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**Genome-wide profiling of methylation identifies novel targets with aberrant hyper-methylation and reduced expression in low-risk myelodysplastic syndromes**

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

4 Epigenetic Regulation in Low-Risk Myelodysplastic Syndromes

5

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29 **Abstract**

30 Gene expression profiling signatures may be used to classify the subtypes of MDS  
31 patients. However, there are few reports on the global methylation status in MDS. The  
32 integration of genome-wide epigenetic regulatory marks with gene expression levels  
33 would provide additional information regarding the biological differences between MDS  
34 and healthy controls. Gene expression and methylation status were measured using  
35 high-density microarrays. A total of 552 differentially methylated CpG loci were  
36 identified as being present in low-risk MDS; hyper-methylated genes were more  
37 frequent than hypo-methylated genes. In addition, mRNA expression profiling identified  
38 1005 genes that significantly differed between low-risk MDS and the control group.  
39 Integrative analysis of the epigenetic and expression profiles revealed that 66.7% of  
40 the hyper-methylated genes were under-expressed in low-risk MDS cases. Gene  
41 network analysis revealed molecular mechanisms associated with the low-risk MDS  
42 group, including altered apoptosis pathways. The two key apoptotic genes *BCL2* and  
43 *ETS1* were identified as silenced genes. In addition, the immune response and miRNA  
44 biogenesis were affected by the hyper-methylation and under-expression of *IL27RA*  
45 and *DICER1*. Our integrative analysis revealed that aberrant epigenetic regulation is a  
46 hallmark of low-risk MDS patients and could play a central role in these diseases.

47

48 **Keywords:** gene expression profile, methylation, low-risk MDS, apoptosis, *BCL2*,  
49 *ETS1* transcription factor targets.

## 50 **Introduction**

51 Myelodysplastic syndromes (MDS) are a heterogeneous group of clonal myeloid stem  
52 cell disorders affecting mainly elderly patients. MDS are characterized by cytopenia of  
53 the peripheral blood (PB), hypercellularity of the bone marrow (BM) and morphological  
54 alterations in one or more hematopoietic cell lineages (1). According to the WHO  
55 classification, MDS can be classified as low- or high-risk depending on the percentage  
56 of blast cells in the BM (2). In most cases, the presence of cytogenetic aberrations,  
57 such as alterations on chromosomes 5, 7, and 8, are the hallmark of MDS, but other  
58 abnormalities in signal transduction, transcription activity, cell-cycle control,  
59 mitochondrial DNA, angiogenesis and epigenetic changes have also been associated  
60 with MDS (3).

61

62 DNA methylation is an epigenetic process that involves the addition of a methyl group  
63 (CH<sub>3</sub>) to the 5-position carbon of the cytosine pyrimidine ring in a CpG dinucleotide.  
64 This process is carried out in an orchestrated reaction that includes DNA  
65 methyltransferases, methyl-binding domain proteins, and histone deacetylases (4).  
66 DNA cytosine methylation is the best characterized epigenetic event leading to the  
67 stabilization of the genome, the remodeling of the chromatin and the regulation of gene  
68 transcription (5;6). In addition, not only the presence of epigenetic marks but also their  
69 location and density play a crucial role in regulating these processes (7;8). A close  
70 correlation between DNA hyper-methylation and transcriptional silencing has been  
71 established in many systems (9).

72

73 Epigenetic alterations are now accepted as having a role in carcinogenesis. DNA  
74 hyper-methylation in cancer is associated with the silencing of tumor-suppressor  
75 genes, whereas hypo-methylation has been described as playing a causal role in  
76 progressive tumor formation and in promoting chromosomal instability (5;7;9-11).

77 Tumor suppressors are not the only genes affected by aberrant methylation;  
78 abnormally methylated genes with other functions are also subject to silencing in  
79 human cancer, including those involved in DNA repair, apoptosis, angiogenesis, cell  
80 cycle regulation and cell-to-cell interaction (12). Hence, epigenetic modifications in  
81 promoter and/or regulatory regions that lead to transcriptional silencing of genes and  
82 development of cancer are important events requiring to be studied in any onco-  
83 pathological state and they are attractive therapeutic targets.

84

85 Gene expression profiling studies have been performed in MDS with the aim of  
86 identifying genes and biological pathways of relevance in these diseases (13;14).  
87 These studies have identified gene expression signatures distinguishing specific  
88 subgroups of MDS and have helped improve our understanding of the biology of these  
89 diseases (15). However, the molecular pathogenesis of MDS is still not fully  
90 understood. Moreover, only part of the cellular information is present at the mRNA  
91 level, and transcriptional activity is dependent on many factors, including epigenetic  
92 modifications. Nevertheless, the methylation patterns of genes have not been as well  
93 explored in low-risk MDS as in other hematopoietic malignancies, and most epigenetic  
94 studies have focused on the analysis of a few tumor suppressor genes (16).

95

96 The underlying mechanisms of altered DNA methylation in low-risk MDS and the target  
97 genes affected by methylation remain unknown. To gain insight into the knowledge of  
98 the molecular mechanisms present in low-risk MDS, an integrative study of methylation  
99 and gene expression profiles was carried out. In this report, we identify genes with  
100 reduced levels of expression in response to increased methylation levels in nearby  
101 CpG islands. Overall, we highlight candidate DNA methylation changes associated with  
102 MDS that may warrant further investigation as potential clinical targets.

