT3 και με RT-PCR οι μεταλλάξεις στο γονίδιο NPM1 σε πάσχοντες με MDS και AML. Η παρουσία της μετάλλαξης V617F/G1849T στο γονίδιο JAK2 στους πάσχοντες με MPD ελέγχθηκε με real time RT-PCR. Επιπλέον τα μεσεγχυματικά και τα αιμοποιητικά κύτταρα που συμπεριλήφθησαν στη μελέτη υποβλήθηκαν σε κυτταρογενετική ανάλυση.

Στο σύνολο των ασθενών με MDS και AML, ανιχνεύθηκαν δυο πάσχοντες από AML που έφεραν μεταλλάξεις στα μυελικά μονοπύρηνα κύτταρα: ο ένας έφερε μετάλλαξη στο γονίδιο NPM1 και ο άλλος στα γονίδια FLT3 και NPM1. Τέσσερις πάσχοντες με MPD έφεραν τη μετάλλαξη V617F του γονιδίου JAK2 στα μυελικά μονοπύρηνα κύτταρα. Εις ό,τι αφορά στα MSCs, σε κανέναν από τους 35 ασθενείς της μελέτης δεν ανιχνεύθηκε κάποια από τις ανωτέρω μεταλλάξεις. Επιπλέον, κανείς από τους μάρτυρες δεν έφερε μεταλλάξεις στα μυελικά μονοπύρηνα κύτταρα ή στα MSCs. 6/28 ασθενείς με MDS ή AML παρουσίασαν χρωμοσωμικές ανωμαλίες στα αιμοποιητικά κύτταρα, και 1/28 και στα MSCs. Τέλος δύο από τα υγιή άτομα έφεραν χρωμοσωμικές ανωμαλίες στα MSCs, ενώ τα αντίστοιχα HSCs ήταν υγιή. Αυτή η μελέτη ενισχύει την άποψη πως τα MSCs δεν φαίνεται να συμμετέχουν στον κακοήθη

κλώνο που εντοπίζεται στις αιματολογικές διαταραχές όπως είναι οι AML, MDS και MPD.

Λέξεις κλειδιά: Μεσεγχυματικά κύτταρα, Μυελοδυσπλαστικό σύνδρομο, Οξεία μυελογενής λευχαιμία, Μυελοϋπερπλαστικό σύνδρομο, FLT3, NPM1, JAK2.

ABSTRACT

In bone marrow (BM) there are at least two types of multipotent stem cells: hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs). Although in general these two cell types are distinct as regards their origin and differentiation potential, there are some indirect evidence supporting that HSCs and MSCs may have their origin from a common ancestor stem cell.

The aim of this study is to investigate whether the abnormal clone in acute myeloid leukemia (AML) -, myelodysplastic syndromes (MDS) - or myeloproliferative syndromes (MPD) – patients, derives from the aforementioned common ancestor stem cell of HSCs and MSCs. To answer this question we assessed whether the associated with myeloid malignancy mutations and chromosomal aberrations detected in hematopoietic cells of patients, were also found in patient MSCs.

We studied 16 MDS patients, 12 AML and 7 MPD, and 10 healthy age- and sex-matched individuals. DNA and RNA were isolated from the mononuclear myeloid cell fraction and cultured marrow MSCs. The presence of mutations in FLT3 gene was evaluated by genomic DNA PCR and the presence of mutations in the NPM1 gene in patients with MDS and AML was assessed by RT-PCR. The presence of mutation V617F/G1849T in JAK2 gene was detected by real time RT-PCR in patients with MPD. Moreover, MSCs and hematopoietic cells were subjected into cytogenetic analysis.

In the total of MDS and AML patients, two AML patients were identified with gene mutations in bone marrow mononuclear cells: one harbored mutations in FLT3 and NPM1 genes and the other harbored a mutation in NPM1 gene. Four MPD patients were positive for the JAK2 mutation in bone marrow mononuclear cells. In respect to MSCs, none of the 35 patients harbored any of these mutations. Furthermore none of the healthy individuals harbored mutations in marrow mononuclear cells or MSCs. In 6/28 of MDS or AML patients, chromosomal abnormalities were identified in HSCs, and 1/28 in corresponding MSCs. Finally, two of healthy subjects were identified with chromosomal abnormalities in MSCs, but the corresponding HSCs were normal. In view of these data, this study gives further confirm to the absence of clonal involvement of MSCs in hematological malignancies, especially in AML, MDS and MPD.

Keywords: Mesenchymal stem cells, myelodysplastic syndrome, acute myeloid leukemia, myeloproliferative disease, chromosomal aberration, genetic mutation, FLT3, NPM1, JAK2

INTRODUCTION

HEMATOPOIETIC AND MESENCHYMAL STEM CELLS

Hematopoiesis is the formation of blood cellular components. This process is dependent upon hematopoietic stem cells (HSCs). HSCs are ultimately the progenitors that become progressively restricted to several or single lineages. They have the ability to regenerate and can differentiate into all the blood cell types from the myeloid (monocytes and macrophages, neutrophils, basophils, eosinophils, erythrocytes, megakaryocytes/platelets, dendritic cells), and lymphoid lineages (T-cells, B-cells, NK-cells) [1]. HSCs reside in the bone marrow (BM) and comprise a small proportion (0.01%) of BM cells [2]. The CD34 surface antigen is commonly used as a marker to identify and isolate hematopoietic progenitor cells from BM, peripheral blood (PB), and umbilical cord/placenta blood [3-5]. Human HSCs are known to exhibit $CD34^{+}/Thy1^{+}/CD38^{lo/-}$/c-kit $^{-/lo}/CD105^{+}/Lin^{-}$ phenotype [5] .

HSCs depend on their microenvironment, the hematopoietic niche. In 1978 Schofield first emphasized the role of the niche which spatially organized the hematopoietic cells within the BM [6]. Niches within the BM preserve specific aspects of hematopoiesis, such as HSC survival, self-renewal, and differentiation, supporting the maintenance of the blood system under normal and stressed conditions [7]. Moreover stem cell niches are protecting the self-renewing/undifferentiated state of HSCs [8]. The various non-hemopoietic cells and extracellular macromolecules present in the hematopoietic niche are collectively referred to as stroma. Stromal cells consist of fibroblasts, macrophages, adipocytes, osteoblasts, osteoclasts, endothelial cells [9]. Stromal cells are producing many paracrine and juxtacrine growth factors, necessary for HSC self-renewal and differentiation. In addition, the stroma plays a role in homing and trafficking of HSCs to hematopoietic organs via ligand-receptor interactions [7-9]. This is of great importance for HSC-engraftment during stem cell transplantation.

Major constituents of the BM stroma are the mesenchymal stem cells or mesenchymal stromal cells (MSCs), which provide most of the cellular components of the BM haemopoietic niche. MSCs represent a rare cell population; approximately 0.001-0.01% of the BM nucleated cells, which can be readily grown in culture [10]. MSCs are also present in many other tissues such as blood, placenta, dental pulp, and adipose tissue. MSCs have the potential to differentiate into multiple phenotypes such as osteoblasts, chondrocytes, adipocytes, and myocytes [11]. In contrast to HSCs, cultured MSCs do not harbor a unique identification marker. The International

Society for Cellular Therapy (ISCT) has provided the following criteria for defining multipotent mesenchymal stromal cells: plastic-adherent fibroblast-like cells under standard culture conditions, characterized by the expression of a variety of cell surface antigens Stro-1, CD29, CD44, CD51, CD73, CD90, CD105, CD106, CD146, CD166, while lacking expression of the hematopoietic markers CD11b, CD14, CD45, CD19, CD79 and HLA-DR [11, 12].

Evidence indicates MSCs as key components of the hemopoietic niche within the BM, ensuring hematopoietic and skeletal homeostasis [13]. Many studies have shown that cultured MSCs support the maintenance of hematopoiesis providing HSCs and their progeny with signals for survival, proliferation and differentiation. MSCs produce stem cell factor (SCF), Flt-3 ligand, thrombopoietin, leukemia-inhibiting factor (LIF), interleukin (IL)-6, IL-8, IL-11, IL-12, IL-14, IL-15, granulocyte-macrophage and macrophage colony stimulating factor (GM-CSF and M-CSF respectively) [14, 15]. Except to direct cell-to-cell contact MSCs can interact with local environment through adhesion molecules such as the intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) and they secrete various extracellular matrix proteins such as fibronectin, collagen and proteoglycans [14, 15].

Apart from their ability to support hematopoiesis, MSCs also display local and systemic immunoregulatory properties, mainly by suppressing T and B-cell activation [16, 17]. Briefly, MSCs suppress the proliferation of T and B lymphocytes, promote functionally the T-regulatory cells, control the production of TNFα from dendritic cells (DC-1), increase the production of IL-10 from the DC-2, reduce the release of INFγ from the helper cells TH_1 and from natural killers and induce the IL-4 production by TH_2 cells [18, 19]. In addition they block the effective maturation of antigen-appearance cells by suppressing expression of co-stimulatory molecules CD40 and CD86 [20, 21]. It is not necessary to have direct cell contact to achieve all the above. The immunomodulatory properties of MSCs is mainly mediated by soluble factors like hepatocyte growth factor (HGF), IL10, transforming growth factor β1 (TGFβ1), indoleamine 2,3-dioxygenase (IDO), prostaglandins E2 (PGE2) and nitric oxide (NO) [20-22].

Within recent years considerable interest has been given to the existing properties of MSCs, i.e. their multilineage potential, the extensive *in vitro* proliferation, their immunosuppressive capacity and their beneficial effect upon local administration and/or systemic infusion. Based to the above data, MSCs are considered as potential candidates for cell-based therapies and this fact is supported

by both pre- and clinical data. However many issues remain open regarding the functional and phenotypic properties of native MSCs in diverse disease states.

Because BM MSCs are classically thought to provide the supportive microenvironment for hematopoiesis [13], it has been reasonable to investigate their potential implication not only in normal function of BM but also in diseases characterized by BM microenvironment defects. Abnormal hematopoiesis is considered to be the result of a multistep process implicating genetic, epigenetic and immune-mediated alterations of an early HSC resulting in excessive apoptosis or proliferation of its marrow progeny [4, 23]. HSC disorders include acute myeloid leukemia, myelodysplastic syndromes and myeloproliferative disorders.

ACUTE MYELOID LEUKEMIA

Acute myeloid leukemia (AML) is the most common leukemia in adults. The pathophysiology of AML can be explained by acquired genetic and epigenetic alterations in bone marrow stem cells that cause a complete or partial block in normal hematopoietic stem cell maturation, abnormal differentiation and uncontrollable proliferation of hematopoietic progenitor cells [24, 25]. The genetic changes may involve mutations that lead to activation of growth-promoting proto-oncogenes, inactivation of tumor suppressor genes, or alterations in transcription factors [26]. For example, mutations in the N-ras gene are common in AML (in up to 30% of AML patients) [27]. Mutations of the Fms-like tyrosine kinase 3 (FLT-3) growth factor receptor are also common, suggesting the possible role of autocrine stimulation. Mutations of the tumor suppressor gene Rb and p53 have been observed and appear to confer a worse prognosis [28, 29]. However, the greatest insight regarding AML pathogenesis has been provided by the identification of genes at cytogenetic breakpoints involved in balanced translocations. The fusion proteins generated by the translocation generally result in disruption of transcription factors believed to be critical in myeloid differentiation. Many of these chromosomal abnormalities (e.g. t(8;21), t(15;17), inv (16)) are associated with specific AML subtypes and carry prognostic importance [30]. But, the mechanisms that account for poor prognosis, when loss of all or parts of chromosomes occur, remain to be elucidated.

