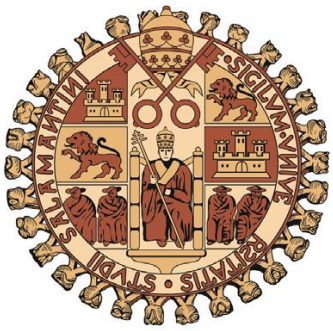
DOCTORAL DISSERTATION



VNIVERSITY OF SALAMANCA

MEDICINE DEPARTMENT

Genomic, Epigenomic and Transcriptomic Characterization of Low-Risk Myelodysplastic Syndromes

With the approval of Salamanca University Faculty of Medicine, this thesis will be defended on March 2013, in the Lecture Hall, Centro de Investigación del Cáncer, Salamanca.

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Abbreviations

ABCB6	ATP-binding cassette, sub-family B, member 6
ABCB7	ATP-binding cassette, sub-family B, member 7
ALAD	Aminolevulinate dehydratase
ALAS	5-aminolaevulinate synthase
AML	Acute myeloid leukemia
AML1 (RUNX1)	Acute myeloid leukemia 1 (Runt-related transcription factor 1)
ANG	Angiogenin
Apaf-1	Apoptosis protease-activating factor-1
ARG1	Arginase
ATG	Antithymocyte globulin
Bad	Bcl-2 antagonist of cell death
Bak	Bcl-2 antagonist/killer
Bax	Bcl-2 associated X protein
BCL2 (Bcl-2)	B-cell CLL/lymphoma 2
Bcl-XL	B-cell lymphoma-extra large
bFGF	Basic fibroblast growth factor
BM	Bone marrow
BMEC	Bone marrow endothelial cells
CCD	Charge coupled device
CDH1	Cadherin-1
CHK2	Checkpoint kinase 2
CPOX	Coproporphyrinogen oxidase
dATP	2'-deoxyadenosine triphosphate
DLK	Delta-like 1 homolog (Drosophila)
DNA	Deoxyribonucleic acid
EBS	ETS- binding site
ENG	Endoglin
EPO	Erythropoietin
ER	Estrogen receptor
ETS1	V-ets erythroblastosis virus E26 oncogene homolog 1
EVI1	Ecotropic virus integration site-1
FAB	French-American-British
Fas	TNF receptor superfamily, member 6
FECH	Ferrochelatase
FLT3	FMS-like tyrosine kinase 3
FN1	Fibronectin 1
FXN	Frataxin 1
FZD9	Frizzled family receptor 9
GATA1	Globin transcription factor 1
GDF15	Growth differentiation factor 15
HGF	Hepatocyte growth factor
HIC1	Hypermethylated in cancer 1
HIF1	Hypoxia inducible factor
HMBS	Hydroxymethylbilane synthase
IL27	Interleukin 27

IL27R	Interleukin 27 receptor
IL27RA	Interleukin 27 receptor, alpha
IL-6	Interleukin 6
IPSS	International prognostic scoring system
IPSS-R	Revised International Prognostic Scoring System
JAK2	Janus kinase 2
KDELR	KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor
LCN2	Lipocalin-2
MAPK	Mitogen-activated protein kinase
MCAM	Methylated CpG islands and microarray
MDS	Myelodysplastic syndrome
MDSu	MDS unclassifiable
miRNA	Micro ribonucleic acid
MMP9	Matrix metallopeptidase 9
NK	Natural killer
PB	Peripheral bood
PPOX	Protoporphyrinogen oxidase
RA	Refractory anemia
RAEB-1	Refractory anemia with excess of blasts- type 1
RAEB-2	Refractory anemia with excess of blasts- type 2
RAEBt	Refractory anemia with excess of blasts in transformation
RARS	Refractory anemia with ring sideroblasts
RCUD	Refractory cytopenia with uniline dysplasia
RBC	Red blood cells
RBM22	RNA binding motif protein 22
RCMD	Refractory cytopenia with multiline dysplasia
RNA	Ribonucleic acid
SCL25A38	Solute carrier family 25, member 38
sENG	Soluble endoglin
SF3B1	Splicing factor 3b, subunit 1
sFLT-1	Soluble Fms-like tyrosine kinase 1
SLC25A37	Solute carrier family 25 (mitochondrial iron transporter), member 37
sVEGF	Soluble vascular endothelial grow factor
TACSTD2	Tumor-associated calcium signal transducer 2
TET2	Tet methylcytosine dioxygenase 2
TF	Transcription factor
TFR2	Transferrin receptor 2
TFRC	Transferrin receptor
t-MDS	Myelodysplastic syndrome in transformation
TNF-α	Tumor necrosis factor
TNNC	Troponin C
TP53	Tumor protein p53
TRAIL	TNF-related apoptosis-inducing ligand
UQCRC1	Ubiquinol-cytochrome c reductase core protein I
UROD	Uroporphyrinogen decarboxylase

UROS	Uroporphyrinogen III synthase
UTR	Untranslated region
VEGF	Vascular endothelial grow factor
WHO	World health organization
WNT	Wingless-type MMTV integration site family
WPSS	WHO Classification-Based Prognostic Scoring System
WT1	Wilms tumor 1
XLSA-A	X-linked sideroblastic anemia and ataxia

General Introduction

1. General characteristics of MDS

Myelodysplastic syndromes (MDS) are a heterogeneous group of clonal diseases characterized by ineffective hematopoiesis in the bone marrow (BM) resulting in peripheral blood (PB) cytopenias (anemia, neutropenia and/or thrombocytopenia in variable degree and combinations). MDS are also characterized by a growing risk of transformation to acute myeloid leukemia (AML)(1).

1.1. Epidemiology

The true impact of MDS is difficult to establish due to several factors which limit a precise assessment. However, MDS is considered a rare disease, with an estimated occurenceof 4 people per 100,000 yearly. The disease becomes more common in older people, so much so that the incidences rise to >30 per 100,000 per year for people over 70 years of age(2-4). A population-based study reports a median age of patients of 77 years old and males are more commonly affected than females(2, 5). Recent studies suggest that MDS patients in Far East countries appearat a lower age than those in Western countries(6, 7).

1.2. Etiology

Several risk factors have been implicated in the etiology of MDS, including age, male gender, alcohol, cigarette smoking, ionizing radiation, immunosuppressive therapy, viral infection, benzene and other environmental or occupational exposure. The main secondary MDS subset comprises those cases occurring from previous chemotherapyor radiotherapy treatments (t-SMD) (5).

1.3. Bone marrow and peripheral blood abnormalities

The marrow is usually hypercellular in MDS patients due to ineffective erythropoiesis. However, normal cellularity is not unusual and some patients have hypocellular MDS. Dysplastic changes may be seen in normal BM, but typically only in a few cells. For this reason, it is important to distinguish this “minor dysplasia” from MDS; a minimum of 10% dysplastic cells per lineage is required to diagnose MDS (8, 9).

Table 1. Morphological abnormalities of BM in MDS patients (8-10)

Dyserythropoiesis	Dysgranulopoiesis	Dysthrombopoiesis
Anisocytosis Poikylocytosis Macrocytosis Basophilic stippling Nucleated RBC	Pelger-Huët-like anomaly Degranulation Hypersegmentation Nuclear sticks Döhle bodies	Large platelets Hypogranulation Hypergranulation

The predominant clinical features of PB (anemia, thrombocytopenia, and neutropenia) are a consequence of BM failure, as previously described.

Table 2. Morphological abnormalities of PB in MDS patients (10-14)

Erythrocyte series	Leukocyte series	Platelets
Multinuclearity Abnormal nuclear shape Megaloblastoid changes Cytoplasmic abnormalities Ringed sideroblasts	Clumping of chromatin Larger granules Ring-shaped nuclei	Micromegakaryocytes Large mononuclear forms Multiple small nuclei

1.4. Classification

Given the heterogeneity of MDS,there are several classifications for these diseases. The FAB classificationof MDS separates five subgroups based on the percentage of blasts in BM and PB, the percentage of ringed sideroblasts and the presence of monocytosis in PB(15).

Table 3. FAB classification

MDS Subtype	% Blasts in PB	% Blasts in BM	% Sideroblasts	Monocytes in PB
RA	<1	<5	<15	$<1x10^9/L$
RARS	<1	<5	>15	$<1x10^9/L$
RAEB	<5	5-20	<15	$<1x10^9/L$
RAEBt	>5	21-30	<15	$<1x10^9/L$
CMML	<5	0-20	<15	$>1x10^9/L$

RA: refractory anemia. RARS: refractory anemia with ring sideroblasts. RAEB: refractory anemia with excess of blasts. RAEBt: refractory anemia with excess of blasts in transformation. CMML: chronic myelomonocytic leukemia

The 2001 WHO classification modified the FAB classification separating as AML the patients with blast countsof over 20% in BM. In addition, in the WHO classification, chronic myelomonocytic leukemia was included in a new category of mixed myelodysplastic/myeloproliferative disorders (MDS/MPD) and incorporated new MDS

subtypes, including for example the 5q-syndrome and the refractory cytopenia with multilineagedysplasia (RCMD) (16).

Table 4. WHO 2001 classification

MDS Subtype	Blasts (%) PB	Blasts (%) BM	Ring Sideroblasts (%) BM
RA	<1	<5	<15
RARS	<1	<5	>15
RCMD	<1	<5	<15
RCMD-RS	<1	<5	>15
RAEB-1	1-10	5-10	<15
RAEB-2	11-20	11-20	<15
5q- Syndrome	<1	<5	<15
MDSu	<1	<5	<15

RA: refractory anemia. RARS: refractory anemia with ring sideroblasts. RCMD: refractory cytopenia with multilineage dysplasia. RCMD-RS: refractory cytopenia with multiline dysplasia and ring sideroblasts. RAEB-1: refractory anemia with excess of blasts-type 1. RAEB-2: refractory anemia with excess of blasts- type 2. MDSu: MDS unclassifiable.

An update of this classification was published in 2008 (17), in which the main change was the addition of a new subtype, refractory cytopenia with unilineal dysplasia (RCUD);thisterm includes simple refractory anemia of the 2001 WHO classification. Nevertheless, this thesis has principally followed the 2001 classification.

Table 5. WHO 2008 classification

MSD Subtype	Blasts (%) PB	Blasts (%) BM	Ring Sideroblasts (%) BM
RCUD (RA, RN, RT)	<1	<5	<15
RARS	<1	<5	>15
RCMD	<1	<5	±15
RAEB-1	<5	5-9	<15
RAEB-2	5-19	10-19	<15
5q- Syndrome	<1	<5	<15
MDSu	<1	<5	<15

RCUD: refractory cytopenia with unilineage dysplasia. RA: refractory anemia. NR: refractory neutropenia. TR: refractory thrombocytopenia. RARS: refractory anemia with ringed sideroblasts. RCMD: refractory cytopenia with multilineage dysplasia. RAEB-1: refractory anemia with excess of blasts-type 1. RAEB-2: refractory anemia with excess of blasts -type 2. MDSu: MDS unclassifiable.

1.4.1. MDS Classification in relation to prognosis

The IPSS is a system based on cytopenias, blasts in BM and cytogeneticsthat allows classification of MDS patients into four risk categories with significantly different prognoses(18).

Table 6. International Prognostic Scoring System (IPSS)

	Score				
Prognosis	**0**	**0.5**	**1.0**	**1.5**	**2.0**
Blasts (%) BM	<5	5-10	-	11-20	21-30
Karyotype*	Good	Intermediate	Poor	-	-
Cytopenias	0/1	2/3	-	-	-

Risk groups: low = 0; Intermediate-1 = 0.5-1.0; Intermediate-2 = 1.5-2.0; High = equal to or greater than 2.5
*Good: normal,-Y, del (5q), del (20q); Poor: complex karyotype (3 or more abnormalities), alterations in 7; Intermediate: other abnormalities.

Due to the limitations of the IPSS system in predicting patient survival and the likelihood of becoming AML, other prognostic systems were proposed. The WPSS system incorporates transfusion dependence, which has proven to be a very important factor in MDS patients. Along with the WHO classification and cytogeneticrisk groups it is possible to separatethe patients into four different prognostic groups (19).

Table 7. WHO Classification-Based Prognostic Scoring System (WPSS)

Variable	**0**	**1**	**2**	**3**
WHO	RA, RARS, 5q-Syndrome	RCMD, RCMD-RS	RAEB-1	RAEB-2
Karyotype*	Good	Intermediate	Poor	-
Transfusions	No	Yes	-	-

Risk groups: Very Low = 0, Low = 1, Medium = 2, High = 3-4; Very high = 5-6
* Good: normal,-Y, del (5q), del (20q); Poor: complex karyotype (3 or more abnormalities), alterations in 7; Intermediate: other abnormalities.
RA: refractory anemia. RARS: refractory anemia with ring sideroblasts. RCMD: refractory cytopenia with multilineage dysplasia. RCMD-RS: refractory cytopenia with multilineage dysplasia and ring sideroblasts. RAEB-1: refractory anemia with excess of blasts-type 1. RAEB-2: refractory anemia with excess of blasts-type 2.

In recent years, new factors have been recognized to have prognostic value in MDS and the IPSS has been modified, resulting in revised IPSS (IPSS-R). The IPSS-R is based on the variables present in the IPSS but stratifies patients into five risk groups with distinct differences in overall survival and risk of progression to AML. Furthermore, the IPSS-R

recognizes the role of age, serum ferritin and lactate dehydrogenase, among other factors, in overall survival (20).

Table 8. Revised International Prognostic Scoring System (IPSS-R)

Prognosis	0	0.5	1	1.5	2	3	4
Karyotype*	Very good	-	Good	-	Intermediate	Poor	Very poor
% Blasts inBM	≤2	-	>2% - <5%	-	5-10%	>10%	-
Hemoglobin	≥10	-	8 - <10	<8	-	-	-
Platelets	≥100	50 - <100	<50	-	-	-	-
Neutrophils	≥0.8	<0.8	-	-	-	-	-

Risk groups: Very Low = 0-2, Low => 2-3.5; Intermediate => 3.5-5, High => 5-6; Very high => 6
*Very Good: del (11q),-Y; Good: normal, del (5q), del (20q), del (12p); Poor: der (3) q21/q26, complex karyotype (3 changes), changes in 7; Very poor: Complex (more than 3 abnormalities) Intermediate: other abnormalities.

Our studies in this thesis will mainly be focused on the low-risk category, specifically in patients with RA, RARS and RCMD.

1.5. Cytogenetics in MDS

Cytogenetic abnormalities have been found in 40-70% of MDS patients at diagnosis. The most frequent single cytogenetic abnormalities are del(5q), monosomy 7 or del(7q), trisomy 8, and del(20q)(21-23). Deletions onthe long arm of chromosome 5 are the most frequent abnormality in MDS and can be present alone or accompanied by other changes. The loss of Y chromosome is also prevalent in MDS patients but is usually considered an age-related phenomenon and not always indicative of a clonal disorder (Figure 1) (24).

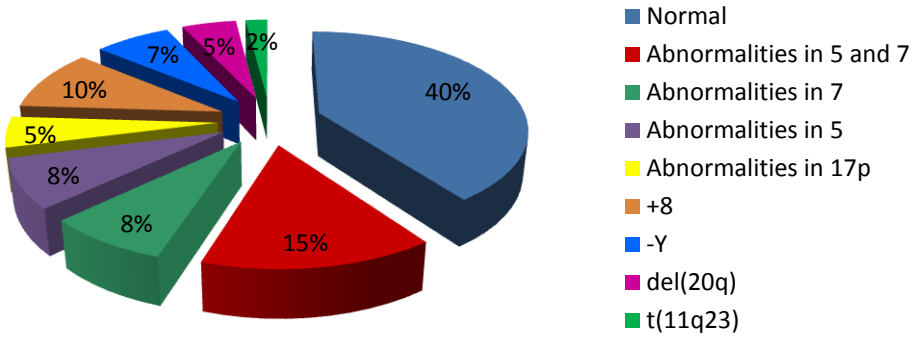

Figure 1. Recurrent chromosomal abnormalities in MDS. *From "Hematologic Malignancies: Myelodysplastic Syndromes." Springer. Deeg, H. J./ Bowen, Dana T./ Haferlach, T./ Le Beau, H./ Niemeyer, C./ Deeg, H. Joachim (Editor)*

The frequency of cytogenetic abnormalities is considered an independent variable that increases with the severity of the disease and the risk of leukemic transformation. The highest frequencies are found in patients with RAEB-1 and 2 and the lowest in those with RARS (18, 25, 26).