## 103 **Materials and Methods**

### 104 **Samples collection and cell separation**

105 A total of 83 low-risk MDS patients and 36 age-matched controls without  
106 haematological malignancies were included in the study (Supplementary Table 1).  
107 MDS were classified according to the World Health Organization (WHO) criteria (2).  
108 Mononuclear cells were isolated from BM samples of low-risk MDS patients and  
109 controls by density gradient (Ficoll). A cohort of 18 patients with low-risk MDS and  
110 seven controls were included in a simultaneous integrative study of methylation and  
111 expression, while the whole series was used as a control group of expression data.  
112 The study was approved by the Local Ethical Committees and written informed consent  
113 was obtained from each patient.

114

### 115 **DNA and RNA isolation**

116 Genomic DNA from subject samples was isolated using a DNeasy blood and tissue kit,  
117 following the manufacturer's protocol. DNA was eluted in AE buffer (Qiagen, Hilden,  
118 Germany).

119 Total RNA from cells was extracted by homogenization in TRIZOL (Invitrogen,  
120 Carlsbad, CA, USA), following the manufacturer's protocol, then treated with RQ1  
121 RNase-Free DNase (Promega, Madison, USA) to eliminate genomic DNA  
122 contamination, and finally purified with RNeasy Minikit (Qiagen). The quantity and  
123 quality of the RNA were determined with an Agilent 2100 Bioanalyzer (Santa Clara,  
124 CA, USA).

125

### 126 **Methylation CpG island amplification and microarray studies (MCAM)**

127 Methylated CpG Island Amplification and Microarray (MCAM) is a two-color array  
128 technique that quantifies methylation by hybridizing equimolar amounts of subject  
129 *versus* control DNA to an array (17). The University Health Network human 12K CpG

130 microarray (UHN, Toronto, Canada) contains 12,192 CpG island clones. Each clone  
131 can be annotated with up to three gene symbols, depending on whether the CpG site  
132 lies upstream, downstream or within the gene. The methylation assay was done largely  
133 as described previously (17), but with the following modifications: DNA was purified  
134 after double digestion, methylated CpG amplification (MCA) reaction and labeling were  
135 done using the QIAquick PCR purification kit (Qiagen) without any indicator in the  
136 buffer PB. RMCA primers (Eurogentec, Southampton, UK) were used at ligation and  
137 MCA reaction stages. The MCA reaction was performed using 5 U HotStarTaq+  
138 (Qiagen) and samples were aliquoted without primers before being heated to 82 °C.  
139 The primers were held at 95 °C for 1 min before adding 4 µL to each tube. Cycling  
140 conditions were: 95 °C for 10 min before 30 cycles of 95 °C for 1 min, 65 °C for 90 s,  
141 72 °C for 2 min, and finally 72 °C for 10 min, and before holding at 4 °C. Samples were  
142 hybridized to UHN HCGI12K CpG microarrays. After hybridization, microarrays were  
143 washed as follows: 3 × 15 min at 55 °C with wash 1 in a rotating oven, then on a  
144 gyrating platform 2 × 3 min at room temperature (R.T.) 1x SSC, 2 × 3 min at R.T. Wash  
145 2: 2 × 3 min at R.T. 0.1 × SSC, 2 × 3 min at R.T. Millipore H<sub>2</sub>O. Finally, slides were  
146 rinsed with H<sub>2</sub>O and centrifuged for 7 min at 370 g to dry. They were scanned with an  
147 Axon GenePix 4400A scanner (MDS Analytical Technologies, Molecular Devices,  
148 Sunnyvale, CA, USA) using Genepix Pro 7 software (MDS Analytical Technologies).

149

#### 150 *Bioinformatic analysis*

151 The output GPR files from Gene Pix Pro 7.15 were imported into the R/Bioconductor  
152 Marray program and quality control diagnostic plots were generated and assessed.  
153 Poor-quality arrays were removed from the analysis and repeated. The log ratio of  
154 median red (Cy5-labeled subject sample) to median green (Cy3-labeled universal  
155 control) processed (dye-normalized) signal intensities were computed using the LIMMA  
156 R/Bioconductor program. Probes that had been flagged by Gene Pix Pro 7.15 as bad,



157 absent or not found were removed. A genomic smoothing step was then performed in  
158 which a weighted average across 1000 bp was calculated for each CpG clone. In order  
159 to determine the degree of enrichment, the Partek Genomics Suite ANOVA tool was  
160 used and the n-fold change using the geometric mean (for log-transformed data).  
161 Probesets that differed significantly ( $p < 0.10$ ) between the low-risk MDS and control  
162 groups were selected for further analysis.

163

#### 164 **Gene expression microarray studies**

165 Gene expression profiling (GEP) studies were done as part of the Microarray  
166 Innovations in LEukemia (MILE) study (18). GeneChips Human Genome U133 Plus 2.0  
167 arrays (Affymetrix, High Wycombe, UK) are gene expression arrays containing 54,613  
168 oligonucleotide probesets that map onto 18,950 human gene loci, following gene-  
169 based remapping of the probes (19). Labeling and hybridization were performed  
170 according to protocols from Affymetrix. Briefly, 100 ng of total RNA was amplified and  
171 labeled using the GeneChip two-cycle cDNA synthesis kit and GeneChip IVT labeling  
172 kit (Affymetrix Inc.) and then hybridized to the Human Genome U133 Plus 2.0  
173 microarray, after quality checking on GeneChips Test3 Arrays. Washing and scanning  
174 were done using Fluidics Station 400 and GeneChip Scanner (Affymetrix Inc.). In  
175 addition, the Human Exon 1.0 ST microarrays (Affymetrix) were used in the study.