While the acquired genetic lesions that lead to leukemia are being rapidly defined, DNA damage from a known cause accounts for only a small fraction of patients with AML. Nonetheless, leukemias clearly occur with increased frequency after irradiation exposure, after certain types of chemotherapy, and with heavy and continuous occupational exposures to benzene. Generally there are two types of chemotherapy-related leukemias: (1) the classic alkylating agent-induced type in

which the leukemia is preceded by a myelodysplastic prodrome and is characterized by clonal abnormalities often with loss of chromosome 5 and/or 7, and (2) an epipodophyllotoxin topoisomerase II associated type with a shorter incubation period that is usually associated with myelomonocytic or monocytic differentiation and abnormalities at the 11q23 region. AML has long been appreciated as a clinically heterogeneous disease, with marked differences in survival following intensive chemotherapy based on age, blast cell morphology, and cytogenetic abnormalities. Because of that significant biological and clinical heterogeneity in the AML patients, the AML cases are classified in one of the AML subgroups (M1-M7) according to FAB classification [31].

Over 60% of the pretreated patients with *de novo* AML display clonal chromosomal aberrations [32]. In 10-20% of AML patients, the abnormal karyotype is complex, containing more than one chromosomal aberration [33]. For that cytogenetics remain the most important disease-related prognostic factor in AML, and the observed aberrations are characterized as favorable, intermediate or adverse risk [33]. For example patients with cytogenetic abnormalities as t(15;17)(q22;q12-21) have an excellent prognosis and those with t(8;21)(q22;q22) or inv(16)(p13q22)/t(16;16)(p13;q22) a relatively favorable prognosis.

The largest cytogenetic subgroup of AML patients (40-45%) appear with a normal karyotype, but do harbor specific gene mutations [34, 35]. Several studies have shown that the presence of specific gene mutations and changes in gene expression have an effect on patients' prognosis [36]. Many new important prognostic factors have been identified lately in cytogenetically normal AML (NC-AML), including gene mutations in FLT3, nucleophosmin 1 (NMP1) and CCAAT enhancer-binding protein-alpha (CEBPA).

MYELODYSPLASTIC SYNDROMES

The myelodysplastic syndromes (MDS) constitute a heterogeneous group of malignant bone marrow disorders, characterized by ineffective hematopoiesis, leading to progressive cytopenias, and increased risk of acute leukemic transformation [37, 38]. The pathogenesis is complex and involves genetic, epigenetic and immune mediated mechanisms. Some of the molecular abnormalities include alterations of the cell cycle control and apoptosis, increased DNA methylation, oncogenic aberrations and bone marrow microenvironment changes such as stromal dysregulation and medullary angiogenesis [39].

Accelerated apoptosis of hematopoietic progenitors and abnormal regulation of apoptosis in their progeny represent important mechanisms underlying the

development of MDS. Some studies have indicated that large number of hematopoietic cells are rapidly proliferating in the bone marrow but also undergo apoptosis, a process mediated by cytokines such as TNF-α, IL-1β, IL-6 and others [40]. In addition, the presence of stromal cells apoptosis and the altered distribution of cell types in the marrow suggest an underlying abnormality in the stroma. Aberrant cytokine production and altered interactions of hematopoietic cells within the extracellular matrix have both been demonstrated in MDS.

Some cytopenic MDS patients respond to immunosuppressive treatment leading to hypothesis that MDS may be in part, autoimmune mediated. Immune-mediator cells, particularly T cells, are part of the hematopoietic microenvironment regulating both hematopoietic proliferation and differentiation. MDS patients exhibit high percentage of $CD8^{+}/CD28^{-}/CD57^{+}$ T-cells, a phenotype consistent with mature cytotoxic T cells. These T cells have limited T-cell receptor (TCR) repertoire representation, leading to the expansion of dominant T-cell clones. The increase in CD57 expression by $CD8^{+}$ cells and the demonstration of TCR variable beta chain families expressing CD57 characterized diseases with activated immune environment like multiple sclerosis, rheumatoid arthritis [41]. Some findings from patients with MDS have led to the hypothesis that MDS may be the result of an autoimmune reaction directed against marrow stem cells. A high percentage of researchers support this popular concept of immune system involvement in the pathogenesis of MDS, based to a study an autoimmune inflammatory manifestation has been observed in 22% of MDS patients [41, 42].

About 40-50% of all MDS patients display clonal chromosomal aberrations [43]. Several cytogenetic abnormalities observed in MDS are also seen in AML, thus supporting a common origin of a function of these two disease categories [37]. Trisomy 8 and deletions of part or all of chromosomes 5 and 7 are common events in myeloid malignancies, however, somatically acquired gains or losses of many other chromosomal segments have been described. MDS are typically associated with loss of chromosomal material, e.g. deletion and/or monosomy of chromosomes 5, 7 and 20 [44, 45].

MDS is considered to require multiple hits and to date, no single genetic lesions has been shown to be sufficient for developing the disease. Many chromosomal translocation-mediated oncogenes and a few of the tumor suppressors have been identified. For example, genes inactivated in MDS comprise a relatively small number of cases and include P53, RB, WT-1, NF1, AML1, C/EBPα, CTNNA1 and nucleophosmin (NPM1). Several genetic alterations have been described especially in high-risk MDS. For example, the first identified molecular lesion in MDS

was an activating mutation of the N-ras oncogene, which correlates to an increased risk of AML evolution. In addition, some of the genetic aberrations regularly occur in AML are less frequent in MDS. FLT-3 mutations are found in <1% of MDS patients, KIT mutations in 1.2% and MLL partial tandem duplication in 2.7% [46, 47]. Furthermore, the nucleophosmin gene (NPM1), one of the most commonly mutated genes excessively in normal karyotype-AML patients, seems to be mutated in a small proportion in MDS patients. Sportoletti *et al.*, have shown that mice heterozygous for NPM1 develop MDS like features and are suspectable to tumor development, particularly myeloid malignancies [48].

Common gene mutations in AML and MDS: NPM1 and FLT3

1. NPM1

Mutations of the nucleophosmin gene NPM1 have recently been described as one of the most frequent genetic lesions in AML and occur in 55% of patients with normal karyotype [49]. NPM1 mutations also appear in a small proportion of MDS patients [50]. The NPM1 gene is located on chromosome 5q35 and encodes a multifunctional nucleolar protein that plays multiple roles in cell growth and proliferation. NPM1 is predominantly localized in the nucleolus and is thought to function as a molecular chaperone of proteins, facilitating the transport of ribosomal proteins through the nuclear membrane [51]. The NPM1 gene regulates the ARF-p53 tumor suppressor pathway. Disruption of NPM1, either by chromosomal translocation or by mutation, leads in the cytoplasmic dislocation of NPM1 [52]. Two types of mutations have been described (figure 1) : the first and most frequent mutations consists of a 4-nucleotide (nt) insertion, downstream from nucleotide 959, the second one is deletion of a GGAGG sequence at positions 965 through 969 and substitution with 9 extra nt. Both mutations lead to aberrant cytoplasmic localization of NPM1, this is caused by open-reading-frameshift mutations that lead to either the disruption of the NPM1 nucleolar-localization signal or the generation of a leucine-rich export motif. In all mutated cases, the resulting frameshift led to a product five amino acids longer with the new C-terminal tail CFSQVSLRK, characteristic to the NPM1-mutated product [53]. Studies in cell lines and knockout mice have shown the involvement of NPM1 in the control of genomic stability and its contribution to growth-suppressing pathways through its interaction with ARF. So, the loss of NPM1 expression can contribute to tumorigenesis [52, 54].

Detection of NPM1 gene mutations is based on molecular and immunohistochemical methods, for example the NPM1 mutations can be detected by direct sequencing, melting point-based PCR assays or fragment analysis [53, 55]. As

regards the aberrant localization of the nucleophosmin protein in the cytoplasm is detectable in NPM1 mutation carriers using immunestaining with anti-NPM1 monoclonal antibodies.

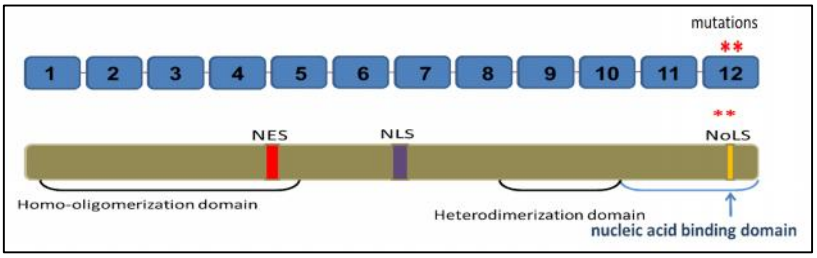


Figure 1. Schematic representation of NPM1 gene and normal NPM protein. NES indicates nuclear export signal; NLS, nuclear localization signal; NoLS, nucleolar localization signal; **, site of mutations in exon 12.

2. FLT3

FLT3 gene mutations are the second most frequent molecular marker in AML. FLT3 is a member of class III receptor tyrosine kinase (RTKIII) receptor family that includes FLT1, FMS, PDGFRβ and KIT [56]. The human FLT3 gene is located on chromosome 13q12 and consists of 24 exons. It encodes a membrane-bound glycosylated protein of 993 amino acids with a molecular weight of 158-160 kDa, as well as a non-glycosylated isoform of 130-140 kDa that is not associated with the plasma membrane. FLT3 consists of five immunoglobulin repeats in the extracellular domain, a transmembrane domain, a juxtamembrane dimerization (JM) domain and a highly conserved intracellular kinase domain (TK) interrupted by a kinase insert. FLT3 is primarily expressed on hematopoietic progenitor cells and functions in the proliferation and differentiation of these cells. Interaction of FLT3 with its natural ligand (Flt3-l) results in activation of the receptor through dimerization and subsequent autophosphorylation of flt3 proteins, followed by induction of multiple intracellular signaling pathways [57, 58]. Similarly, ligand-induced FLT3 activation has been shown to enhance the proliferative capacity of AML cells *in vitro*.

Two types of activating FLT3 mutations have been described in AML: an internal tandem duplication (ITD) of the FLT3 gene (FLT3 ITD) and point mutations of Asp835 within the FLT3 TK domain [59, 60] (figure 2). The FLT3-ITD mutation is the most common mutation with a rate in 25-35% of younger adults with AML. FLT3-ITD results in a constitutive active flt3 protein, which promotes Stat5 phosphorylation leading to an uncontrolled hematopoietic cell proliferation [58, 61, 62]. AML cases

that carry the FLT3-ITD mutation have a poorer clinical outcome, and tend to have higher white blood cells (WBC) counts and an increased percentage of leukemic blasts. Thus, is clear that in NC-AML patients it is important to evaluate the FLT3-ITD status.

The screening of the above mutation is based on PCR with gel electrophoresis; however more sensitive is fragment analysis and sequencing of the cloned PCR products in order to characterize the heterogeneous mutations [63].

The Asp835 missense mutation, located in the activation loop of the second tyrosine kinase domain of FLT3, is found in approximately 5-10% of AML patients. These mutations also lead to constitutive autoactivation of FLT3 receptor [60, 64]. It has been suggested that TKD mutation may both trigger the activation loop and stabilize it in the active state In contrast to the FLT3-ITD they do not seem to be specifically correlated to a certain AML type, but studies indicate that it is also an adverse prognostic marker.