2. Pathogenesis

Although cytogenetic abnormalities are present in a substantial proportion of MDS patients, often with specific clinical features, more than 50% of patients have normal cytogenetics. These findings indicate that many underlying molecular and microenvironmental lesions within the MDS marrow remain to be discerned. There is increasing evidence that haploinsufficiency, epigenetic changes, the immune system, and BM stroma all contribute to the development of MDS(27). In this section, we discuss these and other mechanisms that could be leading to MDSpathogenesis.

2.1. Epigenetic modifications

2.1.1. Methylation

DNA methylation is an epigenetic process that involves the addition of a methyl group (CH_3) to the 5-position carbon of the cytosine pyrimidine ring in a CpG dinucleotide (Figure 2). Regions that are particularly rich in CpGs are called CpG-islands and are often located downstream from or very close to gene promoters. This process is carried out in an orchestrated reaction that includes DNA methyltransferases, methyl-binding domain proteins, and histone deacetylases(28).

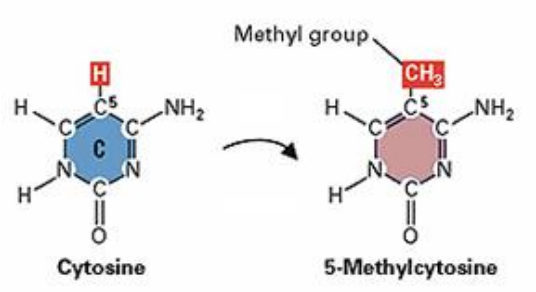

Figure 2. Methylation. Adding a methyl group to the cytosine ring. From http://michaeldomingos.hubpages.com

DNA cytosine methylation is the best characterized epigenetic event leading to the stabilization of the genome, the remodeling of the chromatin and the regulation of gene transcription(29, 30). In addition, both the presence of epigenetic marks andalso their location and density play a crucial role in regulating these processes(31, 32). A close correlation between DNA hyper-methylation and transcriptional silencing has been established in many systems(33). The exact mechanism by which promoter methylation causes silencing of the gene is somewhat unclear but it could include direct interference with the binding of transcription factors, blocking of the transcription machinery by the recruitment of methyl-binding domain proteins to methylated DNA, or alteration of chromatin structure(34).

Epigenetic alterations are now accepted as having a role in carcinogenesis. DNA hyper-methylation in cancer is associated with the silencing of tumor-suppressor genes, whereas hypo-methylation has been described as playing a causal role in progressive tumor formation and in promoting chromosomal instability (29, 31, 33, 35, 36). Tumor suppressors are not the only genes affected by aberrant methylation; abnormally methylated genes with other functions are also subject to silencing in human cancer, including those involved in DNA repair, apoptosis, angiogenesis, cell cycle regulation and cell-to-cell interaction (37). Hence, epigenetic modifications in promoter and/or regulatory regions that lead to transcriptional silencing of genes and development of cancer are important events factorsthat needto be studied in any onco-pathological state and they are attractive therapeutic targets.

Several genes have been described in MDS as targets of DNA methylation. Thus, hyper-methylation of genes involved in cell-cycle control and apoptosis is a common feature, particularly in high-riskMDS. *p15INK4b* (*P15*) and *p16INK4a* (*P16*) are important genes for cell cycle regulation. These two genes are rarelymutated or deleted (38); however, transcription of the *p15INK4b*gene is often silenced due to abnormal methylation of itspromoter region. Hyper-methylation of *P15* gene promoter has been observed in 30-50% of MDS cases and has been shown to correlate with the percentage of BM blasts and the risk of evolution to AML (39, 40).The number of involved loci is increased in high-risk diseases and during disease progression (41). In addition, some epigenetic changes could worsen the possible decreased production of tumor-suppressor proteins in MDS, if they affect haploinsufficient genes, such as *FZD9* on chromosome 7 (encoding the WNT protein receptor) (41)and *RBM22* on chromosome 5 (encoding a RNA-binding protein) (42). Othergenes frequently affected by hyper-methylation inMDS are*HIC1*, *CDH1* and *ER*. Hyper-methylation of these genes was associated with a poor outcome in earlystageMDS (43, 44).

2.1.2. microRNAs

MicroRNAs (miRNAs) are a family of small non-coding RNAs, with 18-25 nucleotides and highly conserved from invertebrate to vertebrate organisms. miRNAs inhibit gene expression by binding to the 3'UTR region of the target gene and modulate the gene expression at post-transcriptional level (45).

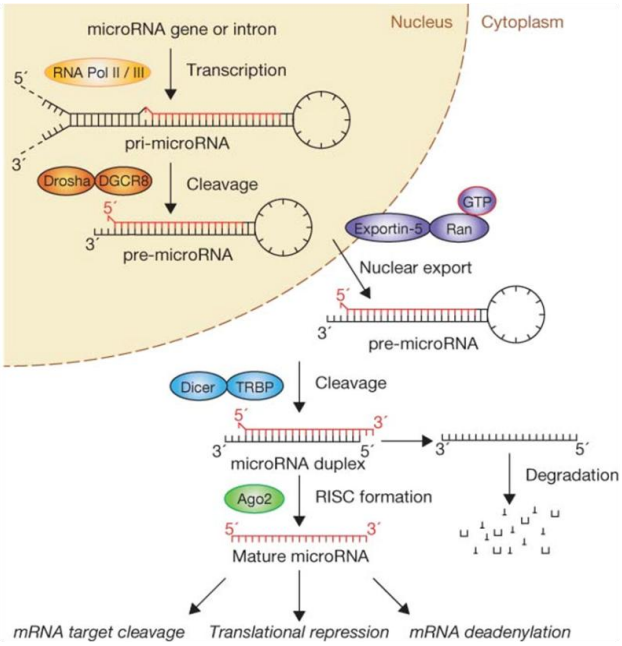

Figure 3.miRNA formation (46)

miRNAs are transcribed by RNA polymerase II as large RNA precursors called pri-miRNAs and comprise of a 5' cap and poly-A tail. The pri-miRNAs are processed in the nucleus by the microprocessor complex, consisting of the RNase III enzyme Drosha, and the double-stranded-RNA-binding protein, Pasha/DGCR8. The resulting pre-miRNAs are approximately 70-nucleotides in length and are folded into imperfect stem-loop structures. The pre-miRNAs are then exported into the cytoplasm by the karyopherin exportin 5 (Exp5) and Ran-GTP complex. Ran (ras-related nuclear protein) is a small GTP binding protein belonging to the RAS superfamily that is essential for the translocation of RNA and proteins through the nuclear pore complex. The Ran GTPase binds Exp5 and forms a nuclear heterotrimer with pre-miRNAs. Once in the cytoplasm, the pre-miRNAs undergo an additional processing step by the RNAse III enzyme Dicer generating the miRNA, a double-stranded RNA approximately 22 nucleotides in length. Dicer also initiates the formation of the RNA-induced silencing complex (RISC). RISC is

responsible for the gene silencing observed due to miRNA expression and RNA interference(45).

The expression profile analysis of miRNAs in cancer patients has shown that miRNAs are differentially expressed in tumor samples versus normal samples (47). The miRNAs abnormal expression is a common characteristic of neoplastic processes (48) and the expression patterns of miRNAs can distinguish cancers according to their diagnosis and developmental stages(49).

In the case of MDS, some recent works have shown that the miRNA expression profile allows differentiation of MDS from controls, patients with chromosomal alterations from those that do not have them and even the different subtypes of MDS from each other (50-53). Other works have also focused on the study of the expression of specific miRNAs in the BM of patients with MDS. Thus, MDS have an over-expression of miR-10a, miR-15a/miR-16, miR-21, miR-126, miR-155, miR-181 and miR-222 with respect to the controls and there is over-expression of miR-15a and miR-16 in low-risk MDS respect to the high-risk patients (54).

Table 9. Under-expression of miRNAs in MDS (55)

miRNA	Chromosome	MDS subtype
miR-197	1p13.3	All MDS
miR-128b	2q21.3, 3p22.3	Del(5q)
miR-1284	3p14.1	All MDS
miR-95	4p16.1	Del(5q)
miR-1305	4q35.1	All MDS
miR-583	5q15	All MDS
miR-143	5q33.1	Del(5q), RA/RCMD (tri8)
miR-145	5q33.1	Del(5q), RA/RCMD (tri8)
miR-146a	5q33.3	Del(5q), all MDS
miR-206	6p12.2	Del(5q), all MDS
miR-93	7q22.1	RA/RCMD(tri8)
miR-182	7q32.2	Del(5q)
miR-335	7q32.2	Del(5q), RA/RCMD (tri8)
miR-124	8p23.1	All MDS
miR-875-5p	8q22.2	All MDS
miR-30d	8q24.22	RA/RCMD(tri8)
miR-661	8q24.3	RA/RCMD(tri8), del(5q)
let-7a	9q22.32, 11q24.1, 22q13.31	All MDS
miR-326	11q13.4	All MDS
miR-940	16p13.3	All MDS
miR-423-5p	17q11.2	All MDS
miR-10a	17q21.32	Del(5q), low-risk MDS, CMML
miR-196a*	17q21.32, 12q13.13	All MDS
miR-150	19q13.33	Del(5q), all MDS
miR-520c	19q13.41	Del(5q)

miR-525-5p	19q13.41	All MDS
miR-507	Xq27.3	All MDS

The differential expression profile that has been described for miRNAs in MDS may reflect analteration in the miRNAs biogenesis. Thus, the enzymes involved in this process, such as DICER1, would be damaged, resulting in deregulation of miRNAs as shown in some studies. Therefore, a better understanding of the mechanisms that control the differential expression of these miRNAs in MDS and its functional consequences could improve the understanding of the pathogenesis of this disease.

2.2. Apoptosis

Apoptosis is an ordered cellular process that regulates cell proliferation size in a variety of conditions. First described in 1980(56), apoptosis is an energy-dependent process morphologically characterized 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 (57-59). This death mechanism is crucial in maintaining a number of cells in an organism. Alterations in apoptosis have been implicated in a variety of medical disorders including MDS.

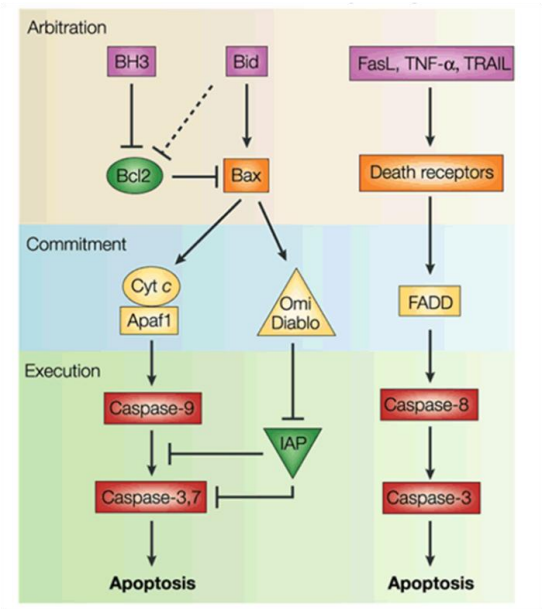

Figure 4. Intrinsic (left) and extrinsic (right) apoptosis pathway(60)

The process of apoptosis may be conceptually divided into **extrinsic** and **intrinsic pathways**.

Extrinsic activation of apoptosis is mediated by the binding of death ligands (e.g., Fas, TNF-α, and TRAIL) to cell surface transmembrane receptors (61-64). By contrast, cellular stress and damage may initiate **intrinsicactivation** of procaspases. In this pathway, mitochondria are stimulated to release cytochrome c into the cytosol where it forms a complex with Apaf-1, procaspase-9 and dATP (65, 66). This complex then triggers downstream effector caspases.

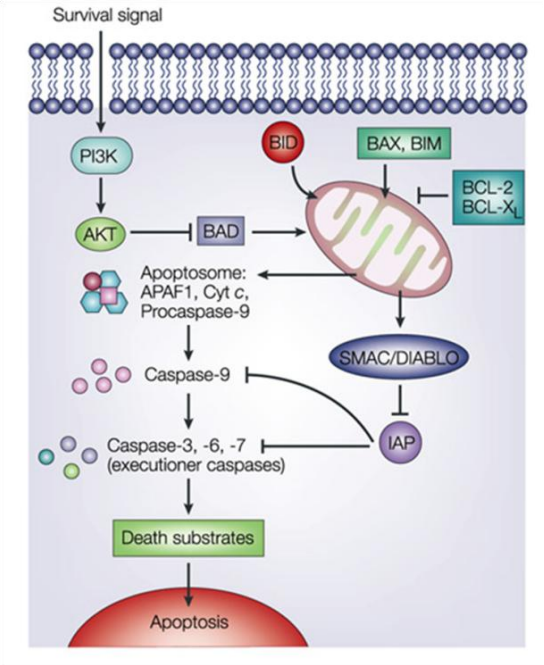

Figure 5. Bcl-2 in the apoptosis pathway (67)

The Bcl-2 family of intracellular proteins includes many of the most important regulators of apoptosis (Figure 5) (68). Some proapoptotic members, such as Bad, act by binding and inactivating death inhibiting members of the family (63). Other apoptotic molecules, such as Bax and Bak, stimulate cytochrome c release from the mitochondria. They form homo- or heterodimers that create membrane pores or ion channels that facilitate the release of cytochrome c and other apoptogenic proteins (69-72). Some members of this family, such as Bcl-2 and Bcl-X_L, inhibit apoptosis by blocking the release of cytochrome c. They may directly bind and sequester cytochrome c and Apaf-1 or interact with Bax or Bak, thereby inhibiting pore formation (73-76).

Increased apoptosis in MDS has been shown by morphology, inmunohistochemistry, flow cytometry and the molecular detection of activated apoptosis-related proteins (77). In 1995, an increased apoptosis inearly MDS patients was proven for the first time (78, 79). These studies were corroborated by the analysis of CD34+ fraction (80) and non-clonal cells(81).

The cause of abnormal apoptosis in MDS is unknown. The question of whether this increased caspase activity is due to the activation of either the extrinsic or the intrinsic apoptotic route has not yet been answered and, for this reason, both pathways are being investigated in ongoing research.

2.2.1. Alterations in the apoptosis extrinsic pathway

Some authors suggest extrinsic signals as the main reason for apoptosis and changed proliferation patterns in MDS. It has been described that death ligands (TRAIL, Fas) are implicated in the apoptosis deregulation of MDS(82). This is the case ofTRAIL which blocks the differentiation of erythroblasts in low-risk MDS determining the severity of dyserythropoiesis and anemia (83).Other studies have reported that Fas is over-expressed in about 40% of early MDS marrows anda negative correlation between the intensity of Fas expression on CD34+ cells and the blast cell percentage has even been found(84). Fas increases apoptosis and decreases colony growth equally in RARS and controls. However, it causes a significantly higher caspases activation in RARS (85). Several other studies reported higher Fas-induced caspase 8 activity in RA and RARS marrows than in normal and advanced MDS (86, 87).

On the other hand, there are conflicting findings regarding the cell death receptors Fas, TRAIL and TNF-α. Some authors have reported that these molecules are over-expressed and trigger apoptosis in erythroid cells inearly stage MDS erythroid cells(88-91). By contrast, other studies suggest that blocking the Fas receptor by antagonists antibodies does not rescue ARSA cells from apoptosis (85).

2.2.2. Alterations in the apoptosis intrinsic pathway

Intrinsic signals have been recently suggested as the main reason for apoptosis and changed proliferation patterns in MDS (92). In addition, the relevance of mitochondria in MDS pathogenesis has also been underlined (93). Some studies have analyzed the expression of pro- versus anti-apoptotic Bcl-2 family members, as these proteins control mitochondrial membrane potentials. The C-Myc:Bcl-2 ratio was higher in early MDS samples and lower in late

MDS and AML samples. The ratio of the pro-apoptotic Bax to anti-apoptotic Bcl-2 was increased in early stage MDS but decreased as the disease advanced(77, 94, 95). This observation supports the hypothesis that the relative balance between cell-death and cell-survival signaling is associated with the increased apoptosis observed in MDS progenitors. Thus, increasing apoptosis in MDS may be related to alterations in Bcl-2 family members; however, the causesof this deregulation remain unknown.

2.3. Transcription alteration

Transcription factors (TF) are proteins that bind to the promoter regions of several genes to modulate their expression and regulate the protein production encoded by these genes. The TF are fundamental in processes such as the choice of lineage, maturation and cell self-renewal. These processes play an important role in hematopoiesis and, therefore, in the development of hematologic malignancies (96).

Some TF may be involved in chromosome rearrangements, like*EVI1*, located in 3q26. MDS patients can suffer translocations or inversions at this level that can lead to inappropriate *EVI1*expression (97). The abnormal *EVI1* expression in hematopoietic cells is involved in the development and progression of MDS (98). Also EVI1 represses *EPO* receptor expression in animal models, which may partly explain the lack of response to EPO in someMDS patients(99).