176

#### 177 *Bioinformatic analysis*

178 The Robust Microarray Analysis (RMA) algorithm was applied to the raw data from the  
179 expression arrays to carry out background correction, intra- and inter-normalization,  
180 and to calculate the expression signal (20). The Significant Analysis of Microarrays  
181 (SAM) algorithm was used to identify genes with statistically significant changes in  
182 expression between different classes (21). For this differential expression analysis,  
183 samples were permuted over 100 cycles using the two-class (unpaired) and multiclass

184 response format, considering variances not to be equal for the genes. Significant genes  
185 were selected on the basis of the false discovery rate (FDR), which was used to correct  
186 the p-values, assuming an FDR threshold of <0.15, which allowed better overlap with  
187 the data from the methylation study. To select each gene, the p-values of the statistical  
188 tests were transformed to q-values using the FDR threshold indicated. All the  
189 calculations described here were done using R and Bioconductor.

190

### 191 **Real-Time PCR**

192 To validate the GEP results, the expression levels of four selected genes were  
193 analyzed by RT-PCR. First-strand cDNA was generated from 1 µg of total RNA using  
194 poly-dT as primers with the M-MLV reverse transcriptase (Promega). Real-time PCR  
195 was performed in triplicate. Each 20µl reaction contained 300ng of cDNA, 400 nM of  
196 each primer, and 1x iQ SybrGreen Supermix (Bio-Rad, Hercules, CA, USA). Standard  
197 curves were run for each transcript to ensure exponential amplification and to rule out  
198 non-specific amplification. The expression level of the glyceraldehyde-3-phosphate  
199 dehydrogenase (*GAPDH*) gene was used to normalize differences in input cDNA. The  
200 reactions were run on an iQ5 Real-Time PCR detection system (Bio-Rad, Hercules,  
201 CA, USA). The primers were designed for specific sequences (Supplementary Table 2)  
202 and checked with the BLAST algorithm (22). In addition, to measure miRNA-145 and  
203 miRNA-196 expression levels, TaqMan qRT-PCR miRNA assay (Applied Biosystem,  
204 Carlsbad, California) was performed. The relative expression levels normalized to  
205 RNU43 endogenous control was determined using the  $2^{-\Delta Ct}$  method. Each  
206 measurement was performed in duplicate.

207

### 208 **Pyrosequencing**

209 Primers were designed for forward, reverse and sequencing using the PyroMark Assay  
210 Design 2.0 program. Primer sequences can be found in Supplementary Table 3.

211 Bisulfite conversion of DNA was done as described by Frommer et al (23). The hot-  
212 start polymerase chain reaction (PCR) was carried out using 2 $\mu$ L (50ng) of bisulfite-  
213 treated DNA. PCR was performed following the manufacturer's instructions.  
214 Pyrosequencing was carried out using the Q24 System (Qiagen), also in accordance  
215 with the manufacturer's protocols.

216

### 217 **Integrative functional analysis of methylation and expression data**

218 To analyze the functional enrichment of the selected gene lists we used the DAVID  
219 bioinformatic resource (<http://david.abcc.ncifcrf.gov/>) (24) and the web-delivered  
220 bioinformatics tool set IPA (Ingenuity Pathway Analysis 9.0; <http://www.ingenuity.com>).  
221 These tools allow the identification of functional modules and the most relevant  
222 biological processes present in the gene lists performing statistical enrichment analysis  
223 based on contingency tests. The Metacore Analytical Suite (Genego Inc., St. Joseph,  
224 MI, USA) was also used for the network analysis of some of the initial data from  
225 differentially methylated/expressed genes. Metacore's shortest path algorithm was  
226 applied to derive a network for the selected genes. Biological processes enriched in  
227 differentially methylated/expressed gene lists were identified and p-values determined  
228 using Metacore's enrichment analysis workflow.

229 The common transcription factor binding sites (TFBSs) were analyzed using search  
230 tools that allowed the sequences upstream of the genes of a given query list to be  
231 explored, for the purpose of finding significant candidate promoter regions. These tools  
232 search for sequence profiles similar to the TFBS defined in JASPAR  
233 (<http://jaspar.cgb.ki.se/>). The bioinformatic tools used were: oPOSSUM  
234 (<http://www.cisreg.ca/cgi-bin/oPOSSUM/oopossum>) (25); TransFind (<http://transfind.sys-bio.net/index.php/home.html>) (26); Pscan (<http://159.149.109.9/pscan/>) (27); and TFM-  
235 Explorer (<http://bioinfo.lifl.fr/cgi-bin/TFME/tfme.py>) (28).