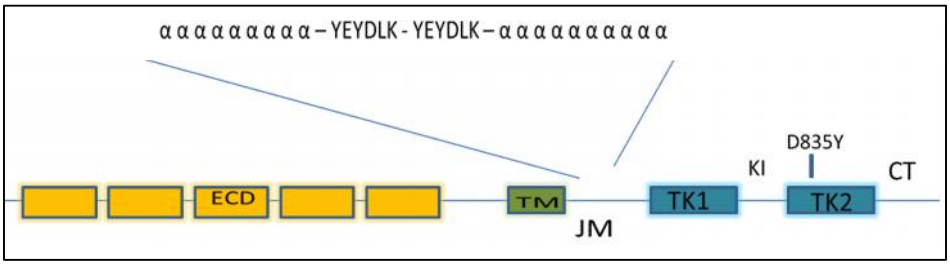


Figure 2. Structure of Flt3 protein

Flt3 consists of five immunoglobulin repeats in the extracellular domain (ECD), a transmembrane domain (TM), a juxtamembrane dimerization (JM) domain, a highly conserved intracellular kinase domain (TK : TK1,TK2) interrupted by a kinase insert (KI) and an intracellular C-terminal domain (CT). Internal tandem duplication (ITD) of the juxtamembrane (JM) domain of flt3is the most frequent mutation in human acute myeloid leukemia, and is significantly associated with leukocytosis and a poor prognosis. The missense mutation in the activation loop of the TK2 of flt3 at Asp835 is found in approximately 5-10% of AML patients. (α: amino acid)

MYELOPROLIFERATIVE DISORDERS

The myeloproliferative disorders (MPDs) represent a range of clonal hematological diseases with overlapping features, characterized by increased proliferation of the erythroid, megakaryocytic, and myeloid lineages. They are related to, and may evolve into, MDS and AML, although the myeloproliferative diseases on

the whole have a much better prognosis than these conditions. The concept of myeloproliferative disease was first proposed in 1951 by William Dameshek [65, 66]. There are four main myeloproliferative diseases, which can be further categorized by the presence or absence of the Philadelphia (Phi) chromosome: chronic myeloid leukemia (CML) is a Phi+ MPD, whereas Phi- MPDs are polycythemia vera (PV), essential thrombocytosis (ET) and primary myelofibrosis (PMF).

CHRONIC MYELOID LEUKEMIA

CML is a clonal disorder in which cells of the myeloid lineage undergo massive clonal expansion. CML was the first malignancy to be linked to a clear genetic abnormality, the reciprocal translocation between the long arms of chromosomes 9 and 22, called Philadelphia chromosome (Phi) [67]. CML patients positive for the Phi chromosome have a much longer median survival than patients Phi negative. The molecular result of this translocation is the generation of the fusion protein BCR-ABL, a constitutively activated tyrosine kinase, which is present in more than 95% of patients with CML. *In vitro* studies and studies in animal models have established that BCR-ABL alone is sufficient to cause CML, and mutational analysis has established that the tyrosine kinase activity of the protein is required for its oncogenic activity. The BCR-ABL transcript does not require activation by other cellular messaging proteins and activates a cascade of proteins which control the cell cycle, speeding up cell division. The BCR-ABL protein inhibits DNA repair enhancing genomic instability and making the cell more susceptible to developing further genetic abnormalities [68].

PHILADELPHIA NEGATIVE MPDs: POLYCYTHEMIA VERA, ESSENTIAL THROMBOCYTOSIS AND PRIMARY MYELOFIBROSIS

The clinical features of the other three myeloproliferative diseases are the overproduction of mature, functional blood cells, specifically characterised by an increased red-cell mass in PV, a high platelet count in ET and bone marrow fibrosis in PMF. The molecular etiology of these myeloproliferative diseases remained elusive till 2005, when the understanding of the genetic basis of these diseases was improved through the identification of a somatically acquired gain-of-function mutation (V617F) in the JAK2 tyrosine kinase in MPD patients [69]. This mutation is present in nearly all patients with PV, approximately 50% of each of those with ET and PMF, and 20% of those with atypical MPDs. It has not been identified in reactive myeloproliferation, lymphoid disorders, or solid tumors [69, 70].

The Janus kinase (Jak) family is one of ten recognized families of non-receptor tyrosine kinases. Mammals have four members of this family: Jak1, Jak2, Jak3 and Tyrosine kinase 2 (Tyk2). JAK2 encodes a non-receptor tyrosine kinase which involves in a specific subset of cytokine receptor signaling pathways [70]. For example, this protein is responsible for signal transduction from type I cytokine receptors that act by phosphorylation and activation of the STAT5 transcription factor. In addition it has been found to be constitutively associated with the prolactin receptor and is required for responses to gamma interferon [71]. Mice that do not express an active protein for this gene exhibit embryonic lethality associated with the absence of definitive erythropoiesis [71, 72]. JAK2 gene is located on chromosome 9p2-5. It is now acceptable that this non-receptor tyrosine kinase is involved in various processes such as cell cycle progression, apoptosis, mitotic recombination, and genetic instability and histone modifications [73].

The JAK2/V617F mutation results in a valine-to-phenylalanine substitution at the amino acid 617, located in the JH2 pseudokinase domain that normally autoinhibits JAK2 kinase activity [69] (figure 3). So, the V617F mutant kinase displays constitutive activity, independent of growth factors. Despite evidence that JAK/STAT pathway activation is common in both hematopoietic malignancies and solid tumors, JAK2/V617F is exclusive to disorders of the myeloid lineage and has not been observed in lymphoid neoplasms or in non-hematopoietic malignancies [73]. Analysis of germline DNA demonstrated that JAK2/V617F is a somatic mutation in hematopoietic progenitors. A subset of patients, most commonly with PV, has homozygous JAK2/V617F mutations, which are the result of mitotic recombination and duplication of the mutant allele [74]. Cells that are heterozygous for the V617F mutation have a proliferative advantage over cells bearing only the wild-type alleles. However, since JAK2 mutation is not present in all patients with MPDs, it is unlikely to be an initial causative event [75].

The development of more sensitive assays, including allele-specific PCR, pyrosequencing, and real-time quantitative PCR [76, 77], has allowed investigators to determine more precisely the frequency of the JAK2/V617F allele in PV, ET, and PMF. Using these more sensitive assays JAK2/V617F can be detected in approximately 90-95% of PV patients, 50-70% of ET patients, and 40-50% of PMF patients [77, 78]. The JAK2/V617F mutation has also been observed in a small number of patients with chronic myelomonocytic leukemia (CMML), MDS, and AML, although most JAK2/V617F mutations in AML occur in patients with a preceding diagnosis of PV, ET, or PMF [77-79].

The genetic basis for clonal hematopoiesis has been studied in MPDs by cytogenetic analysis [80]. Only 10-15% of PV patients have abnormal karyotype at diagnosis, and the most common abnormalities include trisomies (+8, +9, +1) and del(20q) [81]. In PMF, about 35% of patients have an aberrant karyotype, and the most frequent aberrations included del (13q), del (20q), and partial trisomy 1q. However, the functional relevance of these alterations remains questionable, because they are present only in a small proportion of cells [81, 82]. The common MPD deleted region on chromosome 20q is currently under investigation. This deletion is also found in myeloid malignancies such as MDS or AML. A minimal deleted region of 2.7Mb has been defined for MPD and 2.6Mb for other myeloid malignancies, with an overlap between these regions is 1.6Mb. A detailed expression map of this region was assembled, and analyses of candidate tumor suppressor genes within the common deleted region have been initiated, but the precise molecular defect has yet to be identified [83, 84].

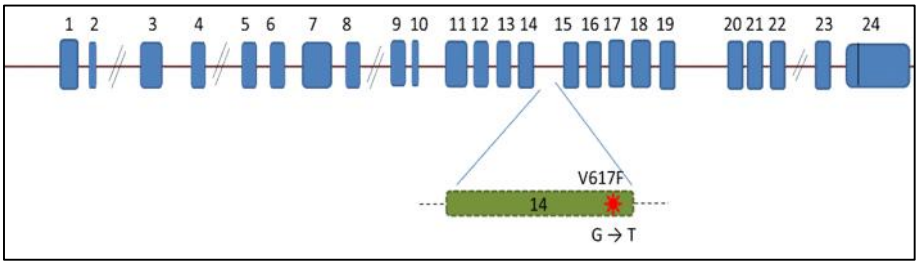


Figure 3. Human JAK2 gene and position of the V617F point mutation on exon 14.

The acquired mutation corresponds to a single-nucleotide change of JAK2 nucleotide 1849, on exon 14, resulting in a unique valine (V) to phenylalanine (F) substitution at position 617 of the protein.

AIM OF THE STUDY

According to the proposed multistep pathogenesis of myeloid malignancies, the hematopoietic progenitor cells are initially damaged by a toxin or a spontaneous mutation and may then undergo several additional alterations that provide them with a growth advantage [85]. However, the biology of hematopoietic malignancies is complex and the pathologic condition encompasses more than one abnormal clone of hematopoietic cell. Many studies provide evidence that important quantitative and functional alterations are present in the stroma of patients with different hematological disorders [4]. Studies have shown increased stromal cell apoptosis and altered distribution of cell types in the marrow of various patients with haemopoietic disorders, suggesting an underlying stroma abnormality. As MSCs and their progeny are the major cellular components of BM stroma, it is reasonable to assume a possible involvement of MSCs in the pathophysiology of haemopoietic disorders. Moreover, based on the hypothesis of a common multipotent stem cell with wide differentiation potential for both BM HSCs and MSCs, the so called Multipotent Adult Progenitor Cell (MAPC), a further assumption can be made that BM MSCs in patients with hematopoietic disorders might belong to the abnormal clone and thus might be primarily defective [86]. Alternatively, BM MSCs in patients might display secondary genetic aberrations and/or functional defects due to the deranged release of pro-inflammatory mediators and inhibitory cytokines by the apoptotic hematopoietic cells and the activated cellular components of the BM microenvironment.

The possible involvement of BM MSCs in the pathogenetic/pathophysiologic process of blood disorders has not been extensively investigated [87-89]. The current study aims to investigate the cytogenetic and genetic characteristics of BM MSCs in comparison to hematopoietic cells (HCs) of AML-, MDS- and MPD-patients. More specifically we aim to assess whether the same chromosomal aberrations and mutations existing in patient HCs also exist in patient MSCs.

PATIENTS AND METHODS

Patients

Sixteen patients with *de novo* MDS, twelve patients with de novo AML, seven patients with MPD and ten age- and sex-matched healthy individuals (Table 1), were studied. Patients were classified according to the French-American-British (FAB), the World Health Organization (WHO) and the International Prognostic Scoring System (IPSS) [90-93] and were studied on diagnosis before receiving any medication. Patient characteristics are summarized in Tables 2-4. The study has approved by the Ethics Committee of the University Hospital of Heraklion and informed consent according to the Helsinki Protocol was obtained from all subjects.