The formation of megakaryocytes and their complete differentiation into platelets depends particularly on theexpression level of *GATA1*(100). *GATA1* expression is deregulated in MDS and it is possible that alterations in methylation could be responsible for this alteration. This event could be contributing to the ineffective erythropoiesis in MDS (101). Finally, mutation of some TF may cause changes in its expression, such as*AML1* (*RUNX1*) in MDS (102).

In short, there are many TF that have not yet been described and may be deregulated in MDS and there are several causes that may lead to this deregulation (chromosomal rearrangements, methylation, mutation ...). Therefore, a study of the possible causes as well as the targets which a specific TF influences could provide new data about the pathogenesis of MDS.

2.4. Immune system deregulation

There is growing evidence that immune deregulation plays an important role in the pathophysiology of MDS. The incidence of autoimmune disorders appears to be increased in these patients (103) and in fact, in a study with a large number of cases, it was found that 63% of the analyzed cases showed an abnormality related to the immune system (104).

The most frequently describedalterations are hypergammaglobulinemia and quantitative and functional alterations of T lymphocytes and NK cells (105).Several studies have demonstrated the existence of polyclonal expansion of T lymphocytes (CD4+) or clonal or oligoclonal expansion of cytotoxic T cells (CD8+) in blood and BM of these patients (106, 107).This data led to propose that myelosuppression observed in MDS could be due to an autoimmune process mediated by T lymphocytes (108, 109).

Some clinical studies have shown that treatment of certain patients with antithymocyte globulin (ATG) and cyclosporine may be effectivefor MDS patients (110-112). The agent ATG has resulted in complete hematologic responses in up to 10-15% of MDS (111) and it has been associated with the disappearance of T cell clones (113). Thus, these and other studies claim that immunomodulation may be effective in the treatment of selected patients with MDS (114).

However, fundamental questions about the mechanisms of autoimmunity in MDS remain unanswered. The hypothesis that T lymphocytes attack the antigen-specific progenitor cells of MDS remains unproven. Furthermore, it is unclear why some patients respond to immunosuppression and others not.

2.5. Angiogenesis alterations

Angiogenesis is the process by which new blood vessels are formed from pre-existing vessels. Under normal physiological conditions, thisoccurs during embryonic development, in wound healing processes, in blood flow restoration of damaged tissue, in the cyclic reconstruction of the endometrium and in the placenta during pregnancy (115). Alterations in this process are associated with the growth, spread and metastasis in solid tumors (116) and with hematological malignancies, although the latter seems to develop differently from solid tumors (117, 118).

Most of the studies show that angiogenesis has a higher expression in MDS than incontrol individualsandsome studies show that the BM microvasculature of these patients appears to be higher than in people without hematological malignancies(Figure 6) (117, 119-121). Furthermore, it has been reported that plasma levels of angiogenic factors such as VEGF, bFGF or HGF are increased in patients with MDS (117, 122), while ANG and IL-6 levels are higher in high-risk MDS with respect to low-risk patients (119).

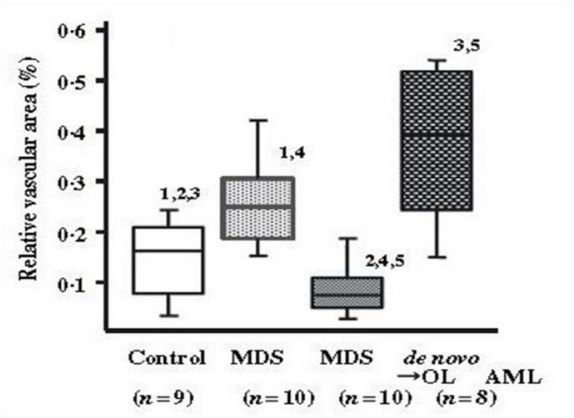

Figure 6. Comparison of microvascular density between control BM, MDS and AML (120)

It has been shown that the BM microvessels of patients with RAEBt are higher than in RA, RARS or RAEB patients (123). By contrast, other studies have described the formation of endothelial colonies for patients with RA, RARS and RAEB but not for RAEBt patients (124) and a negative relation between the microvasculature and the risk of transformation to AML (125). Therefore, the alteration of angiogenic processes is evident in MDS, although no functional works exist that delve deeper into the study of the different entities of MDS.

There are many molecules involved in angiogenesis, but endoglin (ENG) has recently acquired a relevant role in this process, and therefore, in the development of this thesis. ENG is an integral membrane glycoprotein whose properties have become a marker of tumor angiogenesis and a prime target for anti-angiogenic therapy (126, 127). Studies with endothelial cells from vessels of different tumors have shown a correlation between the increase in *ENG* expression and tumor endothelial cell proliferation (128). However in prostate cancer cells, ENG suppresses cell adhesion, motility and invasion and its expression decreases in tumor progression (129, 130).

On the other hand, although ENG is a membrane protein, low levels of soluble protein (sENG)can be found in extracellular medium, probably due to a proteolytic cleavage (131). It has been shown that sENG causes endothelial dysfunction, inhibits the “in vitro” capillary tube formation and increases vascular permeability(132, 133). The levels of the soluble form of ENG can be increased in certain solid tumors such as breast cancer (134). Furthermore, high levels of sENGseem to correlate with the risk of developing metastases in these patients as well as in patients with lung and colon cancer (135, 136); therefore, these studies indicate that sENG could serve as a prognostic marker of tumor progression in several cancer types (124). Other studies show a high concentration of the soluble protein in AML and chronic myeloproliferative neoplasms (137).

2.6. Changes in iron and mitochondrial metabolism in RARS

Refractory anemia with ringed sideroblasts (RARS) is a subtype of MDS that presents isolated anemia, hypochromic erythrocytes, hyperplastic ineffective erythropoiesis and iron accumulation in mitochondrial erythroid precursor cells. Thisiron accumulation (ring shaped sideroblasts around the nucleus), seem to be involved in the increased apoptosis of erythroblasts and, therefore, in ineffective erythropoiesis (138). However, the molecular basis of this abnormal iron accumulation, of the defects in mitochondrial functions and of the ineffective heme biosynthesis in RARS remains unknown.

Iron is essential for heme synthesis and Fe-S cluster biogenesis in the erythroid cell. Both processes take place in the mitochondria. Heme synthesis is initiated in the mitochondrion by the enzyme d-aminolevulinic acid synthase (*ALAS*), which catalyses the first step in the process. Other enzymes, such as aminolevulinic acid dehydratase (*ALAD*), catalyze the intermediate steps of the synthesis and finally, the iron is incorporated into protoporphyrin IX by ferrochelatase (*FECH*) for heme formation (104, 139, 140).It has been speculated that an enzyme defect of the heme synthetic pathway leads to a shortage of heme precursors in sideroblastic anemia. In fact, altered expressions of heme biosynthesis and mitochondrial genes have been discovered. In addition, gene expression profiling showed that RARS patients constitute a relatively homogenous group due in part to the altered expression of this set of genes(Table 10) (141).

Table 10. Genes differentially expressed in RARS(141)

	RA	RAEB	RARS
Heme pathway genes			
FECH	1.35 (0.69-3.89)	1.07 (0.45-2.02)	2.20 (0.79-5.78)
ALAS2	2.00 (0.65-20.44)	1.60 (0.60-29.17)	12.77 (1.16-104.4)
ALAD	1.12 (0.74-2.84)	0.97 (0.66-2.19)	1.93 (0.88-4.90)
HMBS	1.10 (0.60-2.43)	0.94 (0.52-2.02)	2.41 (0.89-8.39)
UROD	1.03 (0.43-2.24)	0.91 (0.30-2.22)	1.73 (0.87-2.83)
Other erythroid genes			
GATA1	1.22 (0.79-2.38)	0.90 (0.52-2.21)	1.91 (0.80-3.36)
CA2	1.15 (0.17-8.97)	0.88 (0.12-2.63)	1.90 (0.28-10.36)
EPO-R	1.40 (0.70-3.36)	1.02 (0.41-2.70)	2.08 (1.19-4.66)
Mitochondrial genes			
CGI-69	1.31 (0.75-3.09)	1.49 (0.77-4.71)	3.03 (1.23-9.33)
TRAP1	0.92 (0.42-1.59)	0.93 (0.43-2.58)	1.62 (0.79-2.75)
TIMM10	0.96 (0.59-1.90)	0.93 (0.39-2.24)	1.64 (0.65-2.79)

The average ratio in each group is given with the range in parentheses.

Upon arrival at the mitochondrion, Fe3+ must be converted into Fe2+. Mitochondria are capable of catalyzing this conversion,and there is experimental evidence thatthe iron reduction is carried out by the respiratory chain complex IV (cytochrome c oxidase)(142, 143). The Fe2 + is the only form that can be used by the enzyme FECH so if this conversion did not occur, iron would accumulate in the mitochondrial matrix (104). Based on this idea, some authors propose a model of the pathogenesis of RARS that postulates a defect in mitochondrial iron metabolism and not in the heme synthesis (144).The first evidence of respiratory chain dysfunction in patients with RARS was described in 1980 (145).It was observed that cytochrome c oxidase and oligomycin-sensitive ATPase, both components of the respiratory chain, had reduced activity in RARS patients.In addition, several mitochondrial DNA point mutations that could be playing a role in the RARS disease have been identified(93, 146-148).

The iron that is not incorporated into the heme synthesis is transported outside the mitochondria by *ABCB7* membrane protein (139).The under-expression of *ABCB7* in RARS patients (146), along with the analogy of this entity with the hereditary syndrome X-linked sideroblastic anemia with ataxia (XLSA-A), in which the gene is mutated, caused*ABCB7*to beproposed as a causative candidate of the sideroblast formation of these patients (149).However, mutational analysis revealed that *ABCB7* does not change in patients with acquired RARS(149, 150).

In addition, some studies have shown mitochondrial Ferritin over-expression and a highly significant relationship between the percentage of mitochondrial Ferritinin erythroblasts and the percentage of ring sideroblasts. These findings suggest that the majorityof the iron accumulation is present in mitochondrial Ferritin form (151, 152).

3.Application of new technologies to the study of MDS

3.1. High density microarrays

3.1.1. Expression arrays

The transcriptome or gene expression profile is the total set of genes expressed or transcribed from genomic DNA and determines the phenotype and cellular function.Expression microarrays allow the whole transcriptome study as well as the study of its alterations because they allow the simultaneous analysis of thousands of genes in a single experiment.

The introduction of microarray technology in the mid-90s represented a turning point in the study of cancer. During the last decade, this type of approach has allowed the classification of some neoplasias and the definition of new entities through differences between expression profiles.Furthermore, the arrays have allowed the correlationof some genetic signatures of tumors with disease prognosis and the response to treatment.The expression profiles have also been applied to the analysis of tumor progression, in order to identify genes specifically activated or inactivated during the different stages of the tumor.Finally this technology has helped to identify therapeutic targets in the management of cancer patients. Investigators have studied the expression of several thousands of genes in a variety of cancers, including diffuse large B cell lymphoma, follicular lymphoma, AML and MDS (153-160).

The gene expression profilestudy has facilitated the differentiation of the MDS from the AML(161), the low-risk MDS from the high-risk (162), patients with t-MDS from those whose disease is stable (163)as well as cases with complex aberrant karyotype from MDS without chromosomal abnormalities. In addition patients with 5q-, trisomy 8 and -7/del (7q) have a characteristic and differential expression profile (Figure 7) (141, 164).

Studies of microarrays have allowed us to know that the most deregulated pathways in MDS include interferon signaling, thrombopoietin signaling and Wnt pathways (164)as well as the genes encoding growth factors, proteins involved in the redox regulation and membrane proteins (161). Patients with low-risk MDS have an increased susceptibility to cellular damage (159) and an under-expression of the genes associated with B-cell lineage (165), while high-risk caseshave over-expression of proliferation genes(162). In addition,apoptosis, immunodeficiency and chemokines are deregulated pathways in MDS while advanced MDS are characterized by deregulation of the response to DNA damage (164).

Expression profiling studies have shown that the *DLK* gene is expressed in most cases of MDS and rarely in AML cases(161). Additionally, some studies suggest that altered expression of *TACSTD2*, *UQCRC1*, *TNNC* and *KDELR* genes in CD34+ cells is predictive in low-risk MDS patients (159) and that there is a correlation between increased expression of *FLT3*and blast percentage, while the increased expression of *ARG1*,*LCN2*and *MMP9*is associated with a lower percentage of blasts.It has been demonstrated that specific expression of some genes in different stages of the disease may have prognostic significance (166)becauseit can predict leukemic transformation in some situations (167).

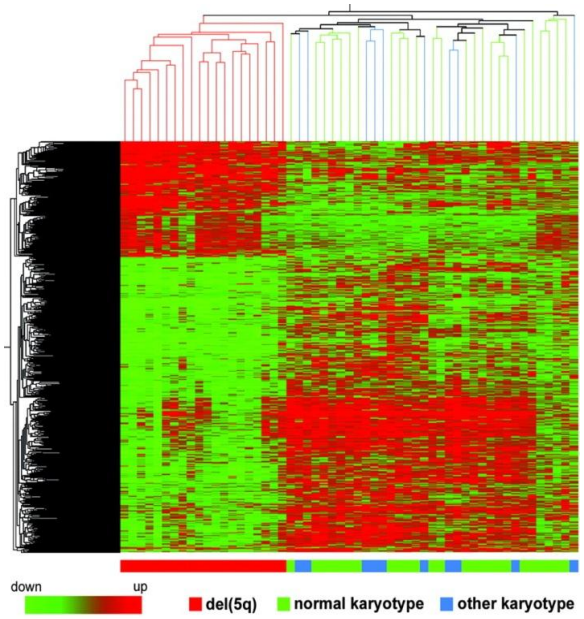

Figure 7. Unsupervised analysis with the differentially expressed genes in patients with del(5q), MDS patients with normal karyotype and MDS with different cytogenetic abnormalities than 5q-, demonstrating a distinct genetic profile in patients with 5q-(141)

In summary, gene expression studies of progenitor cells or neutrophils from MDS patients have underlined the heterogeneity of the disease at a molecular level, differences in gene expression between low-risk and high-risk disease and differences among specific cytogenetic subcategories of the MDS. In addition, microarray analysis can provide sufficient data to detect genes or gene patterns, which are associated with alterations of specific cellular pathways or signal cascades in MDS.Identification of “specific molecular signatures” holds promise for further advances in predicting prognosis and response to therapy.

3.1.2. Methylation arrays

During the last decade, interest in DNA methylation has grown rapidly; therefore, methods of analysis have advanced very well. Thus, the analyses that were restricted to specific locations in a limited number of genes, are now combined with the large-scale analysis of the whole methylome (168).

These high output analyses are currently used to identify methylation signatures of different tumors that may be useful in the early detection of disease or for the diagnosis.These technologies can also help to improve the knowledge of the patients’ progress allowing a rational basis for epigenetic therapy to be established.

In 2007, the amplification technique of methylated CpG islands (MCA) was combined with microarray technology resulting in MCAM methylation arrays. This method, used for the developmentof this thesis, permitsthe analysis ofthe methylation of 12192 CpG islands throughout the whole genome.It also provides information about hyper-methylation and hypo-methylation in promoters and 5 'and 3' regions (169).

Methylated CpG islands are selected using oligonucleotides after two rounds of digestion: First, genomic DNA is digested by SmaI, which eliminates unmethylated sites and, then, XmaI creates sticky ends in the methylated fragments. Later, and thanks to the union of the adapters, the DNA is amplified and the resulting amplicons (methylated fraction of the genome), are marked with fluorochromes. Finally, competitive hybridization is performed between the patient sample and a control sample on a microarray platform (Figure 8) (169).