237

## 238 **Results**

### 239 **Low-risk MDS and normal BM have distinct DNA methylation profiles**

240 The methylation profiles of low-risk MDS patients were compared with those of controls  
241 using the 12K CpG array. Statistically significant changes in the level of CpG island  
242 methylation were identified. A total of 552 CpG loci were sufficiently differentially  
243 methylated between the two groups to give a value of  $p < 0.10$ . These loci were  
244 associated with 817 annotated gene symbols: 457 genes were hyper-methylated in  
245 low-risk MDS, and 360 genes were hypo-methylated. The median fold changes were  
246 1.85 (7.82 to 1.09) and -1.65 (-4.73 to -1.11), respectively (Supplementary Table 4).  
247 The three most representative cellular functions for genes commonly altered by  
248 methylation were GM-CSF signaling (e.g., *LYN*, *GNB2L1* and *ZNF225*), apoptosis-  
249 HTR1A signaling (e.g., *BCL2* and *MAP2K1*) and TGF-beta-dependent induction of  
250 EMT via SMADs (e.g., *SMAD2*, *HN1* and *CDH2*). In addition, four of the top ten cellular  
251 functions deregulated by methylation were related to the immune response (Table 1).  
252 Amongst the genes involved in this response, *IL27RA* and *CD28* were hyper-  
253 methylated whilst *IL6* and *CD96* were hypo-methylated in MDS patients.

254

### 255 **Gene expression profiling distinguishes low-risk MDS from normal BM**

256 The GEP from the BM of low-risk MDS patients was compared with that from the BM of  
257 healthy individuals. 1975 genes showed significant differences (FDR cut-off  $< 0.15$ ) in  
258 mRNA expression levels between the two groups: 764 were over-expressed whilst  
259 1211 genes were under-expressed in low-risk MDS (Supplementary Table 5). This  
260 number was reduced to 1005 genes when an FDR cut-off of  $< 0.10$  (444 up-regulated  
261 and 561 down-regulated genes) was applied. These genes were selected for further  
262 investigation. Hierarchical clustering, selecting for differentially expressed genes,  
263 resulted in a good separation of the two groups analyzed, except in three patients  
264 (Figure 1). These samples had a less differential profile although they were distinct

265 from the controls. Interestingly, two of them displayed chromosomal alterations that  
266 were not present in any other patients: a loss on 5q and a monosomy 7. The most  
267 over-expressed gene in low-risk MDS (R.fold=8.08) was *GDF15*, which has a role in  
268 regulating inflammatory and apoptotic pathways during disease processes. By contrast,  
269 cellular development, post-translational modification and the cell-mediated immune  
270 response were the most frequently deregulated molecular and cellular functions  
271 (Supplementary Figure 1). In addition, cellular growth and proliferation was the function  
272 involving the largest group of genes: 121 molecules, of which *BCL2*, *ETS1* and *FLT3*  
273 were highlighted as down-regulated genes in MDS patients (Supplementary Table 5). It  
274 should be noted that nucleosome assembly, chromatin organization and DNA  
275 packaging were also significant functions that were altered in low-risk MDS. In this  
276 respect, a total of 33 up-regulated histone genes involved in these three functions were  
277 observed in low-risk MDS (Supplementary Table 5).

278

### 279 **Hyper-methylation correlates with decreased gene expression in low-risk MDS**

280 An integrative approach involving methylation and expression profiling was used to  
281 characterize genomic changes between low-risk MDS patients and healthy controls.  
282 Comparison of the 817 putative target genes of differential methylation and the 1975  
283 genes of differential expression allowed the detection of gene loci that experienced  
284 both concurrent changes in low-risk MDS patients. In total, 91 genes were both  
285 differentially methylated and differentially expressed (Figure 2A): 37 of these (41%)  
286 were hypo-methylated, and 54 (59%) were hyper-methylated (Figure 2B). Thirteen of  
287 the genes that were hypo-methylated in low-risk MDS also featured up-regulated gene  
288 expression (35%), all with a value of  $p < 0.10$  in both the methylation and expression  
289 analyses. *UBE2D3*, *ING1* and *RRAS2* were highlighted in this group of genes (Table  
290 2).

291 Interestingly, a high proportion (66.7%) of hyper-methylated genes was also down-  
292 regulated (all with a value of  $p < 0.10$  in the methylation and expression analyses). This  
293 combination represented the highest association between methylation and expression  
294 with respect to the other possible combinations and was consistent with the pattern  
295 expected for silenced genes. For this reason, this group of 36 genes was examined  
296 further (Figure 2B; Table 3). Using functional enrichment, we observed that the most  
297 well represented categories in this gene set were regulation of gene expression, RNA  
298 process, immune response, regulation of cell differentiation, and cell adhesion and  
299 apoptosis (Figure 3). Finally, we externally validated the most significant genes for the  
300 top altered functions: regulation of gene expression (*ETS1*), RNA process (*DICER1*),  
301 the immune response (*IL27RA*) and apoptosis (*BCL2*). The under-expression of these  
302 genes was confirmed in the larger cohort of 83 MDS patients by expression arrays  
303 (Supplementary Figure 2). In addition, the differential methylation and expression of all  
304 four genes from the integrative group was confirmed by pyrosequencing and Q-PCR,  
305 respectively, and there was a 100% correlation between these techniques and the  
306 previous results.