Table 1. Cytogenetic data of healthy BM hematopoietic cells and MSCs

No	Age	Karotype HC	MSCs Passage	Karyotype MSC	FISH HC	FISH MSC
ND-1	data not available	46,XY[5]	P2	46,XY[4]	5 and 7 chromosomes: OK	5 and 7 chromosomes: OK
ND-2	55	46,XY[5]	P2	not done	5 and 7 chromosomes: OK	5 (x3) 7 chromosome: OK
ND-3	64	46,XX	P2	no metaphases found	5 and 7 chromosomes: OK	5 and 7 chromosomes: OK
ND-4	75	46,XX[5]	P2	not done	5 and 7 chromosomes: OK	5 (x3) 7 chromosome: OK
ND-5	74	46,XX[3]	P2	46,XX[7]	5 and 7 chromosomes: OK	5 and 7 chromosomes: OK
ND-6	65	not done	P2	46,XY[4]	5 and 7 chromosomes: OK	5 and 7 chromosomes: OK
ND-7	47	46,XY	P2	46,XY [4]	5 and 7 chromosomes: OK	5 and 7 chromosomes: OK
ND-8	data not available	not done	P2	46,XY[4]	5 and 7 chromosomes: OK	5 and 7 chromosomes: OK
ND-9	56	not done	P2	not done	5 and 7 chromosomes: OK	5 and 7 chromosomes: OK
ND-10	61	not done	P2	not done	5 and 7 chromosomes: OK	5 and 7 chromosomes: OK

Table 2. Clinical and cytogenetic data of BM hematopoietic cells and MSCs of AML patients

No	Age	FAB classification	WHO subtype	Karyotype HC	Karyotype MSC	FISH HC	FISH MSC
Pt-1	72	M7	AML with recurrent cytogenetic translocations	no metaphases available	not done	5 and 7 chromosomes: OK MLL - amplification ETO - 3 signals p53 - deletion ATM - deletion	5 and 7 chromosomes: OK
Pt-2	50	M1	AML not otherwise categorized	46,XX[24]	46,XX[5]	5 and 7 chromosomes: OK	5 and 7 chromosomes: OK
Pt-3	67	M1	AML not otherwise categorized	46,XY[20]	no metaphases found	5 and 7 chromosomes: OK	5 and 7 chromosomes: OK
Pt-4	80	M1	AML and MDS, therapy-related	46,XY[10]	46,XY	5 and 7 chromosomes: OK Rb – deletion IgH-Bcl2 fused gene [t(14;18)]	5 and 7 chromosomes: OK
Pt-5	80	M5	AML with multilineage dysplasia	46,XX[7]	46,XX[3]	5 and 7 chromosomes: OK	5 and 7 chromosomes: OK
Pt-6	53	M1	AML not otherwise categorized	46,XY[12]	46,XY[7]	5 and 7 chromosomes: OK	5 and 7 chromosomes: OK
Pt-7	75	M1	AML not otherwise categorized	46,XY[20]	not done	not done	not done
Pt-8	data not available	M1	AML not otherwise categorized	46,XY[10]	not done	not done	not done
Pt-9	91	M5	data not available	data not available	not done	not done	not done
Pt-10	data not available	M1	AML not otherwise categorized	46,XY[2]/46,XY,del(20)(q11.2)[12]/47,XY,t(1;21)(q42;q22),+19,del(20)(q11.2)[5]/90,XXYY,t(1;21)(q42;q22)x2,-6,17	not done	not done	not done
Pt-11	82	M5	AML not otherwise categorized	46,XX [3]/43~44,XX,-5,-7,add(9q),-10,add(16)(q12.1),-17,-18,-22x2,+4-5mar	not done	not done	not done
Pt-12	59	M5	AML with characteristic genetic abnormalities	46,XY,der(6)t(6;11)(q27;q14~21),inv(16)(p13q22)	not done	chromosome 11(x3) inversion of chromosome 16.	not done

Table 3. Clinical and cytogenetic data of BM hematopoietic cells and MSCs of MDS patients

No	Age	IPSS risk score	WHO subtype	Karyotype HC	Karyotype MSC	FISH HC	FISH MSC
Pt-13	65	Int-2	RAEB-2	46,XY[22]	46,XY[13]	5 (X3) 7 chromosome: OK	5 (x3) 7 chromosome: OK
Pt-14	75	Low	RA	46,XY[23]	46,XY	5 and 7 chromosomes: OK	5 and 7 chromosomes: OK
Pt-15	73	Int-2	RAEB-2	44,XY,del(5)(13q33),ins(6;19)(p?;?),der(7)t(7;18)(q10;?)del(7)(q10),-18,-19[10]	not done	5 (deletion) 7 chromosome: OK	5 and 7 chromosomes: OK
Pt-16	75	Low	RA	46,XY[25]	46,XY	5 and 7 chromosomes: OK	5 and 7 chromosomes: OK
Pt-17	61	Int-1	RA	46,XX[25]	46,XY[7]	5 and 7 chromosomes: OK	5 and 7 chromosomes: OK
Pt-18	54	Low	RA	46,XY[20]	not done	not done	not done
Pt-19	clinical data not available for classifying			46,XY[10]	not done	5 and 7 chromosomes: OK	5 and 7 chromosomes: OK
Pt-20	data not available	Int-1	RA	46,XX[24]/46,XX,del(5q)[1]	not done	not done	not done
Pt-21	75	Int-2	RAEB-2	46,XY [9]/47,XY,+11 [15]	not done	not done	not done
Pt-22	61	Low	RARS	46,XY[17]	not done	not done	not done
Pt-23	86	Int-1	RA	46,XX [20]	not done	not done	not done
Pt-24	71	Int-2	RAEB-2	46,XY[16]	not done	not done	not done
Pt-25	49	Low	RA	46,XX[20]	not done	not done	not done
Pt-26	78	Int-1	RA	46,XX[20]	not done	not done	not done
Pt-27	72	Low	RA	46,XY[20]	not done	not done	not done
Pt-28	78	Int-1	RARS	46,XY[20]	not done	not done	not done

Table 4. Clinical and cytogenetic data of BM hematopoietic cells and MSCs of MPD patients

No	Age	Philadelphia "negative" MPDs – classification	Karyotype HC	Karyotype MSC	FISH HC	FISH MSC
Pt-29	64	PV	46,XY[21]	no metaphases found	5 and 7 chromosomes: OK	5 and 7 chromosomes: OK
Pt-30	61	PV	46,XX[17]	46,XX[9]	5 and 7 chromosomes: OK	5 and 7 chromosomes: OK
Pt-31	37	PV	46,XY[20]	not done	5 and 7 chromosomes: OK	5 and 7 chromosomes: OK
Pt-32	62	ET	46,XX[20]	not done	5 and 7 chromosomes: OK	5 and 7 chromosomes: OK
Pt-33	66	not done	not done	not done	not done	not done
Pt-34	the patient (male) was diagnosed with PV but the other data was not available				not done	not done
Pt-35	the patient (female) was diagnosed with MPD but the data was not available for more classification				not done	not done

MSC cultures

Posterior iliac crest aspirates from patients and healthy individuals were overlaid on Histopaque-1077 (Sigma, Saint Louis MO) to obtain the BM mononuclear cells (BMMCs). BMMCs were cultured in DMEM-LG/10% fetal calf serum (FCS; Hyclone, Logan, Utah, USA)/100 IU/ml PS (MSC medium) at a concentration of $2x10^5$ cells/cm^2 in 25cm^2 culture flasks at 37°C/5%CO_2 fully humidified atmosphere. MSC cultures were expanded as previously described [94]. On 70-90% confluence, cells were detached using 0.25% trypsin-1mM EDTA (Gibco) and re-seeded for a total of two passages (P).

Cytogenetic analysis of BM hematopoietic cells and culture expanded MSCs

Chromosome banding analysis

BM cells from 16 MDS patients, 11 AML patients, 4 MPD patients and 6 healthy individuals were cultured in RPMI-1640 medium supplemented with 5% FCS and 100 IU/ml PS at 37°C for 24 and 48 hours. For chromosome preparation, colcemide (0.1 g/ml) was added for 2 and 24 hours before harvesting. Following treatment with hypotonic KCl solution and fixation with a mixture of glacial acid and absolute methanol (1:3), a modified chromosome-banding technique (GAG; Giemsa bands by acetic saline-Giemsa) was used and 15 to 25 metaphase cells were analyzed and classified according to the International System for Human Cytogenetic Nomenclature (ISCN) [95, 96]. Similarly, MSCs from P2 were exposed to colcemide (0.1 g/ml) for 24 hours and trypsinized. For chromosomal analysis, GAG banding was performed in MSCs of 4 MDS patients, 5 AML patients, 2 MPD patients and 6 healthy individuals. Whenever possible, 15-20 metaphase cells in P2 were analyzed and classified according to the ISCN. A chromosomal aberration was defined as clonal abnormality when at least 2 metaphases were demonstrating the same structural rearrangement or chromosome gain, whereas a chromosome loss had to be identified in at least 3 metaphases.

Fluorescence in situ hybridization

Fluorescence *in situ* hybridization *(FISH)* was performed using commercially available locus-specific probes for chromosome 5 [LSI EGR1(5q31)/D5S23,D5S721], chromosome 7 [LSI D7S486(7q31)/CEP7], for MLL gene in chromosome 11 [LSI MLL Dual Color, Break Apart Rearrangement Probe], for AML1 or RUNX1 gene in

chromosome 21 and ETO or RUNX1T1 gene in chromosome 8 [RUNX1/RUNX1T1 DF FISH Probe Kit], for chromosomes 17 and 11 [LSI p53 / LSI ATM], for chromosomes 13 and 12 [LSI D13S319 / LSI 13q34 / CEP 12 Multi-color Probe] and for chromosomes 14 and 18 [LSI IGH/BCL2 Dual Color, Dual Fusion Translocation Probe], for chromosome 16 [LSI CBFB Break Apart Rearrangement Probe] following the protocol of the manufacturer (Abbott Laboratories, Illinois, USA). The signals of at least 200 interphase nuclei in each case were visualized, counted and documented using a Zeiss Axioskop microscope (Zeiss, Jena, Germany).

Nucleic acid extraction

DNA was extracted from BMMCs, peripheral blood mononuclear cells (PBMCs), and MSCs from patients and healthy individuals using QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany).

Total RNA was extracted from BMMCs, and MSCs of patients and healthy individuals using RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's instructions.

Analysis of FLT3 ITDs

Polymerase chain reaction (PCR) was performed on isolated DNA from mononuclear cells and P2 MSCs of all MDS, AML patients and healthy individuals. Because the location of FLT3 ITDs is restricted to exons 14 and 15 [57, 61, 62], PCR amplification of DNA was carried out using primers 11F (5' –GCA ATT TAG GTA TGA AAG CCA GC-3') and 12R (5'- CTT TCA GCA TTT TGA CGG CAA CC-3'). 500ng gDNA were amplified in a total reaction volume of 25μL, using 10pmol from each primer, 0.2mM of each deoxynucleotide triphosphate, 1X PCR RnX Buffer (Invitrogen), 1.5mM $MgCl_2$ (Invitrogen) and 2.5 units Taq DNA Platinum Polymerase (Invitrogen). Denaturing, annealing, and extension steps were performed at 94^{o}C for 30sec, 55^{o}C for 30sec and 72^{o}C for 1min, respectively, for 34 cycles on a thermal cycler (DNAEngine Peltier Thermal Cycler; BIORAD) including an initial 7min denaturation step at 95^{o}C and a final extension step at 72^{o}C for 10min. PCR products were resolved on a 2% agarose gel stained with ethidium bromide.