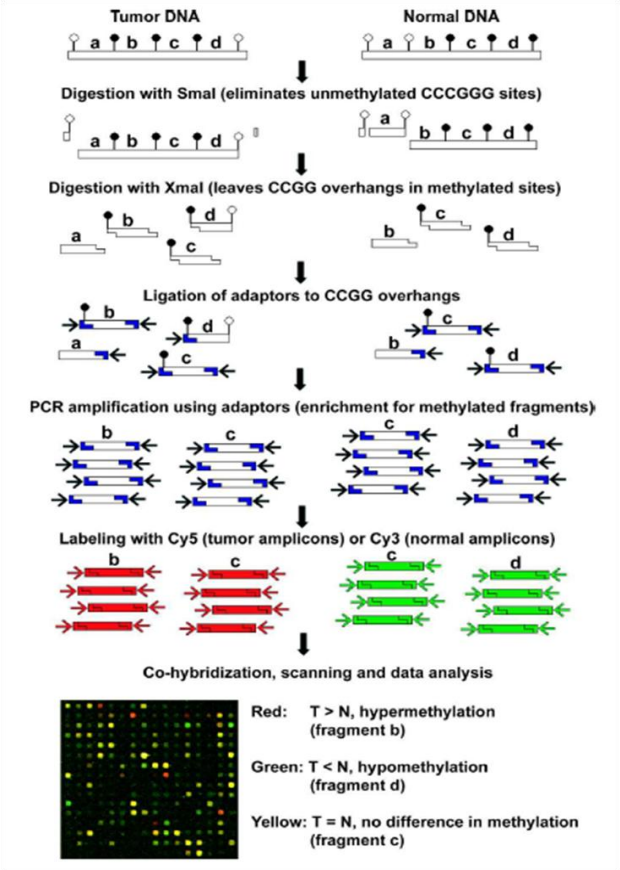

Figure 8. Scheme on MCAM method (169)

The methylation pattern of MDS has not been as well studied as other hematological malignancies and most studies have focused on analyses that are restricted to a limited number of genes. However, it is interesting to note some recently published works on the methylation profile analysis in MDS which highlight the importance of such studies for the understanding of the disease.Specifically, these studies show an increased methylation in MDS in relation to healthy controls (170)as well asin t-MDS in relation to the rest of MDS(41). Differentially methylated genes in MDS seem to be related to WNT and MAPK pathways (167).It has been reported that there is an association between the methylation status of the *P16*, *CHK2*, and *WT1*genes and IPSS (171) and a worse prognosis is attributed to patients who have a loss and methylation of the*FZD9* gene (41).Furthermore, it has been observed that methylation in MDS patients treated with azacitidine decreases with respect to the initial phase (170).

3.2. Massive sequencing

In 2001, the first draft of the human genome was published, which cost nearly 3,000 million dollars. These costs, unacceptable for any lab, stimulated scientists to look for cheaper solutions. In this search, second generation sequencers were developed, capable of generating hundreds of thousands of parallel sequencing reactions due to the immobilization of reactions on a solid surface. Specifically, the technology used in their model by Roche GS-FLX (used in this thesis) allows reading of up to 100 million bases in approximately 4 hours at an affordable cost.

Massive parallel sequencing is based on pyrosequencing DNA and the steps taken with this technology are outlined below.

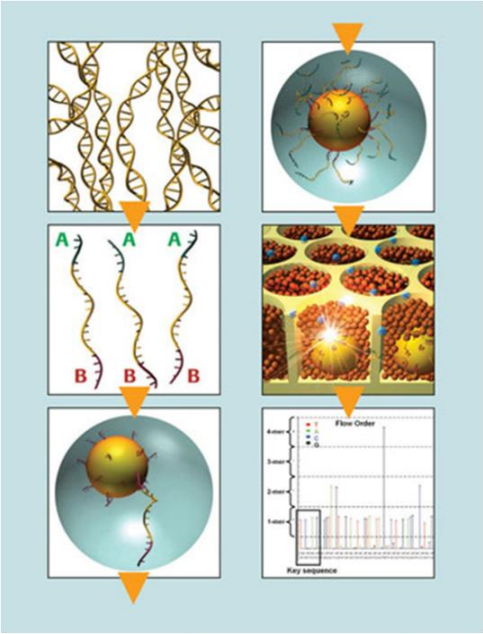

Figure 9. Scheme of massive sequencing technique.
From http://www.genengnews.com

1- DNA library preparation

The preparation of the DNA library is the genomic DNA fractionation (gDNA) into small fragments (from 300 to 500 bp) which are subsequently polished (blunt ends) and linked to adapters (A and B). These adapters provide the necessary hybridization sequences for subsequent amplification and fragment sequencing of the library. Furthermore adapter B is

biotinylated at its 5'end, which allows the immobilization of the library on top of streptavidin coated beads.

2- Emulsion PCR

Each sphere contains a single molecule of single-stranded DNA from the library. The sphere tied to the library is emulsified with the amplification reagents in a water and oil micelle, so that each one is included in its own microreactor, in which PCR amplification is carried out. The result is an immobilized sphere containing clonally amplified DNA fragments.

3- Sequencing

The spheres tied to the DNA fragments are distributed in layers together with enzyme spheres containing luciferase and sulfurylase in the sequencing plate. Each well of the plate contains a single sphere with thousands of copies of the same DNA fragment. During the nucleotides flow, each of the hundreds of thousands of spheres with millions of DNA copies is sequenced in parallel.If a nucleotide is complementary to the template strand in a well, the polymerase extends the existing DNA strand by the addition of nucleotide(s). The addition of one (or more) nucleotide(s) results in a reaction that leads to a light signal that is collected by the CCD camera equipment.The signal intensity is proportional to the number of nucleotides incorporated in a single nucleotides flow. This generates an image which is analyzed and interpreted in the form of nucleotides sequences (Figure 10).

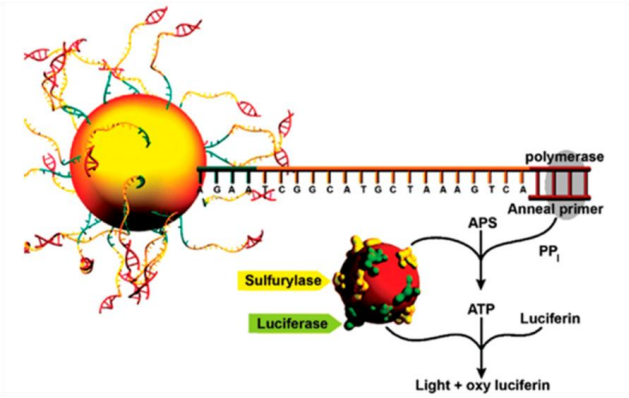

Figure 10. Last step in the massive sequencing technique: Pyrosequencing(172)

Most mutations described to date in MDS are rare and can be found in other myeloid malignancies.There are mutations in tumor suppressor genes, oncogenes, genes involved in

cell cycle regulation, apoptosis mechanisms or "splicing" (173).These mutations may be involved in the development of the disease, although their role in the pathogenesis or progression of MDS is sometimes unclear.

Most known mutations in MDS until today (*TET2*, *AML1*, *TP53*, *JAK2*, *FLT3* or others) were detected using conventional sequencing techniques such as Sanger sequencing.However, in recent years, massive sequencing techniques have been applied to the study of several hematological malignancies, including MDS, and have thus allowed the definition of the presence of new mutations in genes that may be involved in these diseases (174-176). Between 2011 and 2012, more than ten different workshave been published that have been a milestone in the study of MDS (177-189). In all of them, the massive sequencing hasallowed the analysis of hundredsof MDS patients and the description ofthe presence of mutations in the *SF3B1* gene, unrelated to the disease so far.*SF3B1* is part of the "spliceosome" and its mutations are clearly associated with the presence of ringed sideroblasts (frequency of 65-75% in ARSA cases).In addition, these mutations appear to be present in other types of blood disorders, such as chronic lymphocytic leukemia, although in these cases with a much lower frequency (190, 191).

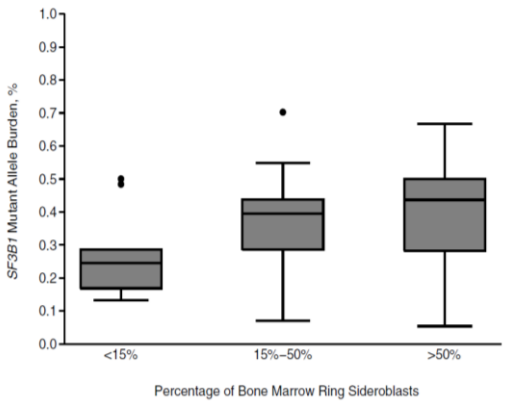

Figure 11. Relation between the *SF3B1* mutation and the proportion of ring sideroblasts(182)

It is important to highlight that the presence of mutations in this gene has been linked to less cytopenias, increased survival and less chance of progression to AML (Figure 12).

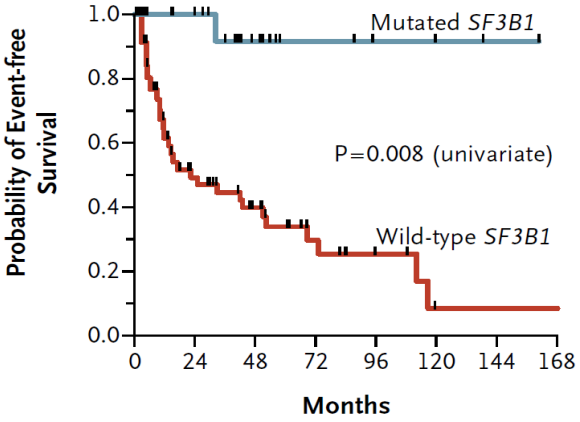

Figure 12. Kaplan-Meier curves that represent the survival of a group of MDS according to the presence or absence of mutations in *SF3B1*(185)

In conclusion, recent findings in SF3B1 by applying massive sequencing are a clear example of how this methodology can help in the study and understanding of the pathology of MDS.

Hypothesis

The low-risk MDS are a heterogeneous and diverse disease due to its complex pathophysiology. They are entities that are sometimes difficult to separate from normality and therefore are difficult to diagnose and a widely variable in clinical behavior, as befits a heterogeneous group of diseases. Therefore, we hypothesize the possibility of differentiating this group of patients by studying transcriptome, genome and methylome beyond morphological and cytogenetic differentiation used for diagnosis. To do this, the application of new techniques for overall genetic and epigenetic analysis could be a useful tool.

In the last decade, the introduction of microarray technology has allowed the differentiation of MDS from AML, from other hematological malignancies and even from those patients with MDS in transformation. However, they have been more difficult to distinguish from normal BM. Our group, as part of the MILE project (Microarray Innovations in Leukemia), has carried out studies in which MDS were differentiated from other fifteen hematological malignancies through its gene expression profile. However, low-risk MDS have been less studied, so the study of the characteristics that distinguish these patients from normality is of great interest, and constituted a part of the hypothesis of this thesis. In addition, our group suggested that the differences found at transcriptional level could be due to changes in their methylation profile. The possibility of doing a combined study, epigenetic and transcriptional, in the same group of patients, can reveal how the differential expression is influenced by aberrant methylation, which has not been investigated in low-risk MDS so far.

One of the cellular functions usually affected in MDS is the alteration in the process of angiogenesis. However, there are still some contradictions about the mechanisms and processes involved in abnormal angiogenesis in these patients. Our group suggested that functional studies in which the BM microenvironment was involved, the differentiation of this process between the different entities of MDS and the analysis of the role of ENG, could help to better understand the biology of the process in these patients.

Massive sequencing technology has recently allowed the identification of *SF3B1* mutations, which have been related to the presence of ringed sideroblasts in MDS. However, there is only one work linking functional alteration of this gene with the occurrence of the ringed sideroblasts. In addition, the presence (in lesser extent) of *SF3B1* mutations in other hematological malignances without ringed sideroblasts has not been able to be justified. Therefore, we decided to analyze the presence of mutations in new genes related to iron and

mitochondrial metabolism that might be involved in the pathophysiology of MDS with ringed sideroblasts.

All these questions have encouraged us to carry out this project, whose objectives are set out below.

Aims

The general aim of this thesis was to improve understanding of the pathophysiological mechanisms of low-risk MDS through a combined analysis of the transcriptome, methylome and genome in the bone marrow of these patients.

Specific aims

1.To analyze the differences at gene expression level between low-risk MDS and patients without hematological malignancies.

2. To identify the functions and signaling pathways mainly deregulated in low-risk MDS.

3. To define the methylation pattern in low-risk MDS.

4. To determine the genes that may have affected their gene expression due to aberrant methylation through a combined study of the expression and methylation profiles in the same group of patients with low-risk MDS.

5.To broadenknowledge of the mechanisms involved in angiogenesis in low-risk MDS and to carry out functional studies to define the differences between the different types of MDS.

6.To analyze the role of endoglin, recently described as an important angiogenic marker in low-risk MDS.

7.To characterize the subgroup of MDS with ringed sideroblasts through gene expression and massive sequencing studies.

8. To determine the presence of mutations in genes involved in iron and mitochondrial metabolism in MDS with ringed sideroblasts.

Results

1

Genome-Wide Profiling of Methylation Identifies Novel Targets with Aberrant Hyper-methylation and Reduced Expression in Low-Risk Myelodysplastic Syndromes

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2

Alteration in Endoglin-Related Angiogenesis in Refractory Cytopenia with Multilineage Dysplasia

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JMHR and ARB contributed equally to this study

Abstract

The functional mechanisms involved in angiogenesis and the potential role of endoglin (ENG), recently described as a new marker for this process, have not been explored in Myelodysplastic Syndromes (MDS). In order to gain insight in MDS angiogenesis a combined analysis in bone marrow (BM) of gene expression levels, angiogenesis-related soluble factors and functional angiogenesis-related studies was carried out. Ninety-seven MDS patients and forty-two normal BM samples were studied. The morphology of the capillary-like structures originated by two endothelial cells lines in the BM environment of patients with refractory cytopenia with multilineage dysplasia (RCMD) was different from those of the remaining MDS. In addition, the BM mononuclear cells fromRCMD patients displayed over-expression of *VEGF*, *HIF1* and *FN1*while they showed reduced expression of *ENG* in contrast to the normal *ENG* expression of the remaining low-risk MDS and the high expression of *ENG* in high-risk MDS subtype. Moreover, higher soluble ENG and soluble FLT-1 levels in BM microenvironment were observed in RCMD cases, which distinguished them from other individuals. Therefore, the present study suggests that the patterns of angiogenesis are different between the MDS subtypes. The differences in angiogenesis observed in RCMD patients could be related to ENG abnormalities.

Introduction

Myelodysplastic Syndromes (MDS) are a heterogeneous group of hematopoietic malignancies, characterized by ineffective hematopoiesis, hypercellular bone marrow (BM), dysplasia of at least one lineage and cytopenias in the peripheral blood [1]. These disorders are classified according to WHO criteria, which take into account types and number of cell dysplasias, percentage of blasts and cytogenetic abnormalities [2,3]. Moreover, based on these parameters, MDS can be divided into four prognostic categories: low, intermediate-1, intermediate-2 and high risk [4]. MDS are stem cell disorders, however, some studies have recently stressed the possibility that the BM microenvironment may play a relevant role in the pathogenesis of these diseases [5]. In addition, abnormalities in signal transduction, transcription activity, cell-cycle control, epigenetic, mitochondrial DNA and angiogenesis have been related to MDS [6].

Angiogenesis is the process by which new blood vessels are formed from pre-existing vessels and it has been associated with growth, dissemination and metastasis of solid tumours [7]. In hematological malignancies, angiogenesis develops in different way than in solid tumours [8,9]. There are conflicting evidences regarding angiogenesis in MDS; some studies have proposed that BM microvascular density (MVD) increases with MDS progression [10], whereas others suggest an increased vascularity in the early but not the latter stages of MDS [11].Differences between MDS subtypes could explain these conflictingresults and hence the importance of the discrimination between the different entities of MDS.

Endoglin (ENG) is an integral membrane glycoprotein whose properties have made it a reliable marker of tumour angiogenesis and a prime target for anti-angiogenic therapy [12]. ENG serves as co-receptor for members of the transforming growth factor beta (TGF-β) superfamily of proteins [13] and a major evidence for the pivotal role of ENG in angiogenesis is that mice lacking *Eng* (*Eng-/-*) die from cardiovascular defects at mid gestation with major defects in yolk sac vasculature [13,14]. *ENG* is mainly expressed in proliferating vascular endothelium and its expression increases during tumour angiogenesis and inflammation [12,13].Elevated expression of *ENG* correlates with the proliferation of tumour endothelial cells[15] and also in hematopoietic tumours such as multiple myeloma[16] and in hairy cell leukemia[17]. The mechanism involved in the *ENG* over-expression is probably multifactorial, being hypoxia one of the most suitable candidates. In fact, many of the pathophysiological settings where *ENG* is upregulated involve hypoxic microenvironments, as is the case of tumour angiogenesis [18].

Although ENG is a membrane protein, low levels of soluble protein (sENG)can be found in extracellular medium.The appearance of this soluble protein formis probably due to proteolytic cleavage of isoform membrane as occurs withbetaglycan, which can be shed by metalloproteinase 1 [19]. sENG interferes with TFG-β signalling causing endothelial dysfunction [20]. It has been demonstrated thatsENG inhibits the capilar tube formation “in vitro” and increases vascular permeability [21].