307

308 ***Hyper-methylation of the ETS1 transcription factor is linked to gene down-***  
309 ***regulation in low-risk MDS***

310 As *ETS1* is a transcription factor, we explored the link between the hyper-methylation  
311 of the transcription factor and the down-regulation gene observed in low-risk MDS  
312 patients. For this purpose, we analyzed the promoter regions of the 561 genes included  
313 in the under-expression signature assigned to low-risk MDS (Table 4). We searched for  
314 the TFBSs within this set of 561 genes. The analysis demonstrated that the *ETS1*  
315 transcription factor, which is hyper-methylated and under-expressed in low-risk MDS, is  
316 involved in regulating 83 target genes included in the down-regulation signature of  
317 these MDS patients. The most significant functions of these target genes were

318 delineated and the cell-to-cell signaling and interaction pathway were found to be  
319 prominently affected. The genes included in this function were *FOXP1*, *ITGAL*, *ZAP70*  
320 and *LCK* (Table 4). In addition, cell death (apoptosis) was identified as the function with  
321 greatest number of down-regulated target genes (*IL7R*, *ITGAL*, *LCK*, *MAP4K1*, *PAK2*,  
322 *PTAFR*, *TNFSF13*, *TOPBP1* and *TRADD*) (Table 4).

323

#### 324 ***DICER1-interacting genes are deregulated in low-risk MDS patients***

325 The identification of *DICER1* as a gene that is quite significantly altered by methylation  
326 and expression in low-risk MDS prompted us to investigate other genes involved in  
327 RNA processing and related to *DICER1*. *ATXN1*, *NFE2L3* and *POP4* proved to have  
328 direct genetic interactions with *DICER1*. *ATXN1* was under-expressed in low-risk MDS  
329 cases while *NFE2L3* and *POP4* were hyper-methylated and under-expressed in this  
330 group of patients (Table 3 and Supplementary Table 5). Moreover, *PIWIL4*, which was  
331 down-regulated in the low-risk MDS group, was involved in protein-protein interactions  
332 with *DICER1*. Interestingly, *POP4* and *PIWIL4* had genetic interactions with the  
333 *RNASE4* gene. This gene was under-expressed in low-risk MDS patients  
334 (Supplementary Table 5). In addition, to analyze the effect of the *DICER1* deregulation,  
335 183 miRNAs expression levels were measured. A general down-regulation of miRNAs  
336 was observed in low-risk MDS cases respect to the control group (Wilcoxon p value:  
337 0.039) (Supplementary Figure 3). However, no significant differences in miRNA-145  
338 and miRNA-196 expression between low-risk MDS and controls were observed.

339

#### 340 ***IL27RA and other immune response-related genes are down-regulated in low-risk*** 341 ***MDS patients***

342 An immune response-related analysis was carried out to compare low-risk MDS  
343 patients with the control group. This study showed that three genes involved in the  
344 histocompatibility complex (*HLA-DQB1*, *HLA-DQA1* and *HLA-DPB1*) were down-

345 regulated in low-risk MDS. We also found that besides *IL27RA*, which was hyper-  
346 methylated and under-expressed in MDS, another nine interleukins and interleukin  
347 receptors were under-expressed in the same cohort of patients: *IL16*, *IL32*, *IL1RAP*,  
348 *IL2RB*, *IL6R*, *IL7R*, *IL10RA*, *IL10RB* and *IL13RA1* (Supplementary Table 5). Three of  
349 them (*IL16*, *IL1RAP* and *IL10RB*) had direct genetic interactions with *IL27RA*.

350

351 ***Hyper-methylation of BCL2 leads to under-expression of the gene and increased***  
352 ***apoptosis in low-risk MDS***

353 The significant alteration of expression and methylation pattern of *BCL2* observed in  
354 low-risk MDS patients suggests a deregulation of the control of apoptosis. The genetic  
355 and epigenetic signatures of apoptosis-related genes in this group of patients were  
356 studied. *BCL2L11* and *MYC* were found to be over-expressed in low-risk MDS patients;  
357 in contrast, *BAX* and *CUX1* were under-expressed in this group of patients with respect  
358 to the control group. In addition, the *SYK* gene, which was hyper-methylated and  
359 under-expressed, was also associated with apoptosis and *BCL2*. In addition, we  
360 integrated all these genes in a simple interaction network to reveal the links and  
361 associations between them (Figure 4).

362

363



364 **Discussion**

365 Aberrant methylation is a potential mechanism for inactivating genes that has been  
366 implicated in several hematological malignancies, including MDS (29;30).  
367 Nevertheless, until now we have not known whether the low-risk MDS cases have a  
368 specific and distinct DNA methylation profile, as has been demonstrated for the gene  
369 expression profile (GEP) (15;31). The present study showed that the low-risk MDS  
370 patients had a different methylation profile involving 817 genes. Moreover, the GEP  
371 study displayed a deregulation of cellular development and post-translational  
372 modification genes in low-risk MDS patients. It should be noted that, in addition to  
373 these cellular functions, our analysis, performed in mononuclear cells, corroborated the  
374 mainly deregulated functions previously described in the GEP analysis of CD34+ cells,  
375 such as cellular proliferation (15) and up-regulation of histones involved in nucleosome  
376 organization (31). It is of particular note that GDF15, which was previously described  
377 as being deregulated in RARS patients (32), was the most over-expressed gene in low-  
378 risk MDS patients.