Analysis of NPM1 mutation

1μg of total RNA was reverse transcribed to cDNA using random hexamer primers and SuperScript II Reverse Transcriptase, according to the manufacturer's instructions (Invitrogen). cDNA was subsequently amplified by PCR. For the amplification of exon 12 of NPM1 gene, cDNA (50ng) was amplified in a total volume of 25 μL. The reaction volume mixture contained 1.5mM $MgCl_2$ (Invitrogen), 1X PCR RnX Buffer (Invitrogen), 0.2mM of each deoxynucleotide triphosphate, 2.5 units Taq DNA Platinum Polymerase (Invitrogen) and 10pmol of each primer. Primer sequences are listed below: cNPM-R (5'- CAC CGC TAC TAC TAT GTC CTA-3') and cNPM-F (5'- GAA GAA TTG CTT CCG GAT GAC T-3'). Primer cNPM-F was linked with the fluorescent dye IDR700. After the mixture was preheated at 94 °C for 7 min, it was subjected to 30 cycles of 30sec at 94°C, 30sec at 62°C, and 1min at 72°C, and a final extension of 10min at 72°C, all carried out on a thermal cycler (DNAEngine Peltier Thermal Cycler; BIORAD). To detect mutated NPM1 forms in patients, PCR products are separated on a high resolution polyacrylamide sequencing gel, LI-COR 6.5% KB^{Plus} polyacrylamide in 1X TBE, following manufacturers recommendations (LI-COR). The ALF-EXPRESS Sequenator, using Licor DNA Sequencer Long Reader 4200 software was used for the analysis

Detection of JAK2-V617F mutation

JAK2/V617F mutation was quantitatively detected in genomic DNA from mononuclear cells and P2 MSCs from all MPD patients and healthy individuals with RQ-PCR. PCRs were performed using JAK2 MutaQuant^{TM} Kit (IPSOGEN, France) according the manufacturer's instructions and reactions were performed on a Rotor-Gene 6000 (Corbett; QIAGEN). Briefly, 25ng of gDNA were used in a final volume of 25μl PCR reaction. Both wild-type and mutant alleles were amplified and two standard curves (JAK2/V617F and JAK2/WT) were generated using absolute quantitation. The standard curve equations were used to calculate V617F and WT gene copy numbers in all patient samples. The results were reported as percentage of mutant alleles in each patient sample, according to the following equation: JAK2/V617F% = [V617F/ (V617F+WT)]*100. If JAK2/V617F% > 0.091% then the mutation is considered detected in the sample.

CD45 expression

The expression of CD45 in MSCs-P2 of MPD patients was demonstrated by real-time RT-PCR using SyBR GreenER qPCR SuperMix Universal (Invitrogen) in a standard PCR buffer containing cDNA prepared from 1μg of total RNA. RNA was reverse transcribed to cDNA using random hexamer primers and SuperScript II Reverse Transcriptase, according to the manufacturer's instructions (Invitrogen). Real-time PCR reactions were performed on a Rotor-Gene 6000 (Corbett; QIAGEN). Products were normalized according to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. Sequences of the primer sets for the CD45 were: forward primer (5' – GAA ATT GTT CCT CGT CTG AT – 3'), reverse primer (5' – CTT TGC CCT GTC ACA AAT AC - 3') and for the house keeping gene (GAPDH): forward primer (5' – GCC CAA TAC GAC CAA ATC C– 3'), reverse primer (5' – AGC CAC ATC GCT CAG ACA - 3'). Briefly, for each specimen we performed two PCR reactions, one for the expression of CD45 and the other for the expression of GAPDH. Each reaction had a final volume of 25μl and it contained 50ng of cDNA, 10μM of the respective pair of primers and SyBR GreenER qPCR SuperMix (Invitrogen). Thermocycling conditions were 50°C for 2min, 95°C for 10min, followed by 40 cycles of 95°C for 15sec and 60°C for 30sec.

RESULTS

Cytogenetic analysis of BM hematopoietic cells and MSCs

Results from the cytogenetic analysis of patient BM hematopoietic cells (HCs) and MSCs are shown in Tables 2-4.

1. Cytogenetics in AML patients

All results are shown in table 2. In total AML patients, karyotype abnormalities were identified in BM HCs in three out of twelve AML patients. All of three AML patients had an aberrantly complex karyotype in their HCs. More specifically, Pt-10 carried a dominant clone with a deletion of long arm of chromosome 20 [del(20)], a second clone with a reciprocal translocation between long arms of chromosomes 1 and 21 [t(1;21)(q42;q22)] and a third clone with hyper-tetraploidy (94 chromosomes), two copies of deficit long arm of chromosome 20 [del(20)(q11;2) x2], two copies from reciprocal translocation between chromosomes 1 (1q) and 21 (21q) [t(1;21)(q42;q22) x2] and monosomy of chromosomes 6 (-6) and 7(-7). In Pt-11, 26 metaphases were counted with numerical and structural defects. 23 out of 26 metaphases consisted of a large cellular clone with hypodiploidy (43~44 chromosomes), monosomies of chromosomes 5(-5), 7(-7), and 17(-17). Finally, Pt-12 carried a derivative of chromosome 6 from a nonreciprocal translocation between long arms of chromosomes 6 (6q) and 11 (11q) implying the partial trisomy of long arm of chromosome 11 (11q) [der(6)t(6;11)(q27;q14~21) and also carried a pericentral inversion of chromosome 16 [inv(16)(p13q22)] (figure 4) . *FISH* analysis implied the existence of trisomy 11 and inversion of chromosome 16.

Cytogenetic analysis in MSCs was performed in six out of twelve AML-patients (Pt-2 – Pt-6) and MSCs did not show any abnormality. FISH analysis (table 2) for chromosomes 5 and 7 was performed in both HCs and MSCs of patients and no numerical abnormality was observed in any of the samples.

Nonetheless in Pt-1 FISH analysis of HCs revealed the existence of three signals of ETO gene (figure 5) and an amplification of MLL gene (figure 6), as a deletion of tumor suppressor gene p53 and ATM gene (ataxia telangiectasia mutated). These signals were not observed in the corresponding MSCs.

Finally FISH analysis in HCs of Pt-4 showed deletion of 13q14.3 (Retinoblastoma gene - Rb) in 8% of interphases and an IgH-Bcl2 fused gene [t(14;18)] in 21% of interphase cells, but MSCs of this patient were negative for both.

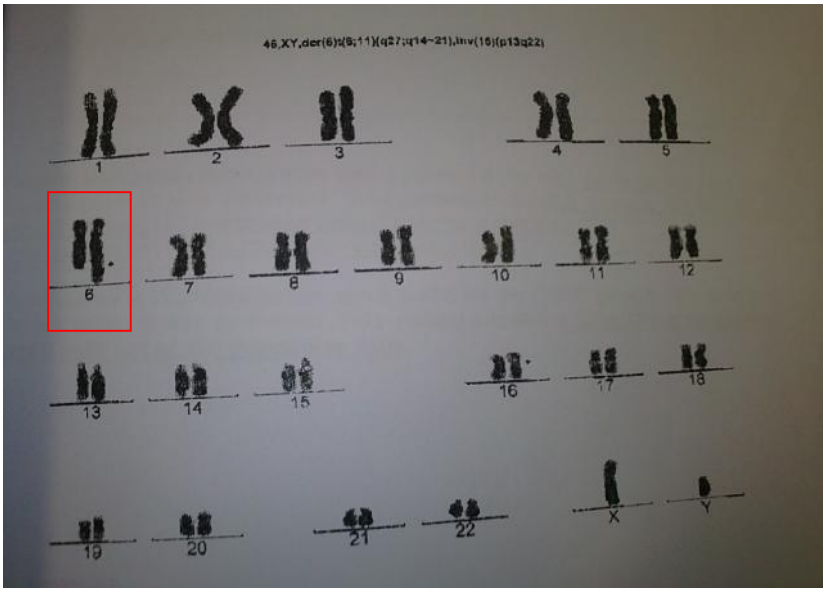


Figure 4. Cytogenetic analysis of AML Pt-12 BM HCs. Pt-12 appears with a derivative of chromosome 6 from a nonreciprocal translocation between long arms of chromosomes 6 (6q) and 11(11q) implying the partial trisomy of long arm of chromosome 11 (11q).

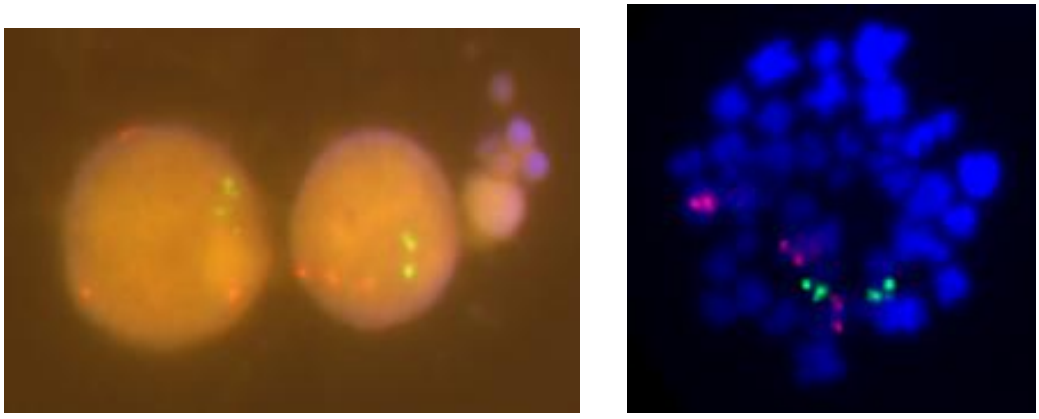


Figure 5. AML Pt-1 FISH analysis. Left: In this interphase cell we recognize 3 green signals for ETO gene implying a trisomy of chromosome 8. Dual Colour-Dual Fusion probe [RUNX1/RUNX1T1 DF FISH Probe Kit] was used, detecting a translocation between AML1 (RUNX1) in 21q22 and ETO (RUNX1T1) in 8q22. We have a green signal from AML1 gene and an orange/red signal from ETO gene. If we had a translocation between two these genes, we could see one red signal for normal chromosome 8, 1 green signal for normal chromosome 21, and 2 green/red signals from the fusion genes. Right: in a metaphase cell, ETO gene is present in three different chromosomes, implying trisomy 8.

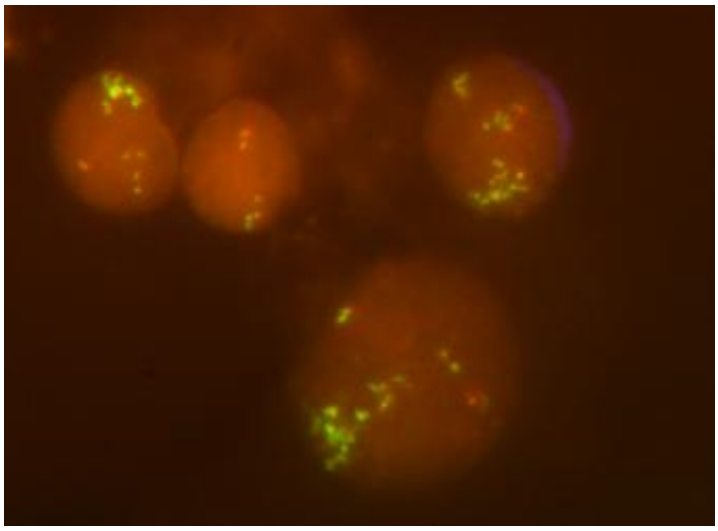


Figure 6. AML Pt-1 amplification of MLL gene. In this picture we have multiple green/red signals showing amplification of MLL gene in the same cell. In this case a break-apart probe for chromosome 11 [LSI MLL Dual Color, Break Apart Rearrangement Probe] was used. In a normal cell we can recognize two yellow signals (each one consists of a red and a green part of probe that hybridizes in adjacent sequences of the MLL gene).