Most of the studies of angiogenesis in MDS have been focused onmalignant hematopoietic cells but there is growing evidence that BM-derived endothelial cells may contribute to tumour angiogenesis [22,23]. In addition, clonal cells may have interactions with these BM endothelial cells and the contact between endothelial cells and normal or malignant hematopoietic cells is mediated by soluble angiogenic factors of the BM microenvironment [24,25]. Therefore the role of endothelial cells in the BM malignant microenvironment and their possible relationship with the malignant clone remains to be clarified by functional studies and not only assessed by immunohistochemistry that so far has been the most used method of analyzing angiogenic activity in MDS [26].

In order to gain insight in the mechanisms involved in angiogenesis in MDS a study of the cellular expression and the BM microenvironment levels of sENG and other angiogenic factors was carried out. The results showed marked differences in the angiogenesis in the MDS subtypes, and could open new approaches in the treatment in MDS patients.

Design and Methods

1. Patients samples

A total of 97 MDS patients and 42 age-matched controls were included in the study. Classification of MDS was performed according to the World Health Organization (WHO) criteria [3]. Twenty-nine patients were diagnosed as refractory cytopenia with multilineage dysplasia (RCMD) and forty-six had other low-risk MDS excluding RCMD: nineteen of them had a refractory anemia (RA), twenty-two had a refractory anemia with ring sideroblasts (RARS) and five patients had a 5q- syndrome. The remaining twenty-two patients had a refractory anemia with excess of blasts (RAEB: high-risk MDS) (Supplementary Table 1). The study was approved by the local ethical committees "Comité Ético de Investigación Clínica, Hospital Universitario de Salamanca". Written informed consent was obtained from each patient before they entered the study.

Mononuclear cells as well as the supernatant fluid were isolated from BM of MDS patients and controls by density gradient (Ficoll). Total RNA from cells was extracted by homogenization in TRIZOL (Invitrogen, Carlsbad, CA, USA) following the protocol supplied by manufactures, and treated with RQ1 RNAse-Free DNase (Promega, Madison, USA) to eliminate genomic DNA contamination, and finally purified with RNeasy Minikit (Qiagen, Hilden, Germany). The RNA quantity and quality was determined by Agilent 2100 Bioanalyzer (Santa Clara, CA, USA). The BM supernatant fluid was collected by centrifugation. The liquid was withdrawn carefully to avoid disturbing the cells and collected in a separate container at -80ºC.

2. BMEC-1 cell culture

The immortalised cell line, BMEC-1 (Bone marrow endothelial cells), kindly donated by Dr. F.J. Candal (Centers for Disease Control and Prevention, Atlanta, Georgia) was used in our studies [27].This cell line was generated by transfecting an early passage of primary BMEC with a vector (pSVT) encoding the large T antigen of SV40. BMEC-1 express vWF/Factor VIII and maintain a phenotype similar to that of primary cells, even at high passage number.

Cells were maintained in culture at 37ºC in a humidified atmosphere of 95% air and 5% CO_2 in Medium MCDB 131 (Invitrogen) supplemented with 15% foetal calf serum (FCS), 10ng/ml endothelial grow factor (EGF) and 1µg/ml hydrocortisone.

3. HMVEC-L cell culture

Lung-derived normal human microvascular endothelial cells (HMVEC-L) were purchased from Clonetics (Lonza Walkersville, MD, USA) and maintained exactly as recommended by the

manufacturer. Cells were cultured with the EGM-2MV bullet kit containing endothelial cell basal medium-2 (EBM-2) and the following growth supplements: hEGF, hydrocortisone, GA-1000, FBS, VEGF, hFGF-B, R^3-IGI-1 and ascorbic acid. The experiments described in this study were performed on cells between three and four passages.

4. Cell proliferation assay

Subconfluent BMEC-1 were plated in 96-well plates to a density of 5,000 cells per well. Twelve hours after plating, cells were serum starved (5% FCS) and the BM supernatant fluid from MDS patients or controls was added (1:10 dilution). After incubation during 24 h, 48 h or 72 h, Thiazolyl Blue Tetrazolium Bromide (MTT, Sigma, Illinois, USA) was added to each well and incubated for 4 h. The formazan crystals formed from MTT by the living cells were dissolved in the lysis buffer (10% sodium dodecyl sulfate (SDS); 5% isopropanol; 0.1M HCl) for 12 h, and the formazan purple solution was detected using a Sunrise plate reader (Bio-Tek, Instruments, Winooski, USA) at 595 nm. All experiments were performed in quadruplicate.

5. Endothelial cell tube formation assay

Endothelial cell tube formation was assessed as previously described Jerkic et al[28]. In briefa total of 8,000 BMEC-1 per well were plated on Matrigel® precoated plates (BD Biosciences, New Jersey, USA) and cultured in medium MCDB 131 with 15% FCS. Half an hour later, the BM supernatant fluid from MDS patients or controls was added in the wells (1:10 diluted). After seeding on Matrigel®, cells spread and aligned with each other to develop hollow, tube-like structures. Endothelial tube formations were observed each hour during seven hours of incubation and the morphological changes were photographed at 5h using a phase contrast inverted Zeiss Microscope (Carl-Zeiss, Jena, Germany). Each experiment was performed in duplicate. As a control in one of each 5 wells just culture medium was added. The experiment was performed in the same way with HMVEC-L. Likewise, a total of 8,000 HMVEC-L per well were plated on Matrigel® precoated plates. However, in this case, it was used its appropriate culture medium as abovedescribed.

6. Real-Time PCR

The expression levels of endoglin *(ENG)*, vascular endothelial grow factor *(VEGF)*, hypoxia-inducible factor 1-alpha (*HIF1*) and fibronectin (*FN1*) genes were analyzed by Real-Time PCR. First-strand cDNAwas generated from 1 μg of total RNA using poly-dT asprimers with the M-MLV reverse transcriptase (Promega). Real-time PCR was performed in triplicate. Each 20 μl

reaction contained 300ng of cDNA, 400 nM of each primer, and 1x iQ SybrGreen Supermix (Bio-Rad, Hercules, CA, USA). Standard curves were run for each transcript to ensure exponential amplification and to rule out non-specific amplification. The expression level of the glyceraldehyde-3-phosphate dehydrogenase(*GAPDH*)gene was used to normalize differences in input cDNA. Thereactions were run on an iQ5 Real-time PCR detection system (Bio-Rad, Hercules, CA, USA). The primers were designed for specific sequences and checkedby BLAST algorithm [29]. Primer sequences were as follows:

GAPDH- forward: 5´-CAG GGC TGC TTT TAA CTC TGG TAA-3´

GAPDH- reverse: 5´-GGG TGG AAT CAT ATT GGA ACA TGT A-3´

ENG- forward: 5´-AGG TGC TTC TGG TCC TCA GT-3´

ENG- reverse: 5´-CCA CTC AAG GAT CTG GGT CT-3´

VEGF- forward: 5´-CGA AGT GGT GAA GTT CAT GG-3´

VEGF- reverse: 5´-CAC AGG ATG GCT TGA AGA TG-3´

HIF1- forward: 5´-GTC ACT TTG CCA GCT CAA AA-3´

HIF1- reverse: 5´-ACC AAC AGG GTA GGC AGA AC-3´

FN1- forward: 5´-TCA CAG CTT CTC CAA GCA TC-3´

FN1- reverse: 5´-TGG CTG CAT ATG CTT TCC TA-3´

7. Enzyme-linked immunosorbent assay

In order to assess the concentration of proangiogenic soluble factors, an enzyme-linked immunosorbent assay (ELISA) was carried out in the BM supernatant fluid from patients and controls. The levels of sENG, solublefms-like tyrosine kinase 1 (sFLT-1) and VEGF were assessed by using commercially available kits from R&D Systems (R&D Systems, Minneapolis, USA). The whole protocolwas performed following the instructions given by the manufacturer.

8. Statistical analysis

The relationship between clinical or biological data and genomic characteristics was analyzed using an independent sample ANOVA test or Mann-Whitney test. All P-values reported were two-sided and statistical significance was defined as P-value < 0.05. Statistical evaluation was carried out using the SPSS 15.0 statistical software. The graphics of the study show the mean ± standard error of the mean or the median of each group that was used for the comparisons in each experiment between the different entities of MDS and the range in each case.

Results

- **BM microenvironment from MDS patients induces endothelial proliferation**

Endothelial proliferation is crucial in the process of angiogenesis. First, we studied the effect of BM supernatant fluid from MDS patients and controls on BMEC-1 proliferation, during eight days. Endothelial cells reached the peak of maximum proliferation at the sixth day (Figure 1A) and the values at this day were chosen for comparison between different subgroups.

At the sixth day, MDS BM supernatant fluid induced a significantly higher proliferation rate in BMEC-1 than the control BM supernatant fluid ($p<0.005$). Specifically, the proliferation was 2.4 times higher in MDS than controls (Figure 1A) and this significant difference ($p<0.05$) was detected in all three MDS subgroups analyzed (low-risk excluding RCMD, RCMD, high-risk) while no statistical differences in the endothelial proliferation were observed between the three groups of MDS themselves (Figure 1B).

- **BM microenvironment from MDS patients induces abnormal tube formation**

Tube formation indicates the extent of angiogenesis and is considered to be an important prognostic factor in this process. To investigate the BM supernatant fluid effects on BMEC-1 (Figure 2A) and HMVEC-L (Figure 2B) tube generation, we used a common method for gauging in vitro angiogenesis, the capillary-like tube formation assay on Matrigel®. As a control,the endothelial tube formation by BMEC-1 and HMVEC-L was maintained in culture medium (Figure 2Ai and 2Bi, respectively). The tubes seem to be completely formed after five hours of incubation, and this time was used to compare the effect of different MDS BM supernatant fluid.

When the endothelial cells lines were cultured with BM supernatant fluid from the control, there was a well organized tube formation. The endothelial tube appearance in cells treated with BM supernatant from controls and cell maintained in culture medium was similar (Figure 2A and B i-ii). By contrast, the tube morphology was strikingly influenced by BM supernatant fluid from MDS patients. Therefore, BM supernatant fluid from MDS patients induces morphogenetic changes in the endothelial tube formation (Figure 2A and B iii-viii). MDS-treated BMEC-1 and HMVEC-L tented to assemble and form aggregates along the tube-like structures, which was not observed in control cells (Arrows in Figure 2). Incubation with different BM supernatant fluid from MDS stimulated the capillary network aggregation of endothelial cells, including increasing areas covered by the cells and lengths of network compared to controls (Figure 2A and B iii-viii). It should be noted that the tubes originated

after the incubation of BMEC-1 and HMVEC-L with the BM supernatant fluid from RCMD patients almost completely disrupted the capillary networks (Figure 2 A and B vii-viii).

- **Endoglin and other angiogenic factors expression differences in RCMD patients with respect to other MDS patients**

Clonal-derived hematopoietic myeloid progenitor cells may facilitate the angiogenesis without directly participating in this process by promoting the activation of normal BMEC. To better understand the role of ENG in the angiogenesis of MDS patients, a gene expression study was performed. RNA obtained from BM mononuclear cells from MDS was used to analyze four angiogenic factors: *ENG*, *VEGF*, *HIF1* and *FN1*. Regarding *ENG* expression, no differences were observed between all MDS patients as a single group and the control group (Supplementary Figure 1A). However, marked differences in the *ENG* levels were observed in the separate analysis of MDS groups. Thus a down-regulation of *ENG* expressionwas patent in RCMD patients (p<0.05). By contrast, *ENG* expression in high-risk MDS cases was higher than in controls (p<0.05). No differences were found between the *ENG* levels of low-risk MDS (excluding RCMD) and healthy controls (Figure 3A).

Overall the expression levels of *VEGF*, *HIF1* and *FN1* in MDS were significantly higher (p<0.05) than in controls (Supplementary Figure 1B-D). Thus, the low-risk MDS groups (including RCMD) showed over-expression of *VEGF* (Figure 3B), *HIF1* and *FN1* (Supplementary Figure 2) with respect to the control group (p<0.05). Moreover, patients with RCMD showed the highest values in the expression of these three genes with respect to the other low-risk MDS (Figure 3B and Supplementary Figure 2). By contrast, no differences in high-risk MDS patients regarding *VEGF*, *HIF1* and *FN1* expression with respect to the control group were observed (Figure 3B and Supplementary Figure 2).

- **Patients with RCMD display high concentrations of anti-angiogenic soluble factors in the BM microenvironment**

To assess the levels of angiogenic and anti-angiogenic factors present in the BM supernatant fluid in different MDS groups, ELISA assays were carried out in the BM supernatant fluid from MDS patients and controls. Therefore, circulating levels of sENG and sFLT-1 as well as VEGF were analyzed.

The Figure 4 summarizes the results: RCDM displayed higher levels of sENG with respect to the controls (p<0.005), the remaining low-risk MDS (p<0.05) and the high-risk MDS patients (p=0.05). Moreover, sFLT-1 concentrations in BM supernatants were higher in RCMD with

respect to the healthy cases (p=0.001), the remaining low-risk MDS and high-risk patients (p<0.005) (Figure 4B). By contrast, the study lacked in detect differences in the concentration of VEGF in the three MDS groups analyzed (Figure 4C).

Discussion

Myelodysplastic syndromes (MDS) are clonal stem cell diseases in which altered angiogenic mechanisms have been described. In the present study, a combined analysis of gene expression, angiogenesis-related soluble factors and functional angiogenesis-related studies were carried out in bone marrow (BM) of patients with MDS. The results demonstrated marked differences in angiogenesis in the subtypes of MDS. Thus, the patients with refractory cytopenia with multilineage dysplasia (RCMD) showed an abnormal angiogenesis characterised by an increased level of soluble endoglin (sENG).

The involvement of the microenvironment in MDS disorders has been stressed. However, most of angiogenesis studies in MDS have been focused in plasma from peripheral blood, while results regarding the BM microenvironment analysis are scarce [8,30,31]. In the present report functional studies in the non-cellular portion of BM were performed. A proliferation assay showed that MDS BM supernatant fluid stimulated bone marrow endothelial cells (BMEC-1) proliferation more than supernatant fluid from controls.Our results support the aberrant angiogenesis in MDS previously analyzed by other techniques [26,32,33]. In addition, the generation of new vessels in MDS is critical in the multistep process of conversion from normal to dysplastic BM [5,33]. The endothelial tube formation by BMEC-1 and HMVEC-L in Matrigel® was performed in the presence of BM supernatant fluid from MDS patients and differences in thickness, structure and density of the formed tubeswere observed. In addition, the differences between the capillary-like structures originated by BMEC-1 in RCDM and the remaining patient groups were evident. RCMD cases showed less extensive capillary network and reduced vessel formation(Figure 2vii-viii).BM angiogenesis in MDS has been usually studied by measuring the microvascular density by immunohistochemistry [8,26,31,34]. However, this is the first time, to our knowledge, that the formation of pseudocapillaries in MDS BM supernatant fluid by means of functional techniques has been carried out. Thus, the present studies demonstrated that MDS patients displayed an abnormal angiogenesis characterized by a high endothelial proliferation and aberrant pseudocapillary formation. Therefore the BM microenvironment plays an important role in this aberrant angiogenesis.

It has been demonstrated that leukemic cells may have intimate interactions with bone marrow endothelial cells (BMEC) and can elicit the sprouting of new blood vessels from pre-existing capillaries by the active release of angiogenic factors [32,33,35]. Based on this consideration, we focused our attention on the analysis of several molecules in BM

mononuclear cells from MDS that have been reported to be involved in the angiogenesis processes and could be influencing on BMEC behavior. Thereby, we demonstrated that vascular endothelial grow factor (*VEGF*), hypoxia-inducible factor 1-alpha (*HIF1*), and fibronectin (*FN1*) expression were differentially over-expressed in low- risk MDS patients, including RCMD cases. These findings are supported by some studies where *VEGF* and other angiogenic factors were significant increase in overall MDS group [34]. In addition, the over-expression of these molecules could explain the abnormal proliferation and tube formation by endothelial cell lines in low-risk MDS.

However, angiogenesis involves two stage of vascular development: the differential growth and sprouting of endothelial tubes and the remodeling the primary endothelial network into a mature circulatory system. Endoglingene (*ENG*)encodes an endothelial transmembrane protein that is required for both processes [14,21]. As ENG stainingrepresents a powerful marker to quantify tumor angiogenesis [13] we have evaluated the expression of *ENG*in MDS cells and we have demonstrated an over-expression in the high-risk cases. *ENG* expression is elevated during alterations in vascular structure and has been associated to many cancers, including breast, ovary, prostate and cervical cancer [20]. Ascellular *ENG* levels regulatethe formation of new blood vessels [14], ENG antibodies have been successfully used to elicit anti-angiogenic effects in tumor-associated endothelium mouse models where *ENG* was highly expressed [36]. These advances will provide new approaches for the development of new therapies for high-risk MDS patients.