379

380 Only a few reports concerning MDS have established a connection between  
381 methylation and expression, and most of these epigenetic studies have focused on the  
382 analysis of a small number of tumor suppressor genes. For this reason, our study  
383 aimed to carry out a combined analysis of the methylation and the GEPs in low-risk  
384 MDS patients. To our knowledge, this is the first time the same cohort of patients has  
385 been used to analyze both profiles in MDS. The integrative study identified DNA  
386 methylation markers that could lead to the down-regulation of some genes involved in  
387 important cellular functions in low-risk MDS: *BCL2*, *ETS1*, *IL27RA* and *DICER1*.

388

389 MDS are characterized by ineffective hematopoiesis that results in peripheral blood  
390 cytopenias, despite the hypercellular dysplasia in bone marrow. Previous studies

391 suggested that the increased apoptosis of the bone marrow myeloid precursors is an  
392 important factor in the ineffective hematopoiesis of MDS patients. These studies also  
393 showed that the increased programmed cell death probably represents a  
394 pathophysiological mechanism rather than a compensatory process to counteract  
395 increased cell growth (31;33;34). Members of the *BCL2* family are major regulators of  
396 these apoptotic pathways. The present study shows that *BCL2* expression was  
397 significantly weaker in mononuclear low-risk MDS cells than in normal individuals.  
398 These results are in accordance with previous studies that showed reduced *BCL2*  
399 expression in CD34+ cells of patients with early MDS subtypes (35). Furthermore, our  
400 study showed that *BCL2* had significantly higher methylation levels in low-risk MDS  
401 samples. Consistent with the increasing evidence for a fundamental role of epigenetic  
402 silencing of apoptotic pathways in cancer (36-38), the hyper-methylation and the  
403 inverse correlation of mRNA expression of *BCL2* would be expected to promote  
404 apoptosis in MDS patients. The under-expression of *BCL2* in low-risk MDS due to  
405 aberrant methylation deserves further investigation as a low-risk MDS biomarker and  
406 supports a role for apoptosis-targeted therapy in these patients in the future.

407

408 Our study found hyper-methylation and under-expression of the *ETS1* gene in the  
409 same group of low-risk MDS patients compared with the control group. Several studies  
410 have indicated that the level of *ETS* expression is reduced during tumorigenesis. These  
411 analyses show that *ETS1* suppresses tumorigenicity and the cases with a high level of  
412 *ETS1* expression had better outcomes for disease-free survival than those with a low  
413 level (39). These findings suggest that under-expression of *ETS1* could have a crucial  
414 role in tumor promotion in MDS patients, especially during their early phases. *ETS1* is  
415 a nuclear phosphoprotein that functions as a transcription factor by binding the target  
416 DNA sequences containing a central GGAA/T core motif (ETS-binding site, EBS) (40).  
417 The *ETS* protein influences the expression of genes that are involved in various

418 biological processes, including hematopoiesis, cellular proliferation, differentiation,  
419 development, transformation and apoptosis (41). Over 400 *ETS1* target genes have  
420 been defined to date, based upon the presence of functional EBS in their regulatory  
421 regions (41). To investigate whether a decrease in *ETS1* expression in low-risk MDS  
422 patients had a functional effect, the expression levels of *ETS1* target genes were  
423 examined and a significant difference in the level of expression of 83 target genes in  
424 the patient group relative to control group was observed. Likewise, several other  
425 studies have demonstrated co-expression of *ETS* factors and presumptive *ETS* target  
426 genes in solid tumors (42-44).

427

428 Several approaches have been used to demonstrate that *ETS* and/or the genetic  
429 pathways that this gene regulates could be potential targets for therapy. In addition, the  
430 methylation and decreased expression of *ETS1* has been involved in silencing several  
431 genes during cellular senescence (45). Therefore, *ETS1* deregulation could be related  
432 to cellular senescence. In the same study, the mRNA expression levels of *ETS1* in the  
433 senescent cells increased significantly with the 5-aza-2'-deoxycytidine treatment.  
434 These findings could partially explain the response to 5-aza-2'-deoxycytidine treatment  
435 in MDS patients as a result of the possible induction of *ETS1*.

436

437 Apoptosis was the most widely affected function, with nine down-regulated *ETS1*  
438 targets. The overall apoptosis pathway could be affected in low-risk MDS patients in  
439 two ways: (1) methylation and decreased expression of *BCL2* with the deregulation of  
440 related genes (*BCL2L11*, *MYC*, *BAX*, *CUX1* and *SYK*), and (2) methylation and  
441 decreased expression of the *ETS1* transcription factor with the deregulation of its  
442 apoptosis-related targets. The molecular basis of apoptosis in MDS is largely unknown  
443 and comprehensive characterization of epigenetic disruption of apoptosis-related  
444 genes in MDS cases is lacking. For this reason, these findings may shed some light on

445 this matter. In addition, a clearer understanding of the molecular events leading to the  
446 deregulation of cell death in MDS should allow us to identify therapeutic targets and  
447 diagnostic markers.