2. Cytogenetics in MDS patients

In MDS patients, karyotype abnormalities were identified in HCs in three out of sixteen MDS patients (all listed in table 3). In more details, Pt-15 had an aberrantly complex karyotype. This patient had a deletion of chromosome 5 [del(5)], a derivative of chromosome 7 from a translocation between chromosomes 7 and 18 [der(7)t(7;18)], a deletion of the long arm of chromosome 7 [(del(7)(q10)] and monosomy of chromosomes 18 (-18) and 19 (-19) (figure 7).

In Pt-20, 25 metaphases were counted. In one of the metaphases there was a deletion of the long arm of chromosome 5 [del(5q)] (figure 8). Finally a trisomy of chromosome 11 (+11) was observed in 15/24 of metaphases in Pt-21 (figure 9).

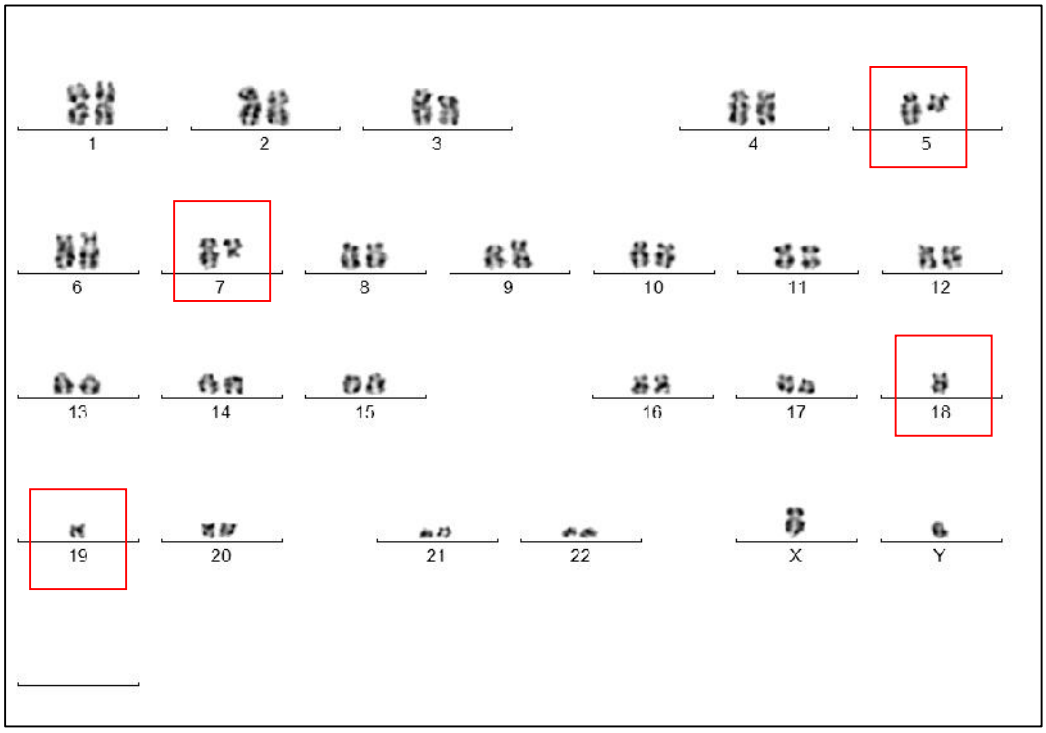


Figure 7. Cytogenetic analysis of BMHCs derived from the MDS Pt-15. Patient appears with an aberrantly complex karyotype: a deletion of chromosome 5 [del(5)], a derivative of chromosome 7 from a translocation between chromosomes 7 and 18 [der(7)t(7;18)], a deletion of the long arm of chromosome 7 [(del(7)(q10)] and monosomy of chromosomes 18 (-18) and 19 (-19).

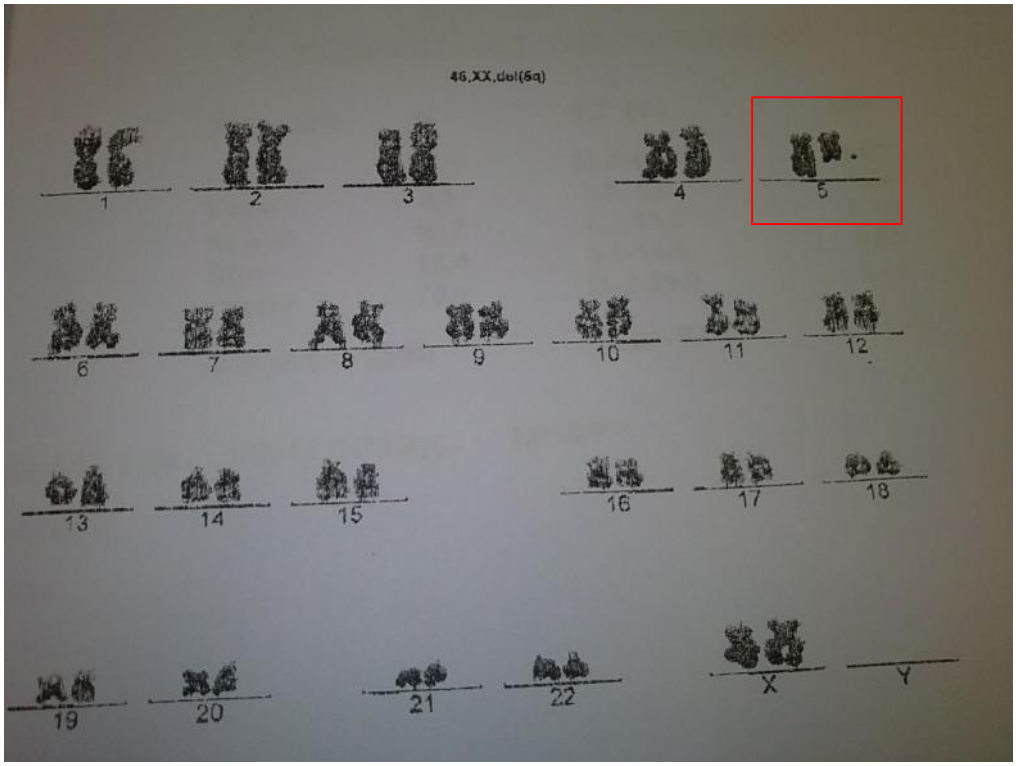


Figure 8. Cytogenetic analysis of BMHCs derived from the MDS Pt-20, with a deletion of the long arm of chromosome 5 [del(5q)].

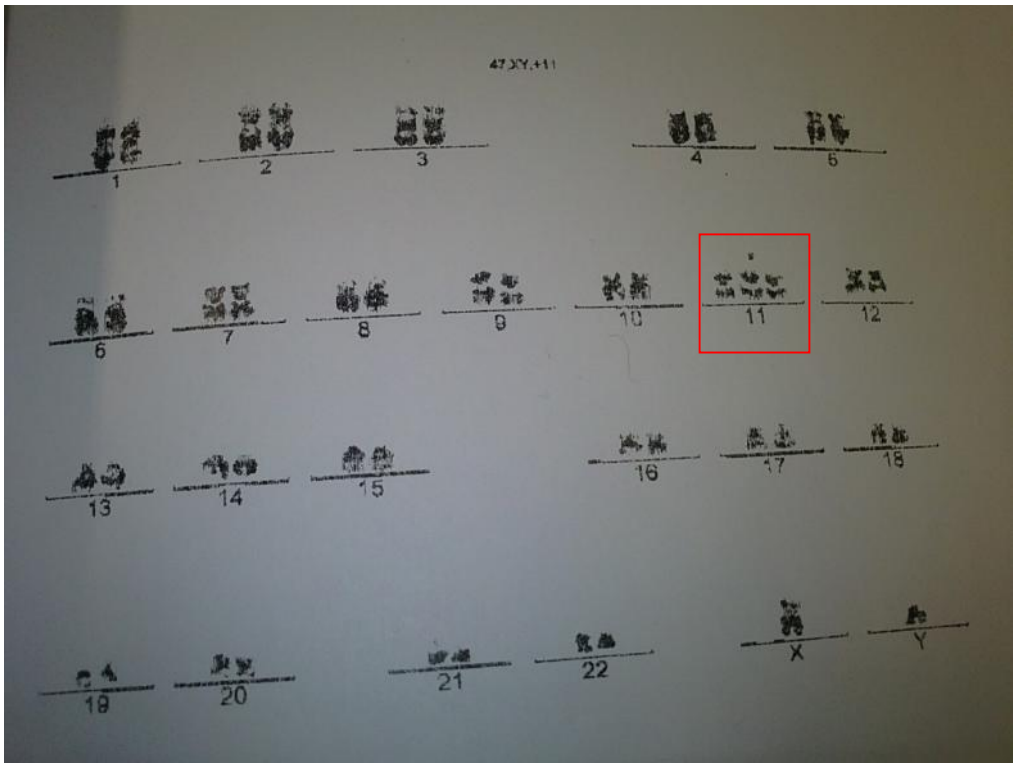


Figure 9. Cytogenetic analysis of BMHCs derived from the MDS Pt-21, with a trisomy of the chromosome11 (+11).

Cytogenetic analysis in MSCs was performed in four out of sixteen MDS-patients (Pt-13/14/16/17) and MSCs did not show any abnormality.

FISH analysis for chromosomes 5 and 7 was performed in HCs and MSCs of Pt-13-17 and Pt-19 (table 3) and there were no numerical abnormalities, except from Pt-13, where both HCs and MSCs harbor trisomy 5 (figure 10). On the contrary, the observed deletion of chromosome 5 in BM HCs of Pt-15 was not observed in the corresponding MSCs by *FISH*.

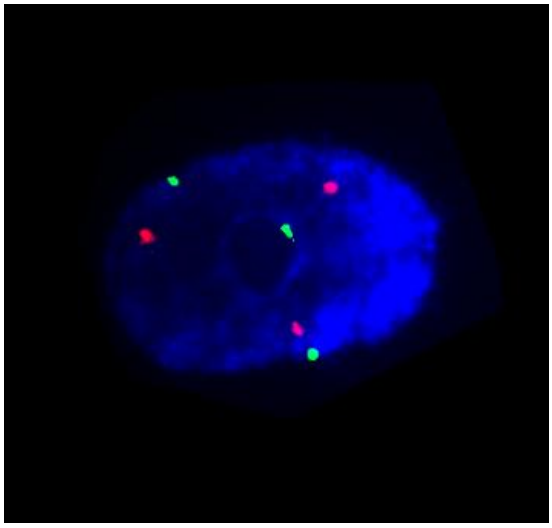


Figure 10. MDS Pt-13 MSC with trisomy 5. FISH analysis with a dual color probe LSI EGR1(Spectrum Orange 5q31)/D5S23,D5S721(SpectrumGreen, 5p15.2) showing three green signals on an interphase nucleus from Pt-13 MSC. Green signals correspond to 5p (small arm) of chromosome and orange/red signals for 5q (large arm) of chromosome 5. In a normal cell we would expect two signals of each colour (2 green, 2 red/orange).