Interestingly, *ENG* expression was significantly lower in RCMD patients than in cell from healthy controls. This event may resemble other vascular diseases, such as the hereditary hemorrhagic telangiectasia type I (HHT). HHT patients have significantly lower ENG levels and are characterized by arteriovenous malformations and focal loss of capillaries [37]. In addition, it has been reported that isolated murine *Eng*+/**-** cells display impaired capillary tube formation and significantly less vascular structures compared to wild type mice [28]. Based on these findings, we suggest that the under-expression of *ENG* in RCMD patients could be associated with the decreased blood vessel formation *in vitro* models of angiogenesis observed in the same group of patients. Furthermore, the expression variations in the diverse angiogenic factors could play different roles in the MDS subtypes suggesting different mechanisms involved in the pathogenesis of these diseases leading to a different angiogenesis in patients with RCMD with respect to the other MDS patients.

The resultsof *ENG* gene expression led us to investigate their presence in the extracellular medium as well as the levels of solublefms-like tyrosine kinase 1 (sFLT-1) (anti-angiogenic factor) and VEGF (angiogenic factor). RCMD patients showed the highest levels of sENG and sFLT-1 in BM supernatant fluid with respect to both the other MDS and the control group (Figure 4).By contrast, the VEGF levels were similar to the controls. A high concentration of sENG has been also described in acute myeloid leukemia and chronic myeloproliferative disorders[38] and in patients with pathologies associated to vascular dysfunction[39]. Elevated circulating concentrations of sENG and sFLT-1 have been showed in the maternal endothelial dysfunction called preeclampsia. In fact, some authors suggest that sENG may act in concert with sFLT-1 to induce severe preeclampsia [21,40]. In addition, some studies have displayed that sFLT-1 binds to and neutralizes the pro-angiogenic actions of VEGF and the contributions of sENG and sFLT-1 to the pathogenesis of maternal preeclampsia are, at least in part, related to their inhibition of TGFβ and VEGF, respectively [41]. This finding could explain the mitigated VEGF secretion observed in RCMD patients in relation to the expression gene in the same group of patients. Based on our results, we suggest that the soluble form of ENG antagonizes the membrane bound form in RCMD patients and therefore potentiates the anti-angiogenic actions of sFLT-1, by disrupting the capacity to form capillary tubes of BMEC-1 and HMVEC-L as we have previously showed in this group of patients.

Angiogenesis is a balanced process between pro and anti-angiogenic factors. In MDS patients, our results suggest the presence of an altered balance that could be involved in RCMD patients. In fact, RCMD patients showed high expression levels of pro-angiogenic factors such as *VEGF*, *HIF1*, and *FN1*. In contrast, this group of MDS had low *ENG* expression, high levels of sENG and sFLT-1 in BM microenvironment, a decrease level of VEGF with respect to the expression gene and the reduced vessel formation by endothelial cell lines. Regarding the increased BMEC-1 proliferation observed in RCMD there are conflicting evidences: endothelial cell proliferation is key early event in angiogenesis, but some studies have demonstrated that myeloid malignancies with high levels of sENG are characterized by a high cellular proliferation rate in BMEC and even, in myelopoietic lineage what could explain the high proliferation in an anti-angiogenic environment [28,38]. Therefore we suggest that the RCMD display features that tip the balance of angiogenesis and appear to be impairing this process.

Previous studies have demonstrated an abnormal angiogenesis in MDS. However most of them have analyzed the differences between the low-risk and high-risk patients while the RCDM patients were not included as an independent group [26,34]. The RCMD has been recently

proposed by the WHO classification as a specific MDS disorder [2] and the present study showed these patients had a different pattern of angiogenesis. These results provide new insights in the molecular mechanisms of RCMD patients that could be ENG-related. Furthermore, recently, it has been suggested that the inhibition of putative protease involved in sENG shedding may be of therapeutic benefit in the treatment of preeclampsia [20]. These observations could provide new therapeutic approaches for this specific subtype of MDS.

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Figures

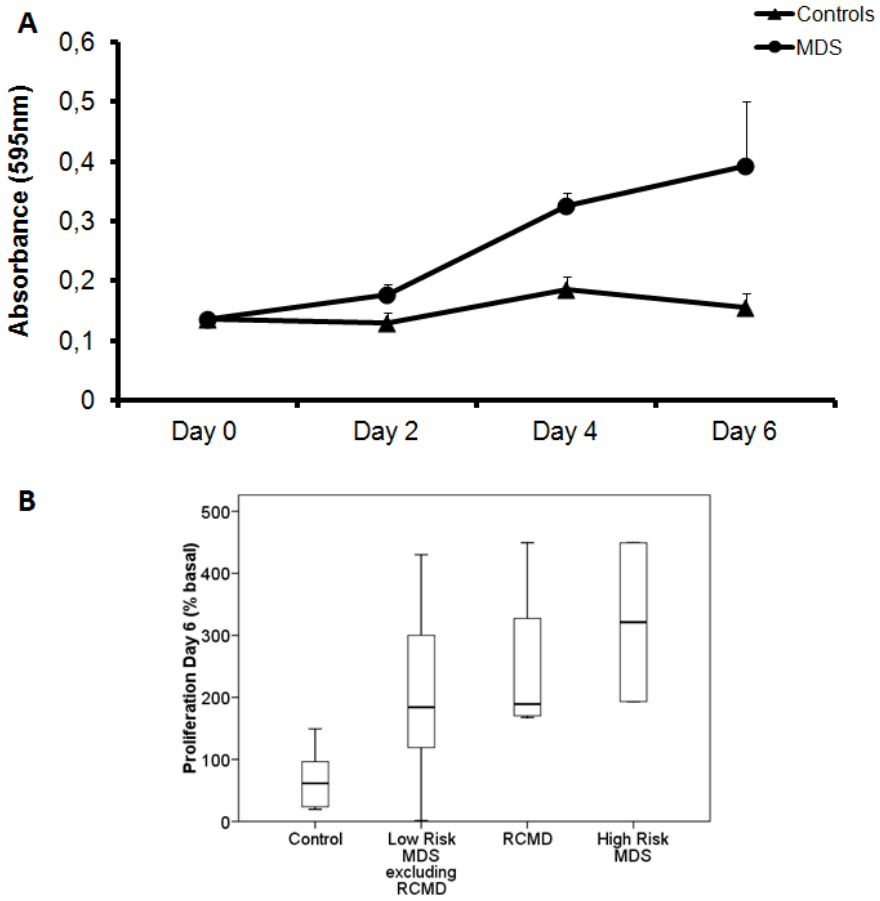


Figure 1. Effect of MDS BM microenvironment on BMEC-1 proliferation. (A) BMEC-1 proliferation curve. To analyze the effect of the BM supernatant fluid from MDS patients and controls on BMEC-1 proliferation, the cell line was incubated with BM supernatant fluid. The cell number was estimated by MTT at two, four or six days. The measurement of absorbance is indicative of the rate of cell proliferation and each value of each patient is the mean of four independent experiments.Each point is the mean of these values ± SEM.The graphics show the increase of proliferation in MDS patients. ANOVA test was used to analyze the overall MDS results at sixth day. The proliferation was 2.4 times higher in MDS than controls (p<0.005). (B) The box plot compares median levels of BMEC-1 proliferation at sixth day in the different subtypes of MDS. Whiskers represent the range. Significant differences between RCMD and the control group (p<0.01), the other low-risk MDS and the control group (p<0.05) and high-risk MDS patients and the controls (p<0.05) were observed by Mann-Whitney test.

MDS: myelodysplastic syndrome; BM: bone marrow; BMEC-1: bone marrow endothelial cells; MTT: Thiazolyl Blue Tetrazolium Bromide; SEM: standard error of the mean; RCMD: refractory cytopenia with multilineage dysplasia.

(Controls n= 8; MDS n= 14; Low-Risk MDS excluding RCMD n= 6; RCMD n= 4; High-Risk MDS n= 4)

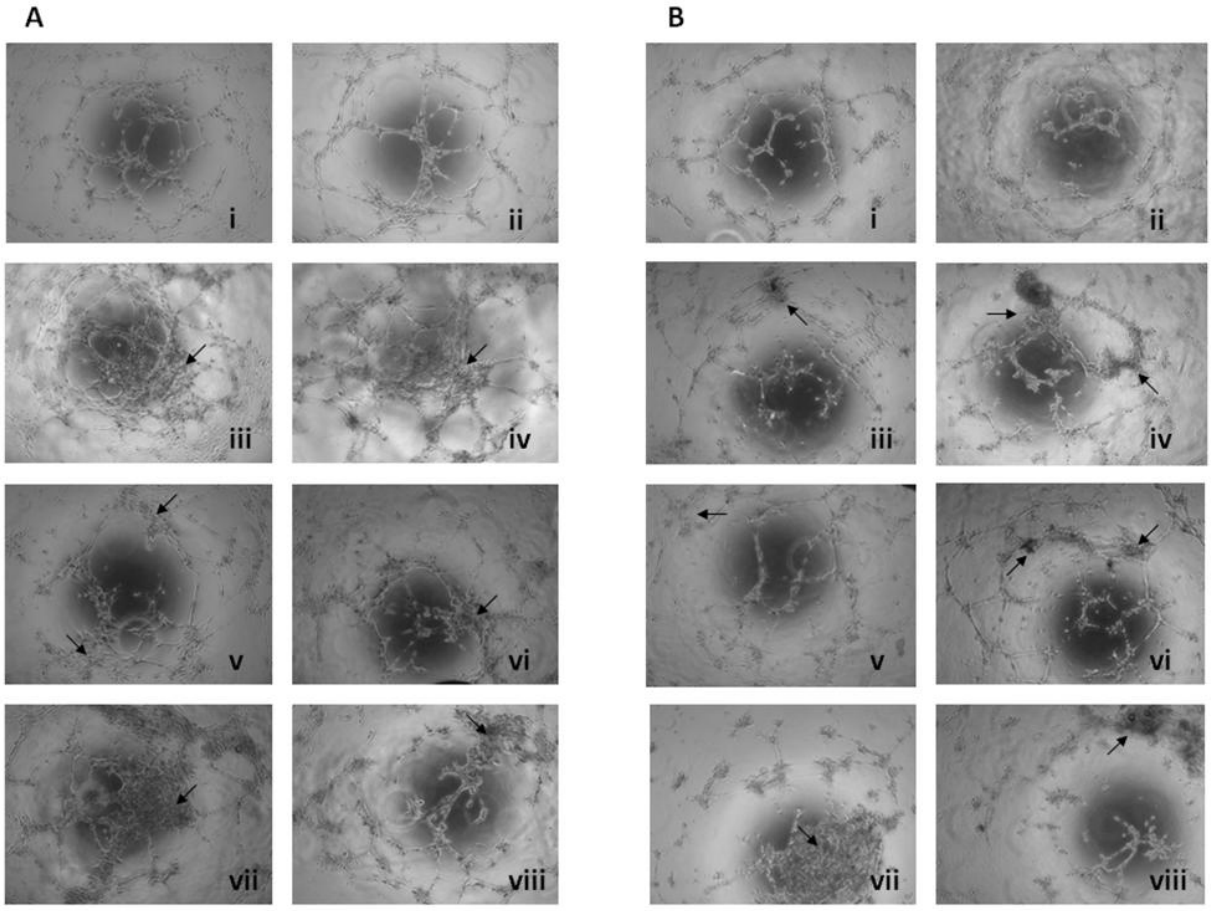


Figure 2.Effect of the MDS BM microenvironment on BMEC-1 and HMVEC-L tube formation. BMEC-1 (A) and HMVEC-L (B) were seeded at a concentration of 8,000 cells per well of 96-well plate and incubated for 7h at 37°C in 5% CO_2. The endothelial tube formation was photographed at 5h using a phase contrast inverted microscope. Each experiment was performed in duplicate. The picturesshow the appearance of endothelial cell tubes on Matrigel® precoated plates in culture medium (i) and BM supernatant fluid from healthy control (ii), RA (iii), RARS (iv), 5q syndrome (v), RAEB (high-risk MDS) (vi) and RCMD (vii-viii) patients at 1:10 dilution in culture medium. As the arrows show in the figure, the tube morphology was strikingly influenced by BM supernatant fluid from MDS (iii-viii) with respect to the controls (ii). The tubes originated after the incubation of BMEC-1 or HMVEC-L with the BM supernatant fluid from RCMD patients (vii-viii) were almost completely disrupted and formed closed capillary networks.

MDS: myelodysplastic syndrome; BM: bone marrow; BMEC-1: bone marrow endothelial cells; HMVEC-L: lung-derived normal human microvascular endothelial cells; RA: refractory anemia; RARS: refractory anemia with ring sideroblast; RAEB refractory anemia with excess of blasts; RCMD: refractory cytopenia with multilineage dysplasia.

(Controls n= 13; RA n= 5; RARS n= 6; 5q syndrome n= 2; RAEB n= 4; RCMD n= 7)

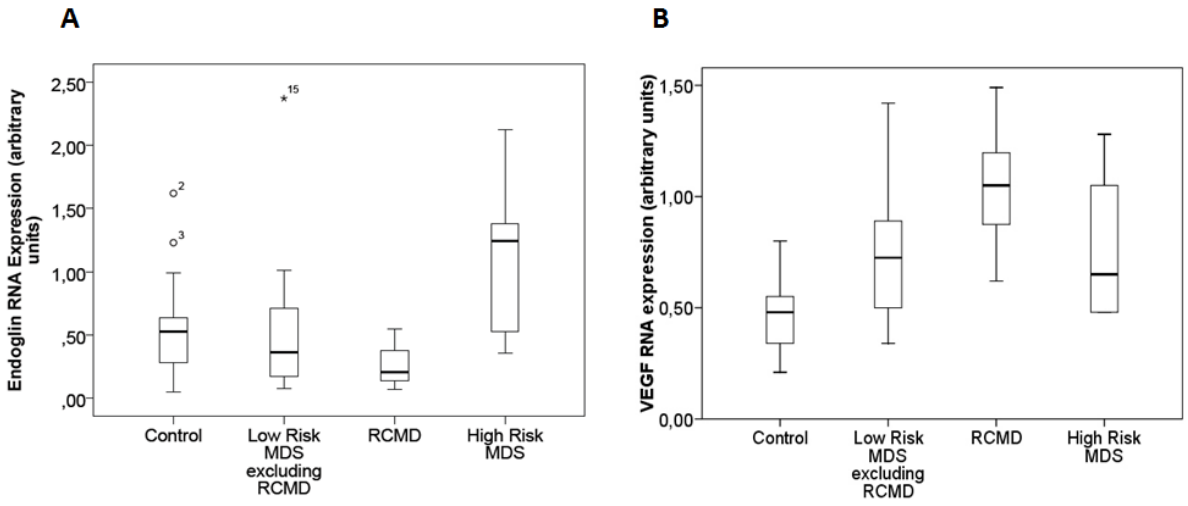


Figure 3.*ENG* and *VEGF* RNA expression in mononuclear BM cells of MDS subtypes. The box plot compares median of *ENG* and *VEGF* expression levels in BM mononuclear cells between the different MDS groups and controls. The gene expression levels were analyzed by RT-PCR. Each sample was performed in triplicate. Value of each patient is the mean of these three experiments. Mann-Whitney test was used to analyze the results. The box plot compares the RNA expression in BM mononuclear cells of subtypes of MDS. Whiskers represent the range. A down-regulation of *ENG* was showed in RCMD cases (p<0.05). By contrast, *ENG* expression in high-risk MDS patients was higher than in controls or in the other MDS (p<0.05). No significant differences in low-risk MDS excluding RCMD patients in *ENG* expression with respect to the healthy controls were found (A). The low-risk MDS groups showed over-expression of *VEGF* with respect to the control group (p<0.05). Moreover, patients with RCMD showed the highest values in the expression of this gene with respect to the other low-risk MDS. No significant differences in high-risk MDS patients in *VEGF* expression with respect to the healthy controls were found (B).

ENG: endoglin; VEGF: vascular endothelial grow factor; BM: bone marrow; MDS: myelodysplastic syndrome; RCMD: refractory cytopenia with multilineage dysplasia; RAEB: refractory anemia with excess of blasts.