448

449 IL27RA is a component of the heterodimeric complex receptor IL27R that is involved in  
450 immunosuppression by inducing a signal transduction in response to IL27 (46). Our  
451 studies identified a marked difference in *IL27RA* methylation levels between low-risk  
452 MDS patients and healthy controls that may be responsible for the under-expression  
453 shown by these patients. These results are consistent with recent studies in which  
454 *IL27RA* has been shown to be a promoter of hematopoietic stem cell differentiation,  
455 which appears to enhance myelopoiesis in a transgenic mouse system (46). According  
456 to this, down-regulation of *IL27RA* could lead to the ineffective differentiation of  
457 hematopoietic progenitors already described in MDS patients by other authors (33).  
458 Moreover, animal models with defects in IL27 or its receptor (IL27RA) display  
459 enhanced immune responses in a range of infectious and noninfectious situations (47).  
460 Therefore, our results are also consistent with these features and with the deregulation  
461 of the immune response known in MDS (33). Furthermore, immune response  
462 deregulation could be enhanced in low-risk MDS patients due to the genetic  
463 interactions between IL27RA and IL16, IL1RAP and IL10RB, and the lower level of  
464 expression of histocompatibility complex genes.

465

466 DICER1 is an RNase III endonuclease essential for microRNA (miRNA) biogenesis and  
467 RNA processing (48). Altered miRNA expression can be expected to occur as a result  
468 of variations in pre-miRNA processing by DICER1. Fluctuations in miRNA expression  
469 regulate the expression of key tumor suppressor genes and oncogenes (49) and the  
470 fate of hematopoietic cells (48). Their global deregulation by the under-expression of  
471 *DICER1* promotes tumorigenesis. Reduced *DICER1* expression has been associated

472 with multiple solid neoplasias (49). In the current study, we observed that *DICER1*  
473 expression levels were widely lower in patients with low-risk MDS. In addition, *DICER1*  
474 was hyper-methylated in low-risk MDS, which could be responsible for the *DICER1*  
475 under-expression observed in these patients. A deletion in *DICER1* has been recently  
476 described in osteoprogenitors that impairs osteoblastic differentiation and the integrity  
477 of hematopoiesis and induces bone marrow dysfunction with myelodysplasia (48).  
478 These data suggest that the disruption of *DICER1* by methylation or mutation may  
479 cause myelodysplasia in mice resembling important features of human MDS. Our  
480 findings also showed that *DICER1* had direct genetic interactions with *ATXN1*, *NFE2L3*  
481 and *POP4* deregulated genes, which might affect the normal relationship of these  
482 genes with *DICER1* and consequently the deregulation of the functions in which they  
483 are involved. Furthermore, our data showed an overall slight down-regulation of  
484 miRNAs in low-risk MDS (p-value = 0.039) which could be related to the deregulation  
485 of *DICER1*. However, no significant differences were found for two miRNAs (miRNA-  
486 145 and miRNA-196) that were examined individually.

487

488 Recent advances have suggested a potential role for hyper-methylation in cancer  
489 because of the transcriptional silencing (50). Nevertheless, global DNA hypo-  
490 methylation in cancer may be as frequent as hyper-methylation (51). Our study showed  
491 that *ING1*, *UBE2D3* and *RRAS2* genes were hypo-methylated and over-expressed in  
492 low-risk MDS patients. The *ING1* and *UBE2D3* genes are both related to p53. The  
493 *ING1* gene encodes a protein that can induce cell growth arrest and apoptosis by  
494 cooperating with p53, and *UBE2D3* functions in the ubiquitination of p53. *RRAS2*,  
495 previously described as being up-regulated by other authors (33), may play an  
496 important role in activating signal transduction pathways that control cell proliferation.  
497 Thus, the alteration of these three genes could be implicated in functions previously  
498 described as deregulated in MDS (15;33).

499 In summary, we have generated a DNA methylation profile for low-risk MDS patients  
500 that could extend our knowledge of these diseases. RNA expression levels were  
501 analyzed and correlated with methylation status, suggesting that DNA hyper-  
502 methylation events in low-risk MDS are biologically important for gene functions such  
503 as gene expression, RNA processes, the immune response and apoptosis. In addition,  
504 these epigenetic modifications that lead to transcriptional silencing of genes are  
505 attractive therapeutic targets for demethylating agents.

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521

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713 **Figure Legends**

714 **Figure 1. GEP of low-risk MDS patients and normal bone marrow samples.** The  
715 heatmap shows hierarchical clustering of 1005 genes differentially expressed between  
716 low-risk MDS and control patients. This gene-set was obtained for an FDR cut-off <  
717 0.10 and included 444 upregulated genes and 561 down-regulated genes. The  
718 absolute expression signal obtained for each gene in each sample is represented by a  
719 color scale. Green indicates upregulation and red indicates down-regulation, black  
720 being the intermediate signal. Each row represents a single gene and each column  
721 corresponds to a separate patient sample. The distances in the clustering are based on  
722 Pearson correlation coefficients, calculated pair-wise, using the expression signature of  
723 each gene in all samples. The unique low-risk MDS cases that displayed chromosomal  
724 alterations are showed with a color point: blue for a loss on 5q and orange for a  
725 monosomy 7

726 (GEP: gene expression profile; MDS: myelodysplastic syndrome)

727

728 **Figure 2. Integrative epi/genomic analysis of low-risk MDS patients compared**  
729 **with controls. A.** Total number of differentially expressed and methylated genes in  
730 low-risk MDS and healthy controls. 91 differentially methylated genes were also  
731 deregulated. **B.** Quantification of genes identified in a two-way analysis. Bars represent  
732 the differentially methylated genes and the two colors within each bar indicate the  
733 number of differentially expressed genes. The hyper-methylation and under-expression  
734 combination corresponds to the most frequent association between the two analyses  
735 with respect to the other possible combinations.