3. Cytogenetics in MPD patients

Cytogenetic analysis was performed in HCs in four out of seven MPD patients and no abnormalities were observed (table 4). *FISH* analysis was performed in four MPD patients (Pt-29 – 32) in HCs and MSCs for numerical abnormalities of chromosomes 5 and 7. No abnormality was found.

4. Cytogenetics of healthy subjects

All healthy individuals revealed a normal karyotype in HCs and the corresponding MSCs (table 1). *FISH* screening for chromosomes 5 and 7 was performed in all HCs and MSCs from healthy subjects and there were no numerical abnormalities, except two individuals. ND-2 and ND-4 individuals harbor trisomy 5 in MSCs (6% and 13%, respectively), but their HCs are normal.

Mutational analysis of FLT3 gene in patient BM HCs and MSCs

FLT3 ITD mutational analysis was performed for HCs and MSCs in sixteen MDS, twelve AML-patients and ten healthy individuals. In AML patients the FLT3 ITD mutation was observed in 1/12 (8.3%) of HCs. However the mutation was not observed in patient MSCs (figure 11). None of the MDS samples (either HCs or MSCs) were positive for the ITD mutation.

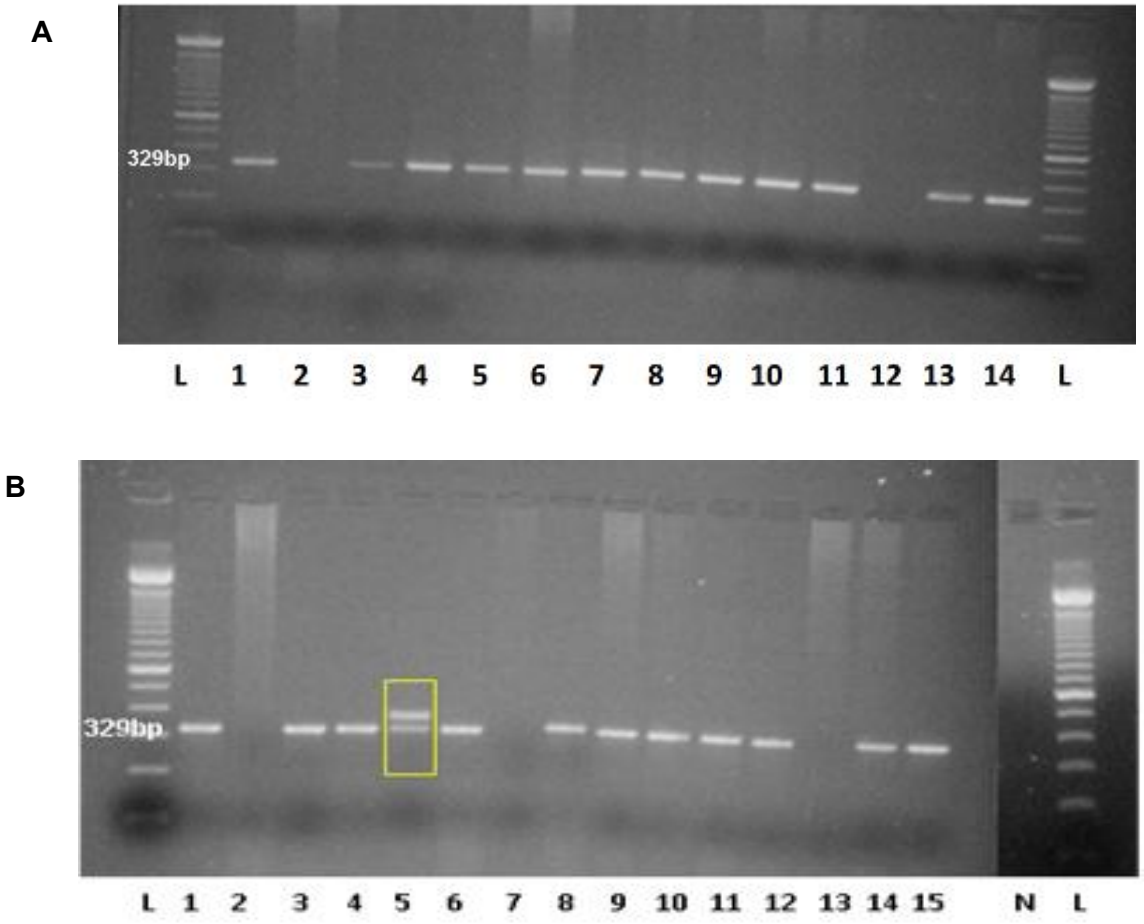


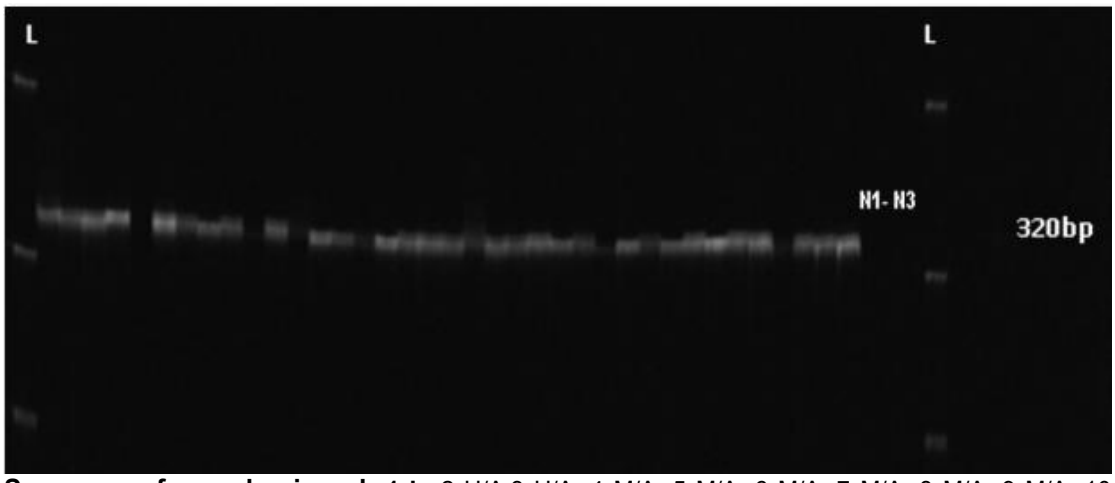
Figure 11. FLT3 ITD mutation analysis in BM HCs and MSCs of AML and MDS patients. **A**: 1 H/M, 2 H/M, 3 H/M, 4 M/A, 5 H/M, 6 M/M, 7 M/A, 8 H/H, 9 M/H, 10 H/M, 11 H/M, 12 M/A, 13 H/A, 14 H/M. All speciments are normal as each one harboring a small band (329bp) **B**: 1 H/M , 2 H/A, 3 M/A, 4 M/A, 5 H/A, 6 M/A, 7 H/M, 8 M/A, 9 H/A, 10 H/A, 11 M/A, 12 M/A, 13 H/M, 14 H/A, 15 H/M. The specimen 5H/A (yellow box) corresponds to HCs of Pt-6 with AML which harbors a mutation for FLT3-ITD. The small band (329 bp) represents normal cell clones and larger band mutated leukemic cell clones.

In any case, small bands (329 bp) have represented normal cell clones and larger bands were mutated leukemic cell clones. [H/M= **HCs/MDS**, H/A= **HCs/AML**, H/H=**HCs/HEALTHY**, M/M=**MSCs/MDS**, M/A= **MSCs/AML**, M/H= **MSCs/HEALTHY**, N=negative control, L=ladder (100bp)]

Mutational analysis of NPM1 gene in patient BM HCs and MSCs

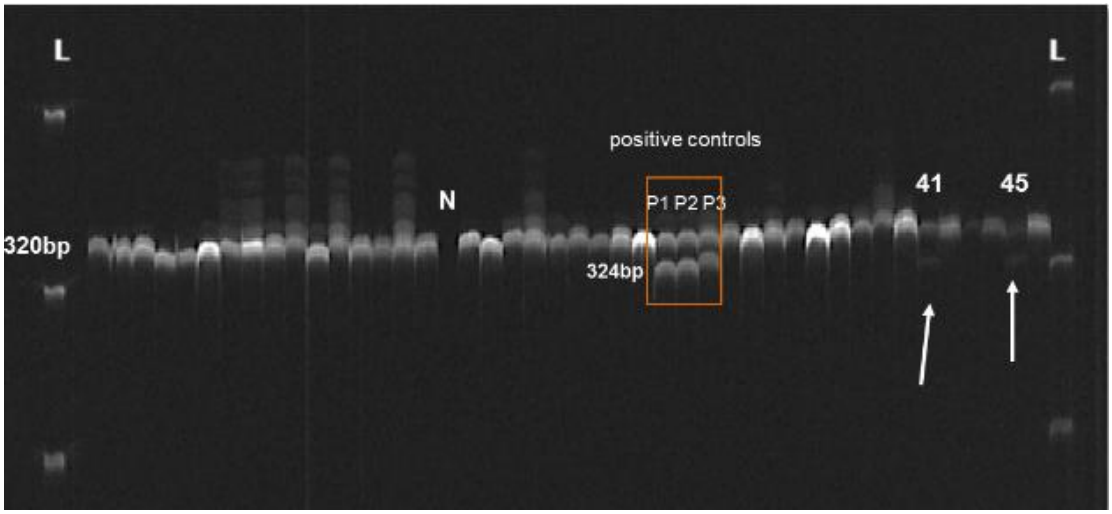
Fourteen MDS-, twelve AML-patients and ten healthy individuals were screened for mutations in NPM1 gene. Small insertions in NPM1 gene were observed in HCs in 2/12 (16.6 %) AML patients, but were not identified in the corresponding MSCs. As shown in figure 10, although HCs of Pt-6 and 9 harbor the NPM1 mutation, the corresponding MSCs do not. The mutation was not observed in either HCs or MSCs of MDS patients (figure 12)

A



Sequence of samples in gel: **1 L**, 2 H/A,3 H/A, 4 M/A, 5 M/A, 6 M/A, 7 M/A, 8 M/A, 9 M/A, 10 M/A,11 M/A, 12 M/A, 13 M/A, 14 M/H, 15 M/H, 16 M/A, 17 M/H, 18 M/H, 19 M/H, 20 M/H, 21 M/A, 22 M/A, 23 H/M, 24 M/M, 25 H/M, 26 H/M, 27 H/M, 28 H/M, 29 H/M, 30 M/M, 31 H/M, 32 M/M, 33 M/M, 34 M/M, 35 M/M, 36 M/M, 37 M/H, 38 H/M, **39 N1, 40 N2, 41 N3, 42 L**

B



Sequence of samples in gel : **1 L**, 2 gap, 3 H/H, 4 H/H, 5 H/H,6 H/H,7 H/H,8 H/H,9 H/H,10 H/H,11 H/H,12 H/M,13 H/M,14 H/M,15 H/M,16 H/M,17 H/M,18 H/M, **19 N**, 20 M/H,21 M/H,22 M/H,23 M/H,24 M/H,25 M/H,26 M/H,27 M/M,28 M/M, **29 P1, 30 P2, 31 P3**, 32 M/H,33 M/M,34 M/M,35 M/M,36 M/M,37 M/M,38 H/A,39 H/A,40 H/A,**41 H/A**, 42 H/A,43 H/A,44 H/A, **45 H/A**, 46 H/A, **47 L**

Figure 12. NPM1 mutations in BMHCs and MSCs of AML and MDS patients **A**: All speciments are normal as each one harboring a small band (320bp) **B**: In lane 41 represents the HCs of Pt-6 and lane 45 the HCs of Pt-9 (white arrows; both AML patients). Three AML - samples positive for Npm1 mutations (lanes 29-31) were used as controls (orange box). After the amplification of NPM1 exon two clearly distinguishable fragments are obvious the upper (320bp) and lower (324bp) band contained the wild and mutated type allele, respectively.
[H/M= **HCs/MDS**, H/A= **HCs/AML**, H/H=**HCs/HEALTHY**, M/M=**MSCs/MDS**, M/A= **MSCs/AML**, M/H= **MSCs/HEALTHY**, N=negative control, L=ladder (50-350 bp sizing standard Licor Biosciences)]

Mutational analysis of JAK2/V617F allele in MPD patients

JAK2/V617F mutation was analyzed by RQ-PCR in both HCs and MSCs in seven MPD patients. All patients were negative for the Philadelphia chromosome, by *FISH* screening. MSCs were negative for CD45. HCs from four out of seven MPD patients were positive for JAK2/V617F mutation (Pt-29, -32, -33 and -35). All corresponding MSCs were negative (figure 13).