(Controls n= 13; Low-Risk MDS excluding RCMD n= 22; RCMD n= 12; High-Risk MDS n= 16)

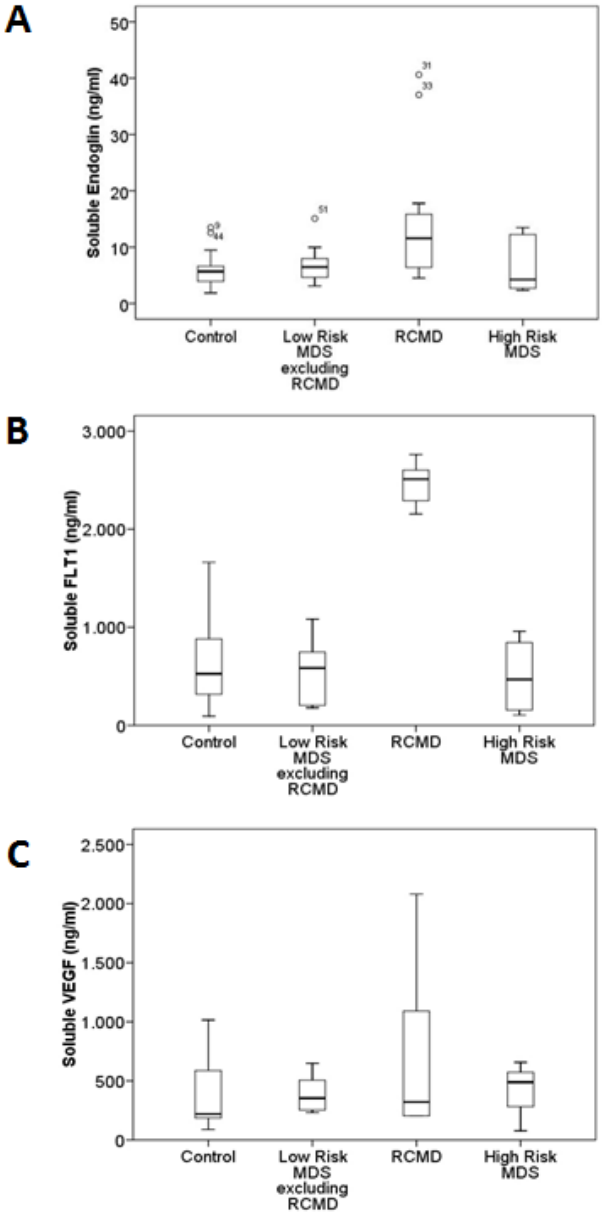
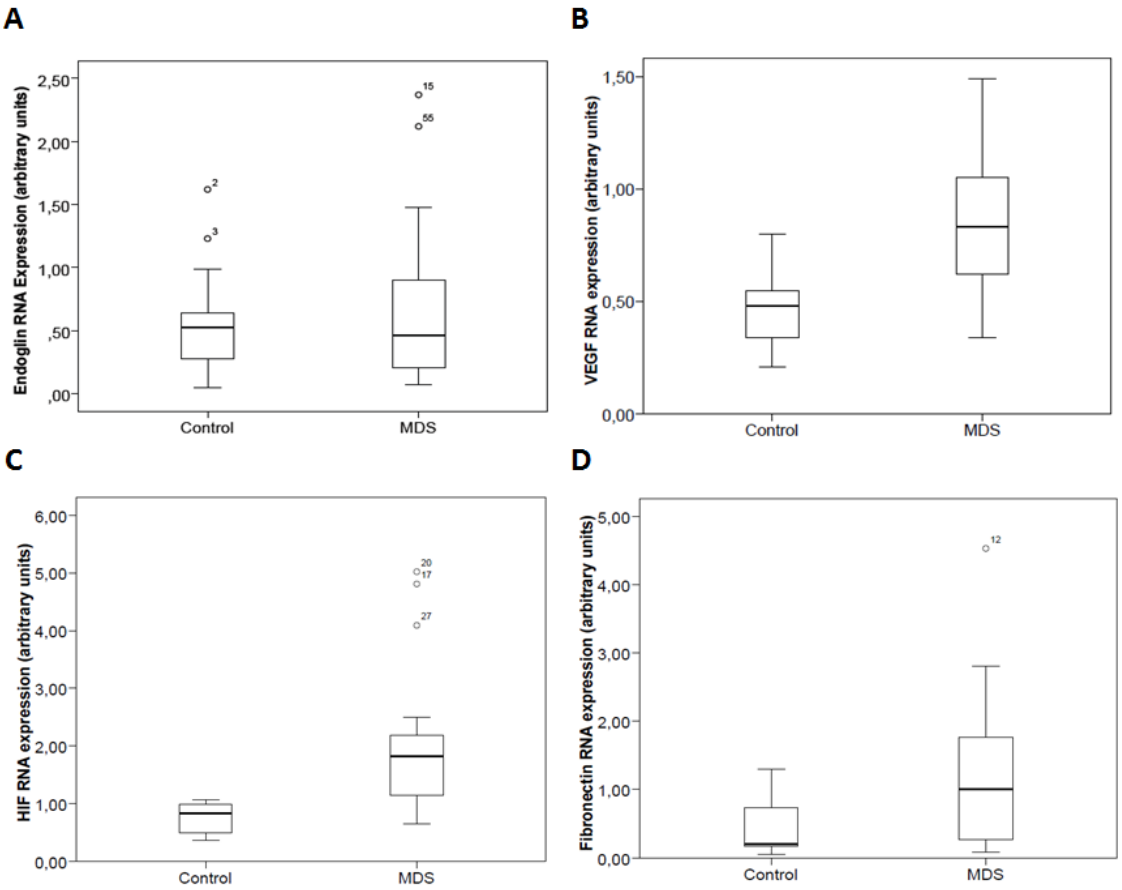


Figure 4.Soluble angiogenic factors in MDS BM microenvironment. The box plot compares median levels of sENG, sFLT-1 and sVEGF in BM supernatant fluid of various types of MDS. To measure the levels of angiogenic factors present in the BM supernatant fluid in the different MDS groups, ELISA assays were carried out in the BM supernatant fluid from MDS patients and controls. Whiskers represent the range. Mann-Whitney test showed that sENG concentrations in BM supernatants was higher in RCMD with respect to the healthy cases (p<0.005), the remaining low-risk MDS (p<0.05) and high-risk patients (p=0.05) (A). RCDM displayed higher levels of sFLT-1 with respect to the controls (p=0.001), the remaining low-risk MDS (p<0.005) and the high-risk MDS patients (p<0.005) (B). No significant differences in sVEGF concentration of MDS groups were found (C).

MDS: myelodysplastic syndrome; BM: bone marrow; ENG: endoglin; sFLT-1: fms-like tyrosine kinase 1; VEGF: vascular endothelial grow factor; RCMD: refractory cytopenia with multilineage dysplasia.

(Controls n= 24; Low-Risk MDS excluding RCMD n= 15; RCMD n= 15; High-Risk MDS n= 6)

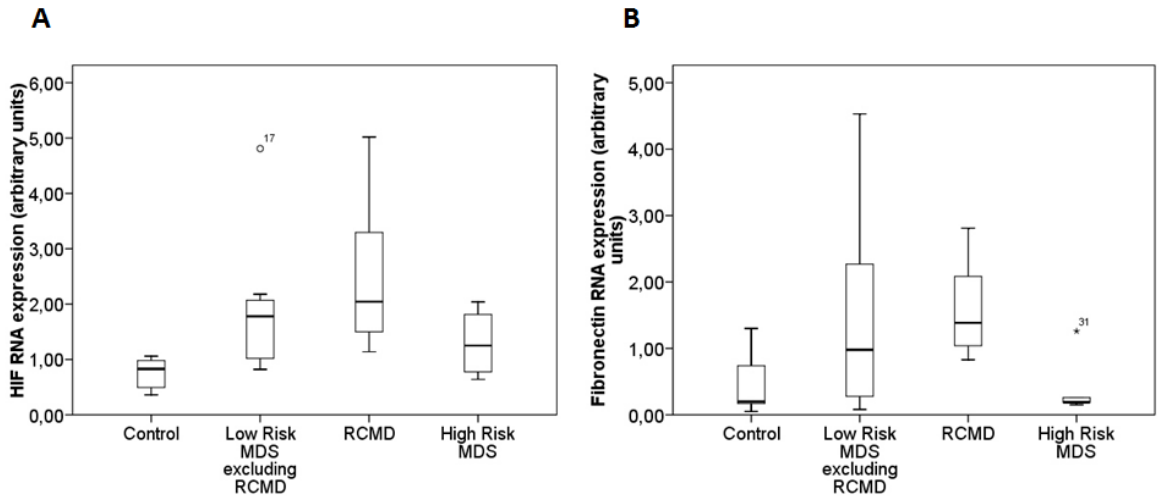
Supplementary Figures



Supplementary Figure 1.*ENG*, *VEGF*,*HIF1* and *FN1* RNA expression in mononuclear MDS BM cells.The box plot compares median of *ENG, VEGF*, *HIF1* and *FN1* expression levels in BM mononuclear cells between MDS and controls. To analyze the gene expression levels of the angiogenic factors we used RT-PCR. Each value of each sample is the mean of three independent experiments. The box plot shows the differences between the samples expression distributions in control and MDS group. Whiskers represent the range. Mann-Whitney test was applied in all cases. No significant differences were found in *ENG* expression between MDS patients and control group (A). Overall the expression levels of *VEGF*, *HIF1* and *FN1* in MDS were significantly higher ($p<0.05$) than levels of controls (B-D).

ENG: endoglin; VEGF: vascular endothelial grow factor; HIF1: hypoxia-inducible factor 1-alpha; FN1: fibronectin; BM: bone marrow; MDS: myelodysplastic syndrome.

(Controls n= 13; MDS n= 50)



Supplementary Figure 2. *HIF1* and *FN1* RNA expression in mononuclear BM cells of MDS subtypes. The box plot compares median of *HIF1* and *FN1* expression levels in BM mononuclear cells between the different MDS groups and controls. The gene expression levels were analyzed by RT-PCR. Each sample was performed in triplicate. Each value of each patient is the mean of these three experiments. Mann-Whitney test was used to analyze the results. The box plot compares the RNA expression in BM mononuclear cells of subtypes of MDS. Whiskers represent the range. The low-risk MDS groups showed over-expression of *HIF1* and *FN1* respect to the control group ($p<0.05$). Moreover, patients with RCMD showed the highest values in the expression of these two genes respect to the other low-risk MDS. Overall no significant differences in high-risk MDS patients in *HIF1* and *FN1* expression respect to the healthy controls were found.

HIF1: hypoxia-inducible factor 1-alpha; FN1: fibronectin; BM: bone marrow; MDS: myelodysplastic syndrome; RCMD: refractory cytopenia with multilineage dysplasia; RAEB: refractory anemia with excess of blasts.

(Controls n= 13; Low Risk MDS excluding RCMD n= 22; RCMD n= 12; High Risk MDS n= 16)

3

Deregulation of Genes Related to Iron and Mitochondrial Metabolism in Refractory Anaemia with Ring Sideroblasts

Under review in British Journal of Haematology

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General Discussion

MDS are a group of clonal diseases characterized by the presence of morphological dysplasias, ineffective hematopoiesis and peripheral cytopenias, which can evolve to AML in 25% of cases. MDS is also a heterogeneous group of diseases whose diagnosis is based on morphological and cytogenetic criteria. Therefore, MDS, especially those with a small population of blasts, normal karyotype or few morphological abnormalities, are sometimes entities that aredifficult to separate from normality, and therefore,are difficult to diagnose. In this thesis,we have sought to deepen the study of the transcriptome, genome and methylome in low-risk MDS in order to know whether other characteristics, different from morphological and cytogenetic characteristics, could help itsidentification. For this, we have used new methods such as microarrays or massive sequencing in the study of these diseases.

The possibility ofanalyzing,in a single experimentandsimultaneously,the transcription ofnearly allknown genes, provided newdata thatallowed theunderstanding of the biologyof MDS to be deepened.Thus, we have been able toidentify a set ofgenesrelated to angiogenesisthatweredifferentially expressedin MDS. It led ustoa more detailed studyof the process.Similarly, we observed a differentialexpression profileinthe subgroup ofRARSthat was the basisfor anoverallmutationalanalysisin whichthe genes involvediniron metabolismandmitochondrialplayed afundamental role.Furthermore, the studyof the methylation statusof MDS,and especiallythe integrated studyofmethylomeandtranscriptome, identifiedgenes with inactivatedexpression that wereinvolved in thederegulationofpathways related to the developmentof MDS.

The gene expression profile study in BM mononuclear cells demonstrated that *GDF15* is the most overexpressed gene in low-risk MDS. GDF15 plays a fundamental role in the regulation of inflammatory and apoptotic pathways in disease processes. However, cell growth and post-translational modification were themost frequently deregulatedmolecular and cellular functions, while growth and proliferation was the process that affected a greater number of genes. The use of unfractionated cells in this type of study has become a subject of debate in recent years. In this sense, our work is supported by previous studies that have been able to identify biological and prognostic characteristics in MDS and AML by studying the gene expression profile and the use of mononuclear cells (167, 192). It is also important to note that these studies showed differences between groups in an unsupervised analysis using mononuclear cells. It is true that hematopoietic defects in MDS derive from the stem cell. Therefore, studies in CD34+ cells are of great scientific value. However, using unfractionated samples allows the identification of the interaction between the different cell types, a fact

which the fractionated samples does not allow. It should be further noted that comparison studies betweenmononuclear cells and CD34+ cells results(164) showed a high level of agreement between the two procedures. Thus, studies on mononuclear cells could be adding interesting additional information forunderstandingof the biology of this disease.

Aberrant **methylation** is a potential mechanism for the inactivation of genes that have been implicated in several hematological disorders, including MDS(193, 194). However, most studies so far have focused on analysis of a restricted number of genes, generally, tumor suppressors. Moreover, the methylation profile of low-risk MDS has never been well defined and there are few studies that have shown that these patients present a differential methylation profile, as happens with the expression profile (141, 163). The first part of our study showed, for the first time, that patients with low-risk MDS had a different methylation profile from healthy individuals, involving deregulation of 817 genes. Furthermore, it was observed that hypermethylation was more frequent than hypomethylation. Also,the use offractionated appears to be controversial in methylation analysis, although a recent study shows that DNA methylation profiles were similar between CD34+ and CD34-cells ($r > = 0.93$) , and that the variability observed between them was less than the variability between patients ($r <= 0.83$) (170). This suggests that aberrant epigenetic profiles within the marrow are transmitted vertically from progenitors to more differentiated progeny. Our analysis strategy with unfractionated cell was based on these findings.

In order to try to explain the deregulation in the expression of certain genes previously identified in our work, we performed a combined analysis expression and methylation profiles in patients with low-risk MDS. Additionally, this study included exactly the same cohort of patients with MDS to analyze both profiles, something that had not been carried out previously. DNA methylation markers that could lead to under-expression of some genes involved in important cellular functions in low-risk MDS were identified in this integrative analysis:***DICER1***, ***BCL2***, ***ETS1*** and ***IL27RA***.

Several studies describe a deregulation in the global set of miRNAs in MDS patients (50-53). Our data corroborated these findings and showed a slight miRNAs under-expression in low-risk MDS ($p = 0.039$). In addition, some studies suggest that many miRNAs are located in fragile sites and genome regions with genetic alterations. The expression of a subset of these miRNAs is significantly altered and is probably important in MDS (55). The acquisition of somatic mutations or polymorphisms in miRNAs is another mechanism by which

theirexpression could be deregulated and therefore the function of their target genes could be altered in MDS. However, Starczynowski et al. presented no evidence of mutations in miRNAs in their study by massive sequencing (195). Finally, abnormal miRNAs expression in MDS may occur due to epigenetic processes such as methylation. This idea is supported by a recent study which shows how the expression of miR-124 is inversely proportional to its methylation status(55). However, the differential miRNAs expression described in MDS miRNAs has been impossible to explain until now.

DICER1is anessential RNase III endonuclease for miRNAs biogenesis and RNA processing (196). Therefore,an altered miRNAs expression can be expected to occur as a result of variations in pre-miRNA processing by this endonuclease. In the present study, it was observed that *DICER1*expression levels were lower low-risk MDS patients compared to the control group. Furthermore, *DICER1* was hypermethylated in these patients, which could explain the observed under-expression of this gene in the same group of patients. The recent publication of a study with *DICER1*knockout mice suggests that global deregulation of miRNAs may be involved in the pathogenesis of MDS. In this model, a suppressionin *DICER1* in osteoprogenitorsimpairedthe differentiation of osteoblasts and the integrity of hematopoiesis as well as an induction of BM dysfunction with myelodysplasia (196). Furthermore, altered expression of mature miRNAs in blood cells can contribute to differentiation defects resulting in neutropenia, thrombocytopenia and / or anemia (55).

The administration of "miRNAs simulators" could be a therapeutic option for MDS in the future. *In vivo* administration has many problems due to its low stability, rapid degradation and inefficient release in "destination cells" (197). Therefore, research on liposome carriers for the release of "miRNAs simulators", can lead tonew approaches in the treatment of MDS.