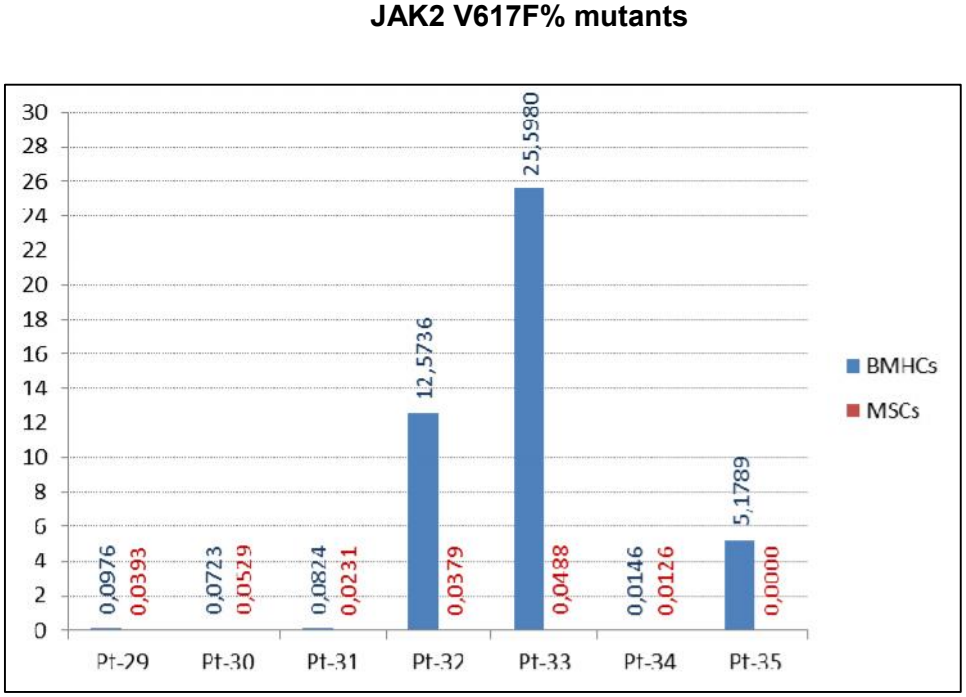


Figure 13. The percentage (%) expression of mutants for JAK2/V617F in MPD patients.Pt-29, -32, -33 and -35 were positive for JAK2/V617F mutation in their BMHCs, as the percentage (%) expression of mutants for JAK2/V617F was >0.091. [blue columns: HCs of MPD patients (Pt), red columns MSCs of MPD patients]

DISCUSSION

The biology of hematopoietic disorders in adults is not entirely known, and there is evidence suggesting that ineffective hematopoiesis results from complex interactions between hematopoietic cells (HCs) and hematopoietic microenvironment (HM) [97, 98]. The pathological condition results not only from intrinsic abnormalities in HCs, but potentially also from an abnormal HM. During the last few years, a great deal of interest has been generated around mesenchymal stem cells (MSCs), not only because of their biological properties, including their capacity to give rise to different mesenchymal cell types but also because of their imminent use in cellular therapy protocols for a variety of human disorders [99].

In recent years, bone marrow MSC research is expanding to enclose the role of stromal cells in the pathogenesis of various hematological disorders. In a mouse model, selection of genetic changes in the stroma has been found to occur in MDS, with occasional transformation to AML. In fact, several studies have reported important quantitative and functional alterations in MSCs of leukemia patients. Some independent studies have documented the existence of genomic alterations in the stroma of leukemia patients [100, 101]. Different groups have shown the extensive variability of the aberrations, such as hypodiploidy, balanced and unbalanced translocations, whole chromosome gains, and deletions [100].

Indirect evidence also suggests that MSCs might share a common precursor with HSCs [86, 102]. Multi-potent adult progenitor cells (MAPCs), which were discovered by chance by Catherine Verfaillie's group, display a higher proliferative and differentiative potential compared to classical MSCs [103]. MAPCs are initially isolated together with MSC, but subsequently grow indefinitely in nutrient-poor medium. It has been demonstrated that human, rat, and mouse MAPCs, obtained from BM, can differentiate into mesodermal (e.g., osteoblast, chondrocytes, adipocytes, myoblasts, and endothelial cells), endodermal (e.g., hepatocytes), and ectodermal (eg, neurons, oligodendrocytes, and astrocytes) cells. MAPCs, produced in vitro from bone marrow, have blood-building capacity in immune-deficient mice [104]. Recently has been shown that these cells can reconstitute hematopoietic compartments in vivo just as well as HSCs and, indeed they can even give rise to HSCs themselves. All these reinforce the opinion that MAPCs represent a more primitive subset of stem cells and are the possible common precursor of MSCs and HSCs [102, 104].

To gain more insight into this field we have evaluated and compared genetic and cytogenetic characteristics of HCs and MSCs from AML, MDS and MPD

patients, in order to investigate whether the pathologic clone(s) observed in HCs of patients also exist in patient MSCs. Cytogenetic examination, *FISH* analysis, and mutational examination of FLT3, NPM1and JAK2 genes were carried out in both HCs and MSCs from patients and ten healthy donors. MSCs from all patients and healthy individuals were successfully expanded *in vitro*. In MSC cultures derived from normal and malignant bone marrows, we observed the typical morphology of spindle-shaped fibroblast-like cells [105].

The chromosome banding analysis of bone marrow HCs revealed clonal abnormalities in three out of twelve AML patients, three out of sixteen MDS patients, none of seven MPD patients, and as expected, normal karyotype in the ten healthy individuals. Chromosomal banding analysis of *in vitro* expanded MSCs did not reveal any karyotypic abnormality in patients or in healthy individuals.

However *FISH* analysis revealed two patients with numerical abnormalities. One MDS - patient (Pt-13), exhibited trisomy of chromosome 5 in both HCs and MSCs. This finding supports the hypothesis that HCs and MSCs might belong to the same abnormal clone, although the possibility of an independent mutational event in MSCs cannot be excluded. HCs from another MDS patient (Pt-15) harbor a deletion of chromosome 5, but the corresponding MSCs appear karyotypically normal. Moreover two healthy individuals (ND-2 and ND-4) MSCs harbor trisomy 5, not observed in the corresponding HCs. In a study [88] a significant proportion of patients with MDS harbored chromosomal abnormalities in HCs, also exhibited cytogenetic aberrations in MSCs, although different from those in HCs. The reason for this finding is not clear; one possible explanation could be that the same agent that caused the genetic damage in HCs (radiation exposure, viruses etc.), also affected MSCs.

The trisomy 5 in MSC cultures from one MDS patient and two healthy subjects, has been described in acute lymphoblastic and myeloid leukemias, and also in neoplastic and non-neoplastic solid tumors [106, 107]. However, other groups have reported that MSCs derived from normal bone marrow *in vitro* expanded for several weeks, retain a normal karyotype even after 40 cell doublings [108]. Because trisomy 5 was identified in both patient and normal MSC cultures and chromosome gain have already been described in cell cultures of normal tissues, we assume that the abnormality probably represents a nonrandom *in vitro* phenomenon.

Moreover, *FISH* analysis revealed the existence of genetic alterations in HCs in two AML patients: one exhibited multi-amplification of both ETO and MLL genes and concomitant deletions of p53 and ATM genes; another AML patient harbored Rb deletion and IgH-BcL2 fusion gene. In both cases MSCs did not harbor any of the

corresponding genetic aberrations. None of the MDS patients or healthy subjects harbor any of the above genetic mutations either in HCs or MSCs.

The genetic profile of patient and healthy individual HCs and MSCs was further evaluated for FLT3 and NPM1 genes in MDS and AML patients. Mutational analysis for JAK2/V617F gene was performed in MPD patient HCs and MSCs. One AML patient harbored mutations in HCs for both FLT3 and NPM1 genes, and another AML patient harbored only the NPM1 mutation in HCs. The corresponding MSCs of the aforementioned cases were negative for all gene mutations tested. In addition four out of seven MPD patients were positive for JAK2/V617F mutation in HCs, but the corresponding MSCs were normal.

In our study, we did not investigate functional effects of chromosomal abnormalities in MSC or their effects on HC. Future studies are needed to assess the functional integrity of leukemia-derived MSC and, more importantly, the interaction between abnormal HC and MSC, which may be crucial to disease biology. Although hematologic malignancies are believed to arise from a stem or progenitor cell abnormality, a primary MSC defect may also lead to or support a hematologic malignancy. Until recently, little evidence maintain the role of primary stromal abnormalities in the pathogenesis of hematologic neoplasms. Based on published studies in mouse models, the microenvironment has been shown to induce malignancy [109, 110]. Because the microenvironment supports leukemic clones as a result of reciprocal interactions, abnormalities in MSC worsen those intrinsic to the neoplastic cells [111].

Our results show that bone marrow MSCs from patients with hematological malignancies may be prone to develop some chromosomal aberrations, although with unknown pathophysiologic significance, as some of the observed alterations may also occur in MSC cultures from normal individuals. Our data indicate that MSCs from patients with blood disorders have distinct genetic profile compared with leukemic/malignant blasts. MSCs of patients with FLT3 and/or NPM1, JAK2/V617F mutations were devoid of these mutations. Detection of genetic alterations in MSCs suggests that unstable MSCs may facilitate the expansion of malignant cells. In view of these data, genetic alterations in MSCs may be a particular mechanism of leukemogenesis.

Overall, this study gives further confirm to the absence of clonal involvement of MSCs in hematological malignancies, especially in AML, MDS and MPD. Because the controversy results of current studies for the MSCs, in many diseases such as bone marrow malignancies, it is necessary more investigation about their characteristics and their function in these defective cases; to understand their

distribution in the disease, so future studies should concentrate on the mechanisms leading to the stromal alterations in these malignancies which probably differ from the mechanisms causing the genetic alterations in hematopoietic cells.

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