Some previous studies have suggested that increased **apoptosis** in BM myeloid precursors is an important factor in ineffective hematopoiesis in patients with MDS. Furthermore, these studies have shown that increased apoptosis probably represents a pathophysiological mechanism more than a process to counteract the increase of cell growth (138, 161, 163). However, the cause of the abnormal apoptosis in MDS is still unknown and whether or not the increase of caspase activity is due to the activation of the extrinsic or intrinsic apoptotic pathway has not yet been resolved. Our study provides more datafor this research. As already mentioned, some members of the Bcl-2 family, such as ***BCL2*** gene are important inhibitors of the intrinsic pathway of apoptosis by blocking the release of

cytochrome c. Our study showedthat *BCL2* expression is significantly weaker in low-risk MDS than in normal individuals. These results are in agreement with previous studies that showed reduced *BCL2* expression in CD34+ cells from MDS patients in early stages of the illness(95). Moreover, our study showed that *BCL2* has significantly higher levels of methylation in low-risk MDS than in the control group. Importantly, there is increasing evidence of the role of epigenetic silencing in apoptotic pathways in cancer (198-200). Therefore, one would expect, that the hypermethylation of *BLC2* and the inverse correlation with its expression could be a mechanism that promotes apoptosis in low-risk MDS patients. A thorough investigation of *BCL2* as a biomarker in low-risk MDS and as a potential therapeutic target for these patients in the future is proposed in this thesis. In addition, the combined study of the intrinsic apoptotic pathway by *BCL2* deregulation along with the extrinsic pathway, as previously described, will provide new data for understanding the aberrant apoptosis in MDS.

Transcription factors are proteins capable of binding the promoter regions of some genes and of regulating their expression. The deregulation of these target genes due to aberrant expression of the corresponding TF could constitute a mechanism involved in the pathogenesis of MDS. Our integrative analysis suggests that ***ETS1***under-expression is due to methylation in its sequence. ETS1 is a nuclear phosphoprotein that functions as a TF by binding the target DNA sequences containing a central GGAA/T core motif (ETS- binding site, EBS)(201). Over 400 ETS1 target genes have been defined to date, based upon the presence of functional EBSin their regulatory regions (202). In the same way that other solid tumor studies have demonstrated co-expression between *ETS* and its possible target genes (203-205), our functional study showed the presence of 83 underexpressed genes presenting the EBS motif in their regulatory regions. The study suggests that methylation of *ETS1* causes its under-expression and this leads to a transcriptional deregulation of 83 target genes. ETS protein controls the expression of target genes involved in diverse biological processes such as apoptosis, hematopoiesis, cell proliferation and differentiation (202). Of these, apoptosis was the most affected process in low-risk MDS, with under-expression of 9 genes.

Therefore, the findings described in this thesis may explain the presence of greater apoptosis in MDS via two pathways: (1) methylation and decresed *BCL2*expression and (2) under-expression of ETS1 target genes that are related to apoptosis, by methylation and decreased*ETS1* TF expression. A clearer understanding of the molecular alterations leading to deregulation of cell death in MDS may allow the identification of new therapeutic targets and diagnostic markers for patients with MDS in the future.

IL27RA is a component of the heterodimeric complex receptor IL27R that is involved in **immune suppression**by inducing a signal transduction in response to IL27 (47). This signal transduction requires the co-expression of both subunits of the complex receptor, so the loss or decreased function of one of them could lead to defects in the immune system (206). Our studies have identified a cleardifference in *IL27RA*methylation levels between low-risk MDS patients and healthy controls, that may be responsible for the low gene expression in these patients. These results are consistent with recent studies that show IL27RA as a promoter of the hematopoietic stem cell differentiation, as appears to enhance myelopoiesis in transgenic mice (47). Accordingly, *IL27RA* under-expression could lead to ineffective hematopoietic progenitor differentiation, previously described in MDS by other authors (33). This means that one component of a cytokine receptor would have the ability to regulate signaling pathways in hematopoietic cells. In light of these findings, our studies suggest that thiskind of receptors may play roles in mediating the immune response or cellular differentiation in myeloid disorders like MDS.

In the second part of this thesis,a functional combined analysis of gene expression and soluble factors involved in **angiogenesis** in the BM of MDS patients was carried out.

The microvasculature measurement has been one of the most commonly used techniques to give evidence forderegulated angiogenesis in MDS (117, 119, 120, 123). However, demostration by functional techniques and the involvement of the microenvironment in this disorder (highlighted in many works), has not been demonstrated in BM, since most of the studies are based on the measure of angiogenic factors levels in PB(117, 119, 122). Based on the above, two functional studies were carried out,where the direct involvement of the microenvironment in angiogenesis in BM of MDS patients could be demostrated. More specifically, in this work we quantified the **proliferation** of endothelial cells in the presence of BM supernatant and the **capillary formation** by these cells also in the presence of marrow microenvironment. It is important to note that we used two endothelial cell lines for carring out these experiments; one of them (BMEC-1) derived from human BM. So far, this cell line had never been used for such experiments and it is possible that their characteristics may make it the most appropriate to demonstrate these processes in the BM of MDS patients. The proliferation assay showed that the MDS BM supernatant stimulates endothelial cell proliferation in comparison with controls without hematological malignancies. Furthermore, MDS showed differences in thickness, structure and density in the capillaryformation as comparison tonormal BM. These results are consistent with previously

described aberrant angiogenesis in these diseases (123-125). Furthermore, these findings provide new data about the role of BM endothelial cells and the marrow microenvironment in angiogenesis in MDS. It should be noted that the capillaryformation was different between the subtypes of MDS: RCMD patients showed less extensive capillary networks and reduced vessel formation. This suggests that the contradictory results published to date regarding angiogenesis in MDS may be due to this process develops differently in the different entities of MDS.

It has been shown that leukemic cells may have intimate interactions with BM endothelial cells (BMEC), and can elicit the sprouting of new blood vessels frompre-existing capillaries by the active release of angiogenic factors (124, 125, 207). In order to know if it could be an explanation for the abnormal behavior of BMEC, previously observed in our functional studies, we focused on the **transcriptional analysis** of several angiogenic molecules in BM mononuclear cells of MDS patients. Thus, we observed that ***VEGF***, ***HIF1*** and ***FN1*** were overexpressed in low-risk MDS (including RCMD subtype) compared to controls. These findings are in line with some studies published to date that showed a significant increase of angiogenic factors in MDS(120). Furthermore, over-expression of these genes may be influencing the aberrant behavior of endothelial cells in low-risk MDS. It should be noted that in RCMD patients showed higher expression values compared to other low-risk MDS patients.

However, angiogenesis involves two stages of vascular development: the differential growth, and sprouting of endothelial tubes and the remodeling of the primary endothelial network into a mature circulatory system. The*ENG* gene encodes a transmembrane protein that is required for both processes (133, 208) making it a good marker for tumor angiogenesis quantification(209). Interestingly,*ENG* expression was significantly lower in RCMD than in cells from healthy controls. This finding could be compared with other vascular diseases such as hereditary hemorrhagic telangiectasia type I (HHT). HHT patients have significantly lower levels of *ENG* and they are caracterized by arteriovenous malformations and focal loss of capillaries (210). In addition, it has been reported that insolated murine Eng+/- cells display impaired capillary tube formation and significantly less vascular structures compared to wild type mice (211). Based on these findings, our study suggests that the "poor" *ENG* expression in RCMD patients could be associated with decreased vessel formation in our *in vitro* model of angiogenesis observed in the same group of patients.

The results of ENG expression in mononuclear cells led us to investigate its **presence in the marrow microenvironment** (**sENG**), in order to assess its direct influence on endothelial cells. Levels of anti-angiogenic factor **sFLT-1** and angiogenic factor **sVEGF** were also measured. Thus, RCMD patients showed higher levels of sENG and sFLT-1 compared with other low-risk MDS and the control group. However, sVEGF levels were similar to controls. It has been shown that the soluble form of ENG is antagonist of the membrane bound form and, thus,enhancesthe action of anti-angiogenic sFLT-1 (133). In addition, some studies have shown that sFLT-1 binds and neutralizes the action of pro-angiogenic sVEGF (212). This finding could explain the "mitigated" secretion of sVEGF in CRDM patients with respect to the gene expression in the same group of patients. Based on these results, we may suggest that anti-angiogenic characteristics of the marrow microenvironment in RCMD patients could explain less extensive and less open capillary networks,and the smaller vessel formation in these patients observed in our functional studies.

Angiogenesis is a balanced process between pro and anti-angiogenic factors. Our findings suggest a loss of this balance in RCMD patients. In fact, these patients displayed high levels of expression of pro-angiogenic factors such as *VEGF*, *HIF1*, and *FN1*in BM mononuclear cells. However, this group of MDS showed low expression of the *ENG* pro-angiogenic factor, high levels of factors with anti-angiogenic properties (sENG and sFLT-1) in the marrow microenvironment, lower sVEGF respect to the gene expression and reduced vessel formation by endothelial cell lines. Regarding the increased proliferation of endothelial cells, there iscontradictory evidence: it is true that the proliferation of endothelial cells is a key event in angiogenesis. However some studies have shown that myeloid neoplasms with high levels of sENG are characterized by a high rate of endothelial cell proliferation, which could explain the high proliferation in a potential anti-angiogenic from RCMD patients (137, 211).

The RCMD has been recently proposed by the WHO classification as a specific disorder in MDS (2)but has never been included as a separate group in angiogenesis studies (120, 123). The present study showscleardifferences in angiogenesis between MDS subtypes and specifically, it presents a different pattern of this process in RCMD cases. These findings provide new insights into the molecular mechanisms of RCMD patients that could be related to ENG. Furthermore, inhibition of the protease responsible for the sENG release to the extracellular medium could provide new therapeutic approaches for this subtype of MDS.

In the third part of this thesis, we tried to identify genes that are commonly deregulated in relation to **iron and mitochondrial metabolism** in RARS patients, in order to determine their involvement in iron accumulation.

RARS patients had a particular **gene expression profile**. Iron and mitochondrial metabolism was the most affected category and the genes showed over-expression in RARS patients with respect to the controls or RCUD group, representing 38% and 33% of the total overexpressed genes, respectively. ***GDF15*** is an iron deplection sensitive gene that showed the most over-expression in RARS patients. This over-expression may be a consequence of the iron accumulation within the mitochondria and therefore the low iron concentration in the cytoplasm. Increased expression of six genes was also observed in our work: *ALAD*, *HMBS*, *UROS*, *UROD*, *CPOX* and *PPOX*, encoding enzymes that catalyze the heme biosynthesis, some of which had already been described as overexpressed in CD34+ cells from RARS patients (141). The ***ABCB6***over-expression is also important because this gene is involved in coproporphyrinogen III transport from the cytoplasm to mitochondria (also as part of the heme formation process) (140, 213). Nine genes were related to cellular iron homeostasis, of which *TF*, *TFR2*, *TFRC*, *FXN*, *SLC25A37* and *SCL25A38* were overexpressed in RARS patients. ***SLC25A37*** contributes to mitochondrial iron acquisition in mammalian cells, since its decrease drastically reduces the iron within mitochondria (214, 215).Therefore, our results suggest that this gene could be involved in the iron accumulation in RARS patients.

The ***ALAD*** gene encodes a cytosolic enzyme which catalyzes the condensation of two molecules of D-aminolevulinic acid (ALA) to form porphobilinogen (PBG) in the second step of the heme biosynthesis pathway (216).The study of this gene identified two polymorphisms in exon 6 located 49 bases from each other and, interestingly, the presence of one of them was always determined by the presence of the other one. The joint occurrence of the two polymorphisms ("variant haplotype") was more frequent in MDS with ring sideroblasts (18%) than in the other groups analyzed (6%). The presence of haplotypes has been linked to the deregulation of some genes in various hematological malignancies such as chronic lymphocytic leukemia and acute lymphoblastic leukemia (217, 218). Based on this data, the presence of this "variant haplotype" might be involved in ALAD gene over-expression in RARS patients, and thus, could be contributing to deregulation of iron and mitochondrial metabolism in MDS with ring sideroblasts.

The massive sequencing technique and subsequent conventional sequencing allowed the identification oftwo un-described changes described in the *ALAD* gene in two RARS

patients. These changes were detected in 4% of cases with ringed sideroblasts and are placed very close together in exon 7. Although these changes were also detected in the CD3+ population from PB of the two patients and in two relatives of one of them, they were not found in controls or in other low-risk MDS analyzed. Furthermore, both variations led to aminoacid changes in the protein sequence. On the other hand, the same cohort of RARS patients was used for a mutational analysis in *SF3B1*, recently described as mutated in a large percentage of MDS with ringed sideroblasts. Interestingly, both patients with *ALAD* variants were among the 30% of cases with ring sideroblasts that did not feature *SF3B1* mutations. These results suggest that these variations may have a potential role in the predisposition to disease as well as contributing to the pathogenesis of RARS.

SLC25A38 is a gene that has been involved in the pathogenesis of congenital sideroblastic anemia due to the presence of mutations in its sequence (219). This gene was included in our study and was analyzed in low-risk MDS and in one case with congenital sideroblastic anemia. The results showed that this case had a different mutation from mutations previously described for the disease(219), even though it was located at the same position asone of them. The patient had a 187R>Q change in the protein sequence, whereas the mutation previously described gave rise to a 187R>P change. Interestingly, when this gene was sequenced in a group of RCUD patients, a new mutation was found in exon 4 of one of the cases studied. The mutation led to an amino acid change in the protein sequence (97V>A). However, no mutations were found in either case with ring sideroblasts. For this reason, we suggest that there are other mechanisms that could be related to the over-expression of this gene in RARS patients. Furthermore, these results indicate that *SLC25A38* gene mutations are not exclusive of congenital sideroblastic anemia and therefore, there are other variations in this gene that may be associated with low-risk MDS.

In summary, the research carried out during the development of this thesis through a global analysis (transcriptome, methylome-genome) has allowed identifying alterations that provide new information about the pathogenesis of low-risk MDS and may have clinical relevance for the disease. First, the integration of the data from methylation and expression arrays has allowed describingepigenetic deregulation of *DICER1*,*BCL2*, *ETS1* and *IL27RA*. Functional studies in low-risk MDS have demonstrated aberrant angiogenesis in CRDM patients that could be related to *ENG*. Finally, the combined study of the expression and mutational status has identified new *ALAD* gene variations in RARS patients that could be playing a role in the predisposition to thedisease. Taken together, the genes described in this

thesis could be contributing to the pathogenesis of low-risk MDS and could be considered as diagnostic markers and even, in some cases, as potential therapeutic targets in the future. Therefore, these results confirm that the MDS is a very heterogeneous disease. They also highlight the use of mass screening techniques (microarrays and massive sequencing) in the study of cancer, in general, and in MDS, in particular.

Conclusions

1. Low-risk MDS patients have a characteristic and different gene expression profile from the bone marrow of patients without hematological malignancies.

2. Gene expression profile analysis of low-risk MDS patients demonstrates that cell development, post-translational modifications and the immune response are the most frequent molecular and cellular functions altered in these patients. Moreover, growth and cell proliferation are processes with the greatest number of differentially expressed genes compared to the group of patients without hematological malignancies.

3. Low-risk MDS patients have a characteristic methylation profile in which the hyper-methylation of genes is more commonthan the hypo-methylation.

4. The integration of data from expression and methylation microarrays shows that 66.7% of hyper-methylated genes in low-risk MDS are also under-expressed. The results of this study reveal the epigenetic deregulation that led to the under-expression of *DICER1*, *BCL2*, *ETS1* and *IL27RA* genes. Disruption of these genes can determine changes in cellular functions where they are involved: RNA processing, apoptosis, gene regulation and immune response, respectively.

5. Functional studies in low-risk MDS reveal the existence of aberrant angiogenesis in the bone marrow of these patients. In addition, the activation of this process is clearly different between subtypes of low-risk MDS.

6. Patients with refractory cytopenia with multilineage dysplasia are characterized by a low expression of *ENG*, high levels of sENG and sFLT1 in the supernatant of bone marrow, sVEGF decreasing as well as a deficient tube formation, which could be producing an imbalance in angiogenic processes.

7. The gene expression profile has allowed us to distinguish the RARS group from patients without hematological malignancies and RCUD patients. A large number of over-expressed genes in RARS are involved in iron and mitochondrial metabolism. This fact may explain the morphological changes observed in this entity.

8. The combined study of the expression and the mutational status has allowed the identification and characterization of new abnormalities in MDS with ring sideroblasts:

- 4% of patients with ring sideroblasts have *ALAD* gene variants that could be involved in its pathogenesis and in predisposition to disease. No mutations in *SF3B1* have been observed in these patients.

- Over-expression of *SLC25A37* in patients with ring sideroblasts is not due to variations in its sequence.

- *SLC25A38* mutations are not exclusive of congenital sideroblastic anemia but also can be present in patients with acquired MDS.

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