736 (MDS: myelodysplastic syndrome)

737

738 **Figure 3. Functional analysis of hyper-methylated and under-expressed genes in**  
739 **low-risk MDS patients.** Identification of processes significantly enriched in the hyper-



740 methylation and under-expression profile of low-risk MDS subjects compared with  
741 control cases. The functional enrichment of the selected genes was analyzed using  
742 DAVID, IPA and Metacore bioinformatics tools. The most representative biological  
743 processes with the highest number of genes are included. The best represented  
744 category was "Regulation of gene expression", which involves 15 genes.

745 (MDS: myelodysplastic syndrome)

746

747 **Figure 4. Schematic representation of the deregulated BCL2-related pathway in**  
748 **low-risk MDS patients.** Red and green respectively denote gain and loss of  
749 expression in the low-risk MDS group relative to control subjects. The yellow genes  
750 BCL2 and SYK were hyper-methylated and under-expressed in low-risk MDS, while  
751 *BCL2L11* and *MYC* were over-expressed and *BAX* and *CUX1* were under-expressed in  
752 the low-risk MDS patients with respect to the control group. An arrow pointing from A to  
753 B signifies that A causes B to be activated. Union between molecules shows protein-  
754 protein interactions which occur when two or more proteins bind together, often to carry  
755 out their biological functions. Many of the most important molecular processes in the  
756 cell are carried out by a large number of protein components organized by their protein-  
757 protein interactions. Solid and dashed lines respectively indicate direct and indirect  
758 interactions between molecules. The gene network was generated with the Ingenuity  
759 bioinformatics tool.

760 (MDS: myelodysplastic syndrome)

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766

**Table 1. Most representative cellular functions corresponding to the 817 genes deregulated by methylation in low-risk MDS patients.**

<i>Cellular Function</i>	<i>p-Value</i>
Development_GM-CSF signaling	5,867E-07
Apoptosis and survival_HTR1A signaling	0,000007751
Development_TGF-beta-dependent induction of EMT via SMADs	0,00001066
Development_VEGF signaling and activation	0,00003635
Development_Regulation of epithelial-to-mesenchymal transition (EMT)	0,00004061
Transcription_CREB pathway	0,00004159
Immune response_CD137 signaling in immune cell	0,00005866
Immune response_Inflammatory response	0,00006636
Immune response_Histamine H1 receptor signaling in immune response	0,00006889
Immune response_Histamine signaling in dendritic cells	0,00008708

767

768

**Table 2. Integration analysis of hypo-methylated and over-expressed genes in low-risk MDS patients.**

<i>Gene Symbol</i>	<i>Expression</i>			<i>Methylation</i>			
	<i>d-Value</i>	<i>p-Value</i>	<i>R.Fold</i>	<i>Genomic Coordinates</i>	<i>CpG Position</i>	<i>p-Value</i>	<i>Fold Change</i>
CDH4	4,61	<b>0,000</b>	1,22	chr20:59839134-59839709	within	<b>0,057</b>	-1,95
RAB8B	4,52	<b>0,000</b>	1,70	chr15:61235998-61237132	downStream	<b>0,052</b>	-2,58
UBE2D3	3,4	<b>0,004</b>	1,18	chr4:104009407-104010129	upStream	<b>0,080</b>	-1,43
ING1	3,19	<b>0,006</b>	1,37	chr13:110249836-110250880	upStream	<b>0,080</b>	-1,52
TBPL1	2,91	<b>0,011</b>	1,49	chr6:134258454-134259172	downStream	<b>0,050</b>	-2,96
CYB5D1	2,84	<b>0,012</b>	1,22	chr17:7702645-7702833	within	<b>0,043</b>	-1,77
FADS2	2,75	<b>0,015</b>	1,26	chr11:61394615-61395519	upStream	<b>0,008</b>	-2,09
HCN3	2,72	<b>0,016</b>	1,18	chr1:153500080-153500610	downStream	<b>0,006</b>	-4,29
H2AFJ	2,59	<b>0,020</b>	1,40	chr12:14847117-14848131	upStream	<b>0,049</b>	-1,88
RRAS2	2,57	<b>0,021</b>	1,43	chr11:14242512-14242599	downStream	<b>0,019</b>	-2,73
SYN3	2,56	<b>0,022</b>	1,22	chr22:31900470-31901416	upStream	<b>0,020</b>	-1,61
AAAS	2,53	<b>0,023</b>	1,29	chr12:52001203-52001932	within	<b>0,040</b>	-1,44
FXD2	2,50	<b>0,024</b>	1,19	chr11:117021453-117021806	downStream	<b>0,064</b>	-1,45

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Table 3. Integration analysis of hyper-methylated and down-expressed genes in low-risk MDS patients.

Gene Symbol	Expression			Methylation			
	d-Value	p-Value	R.Fold	Genomic Coordinates	CpG Position	p-Value	Fold Change
PLAGL1	-4,96	<b>0,000</b>	0,48	chr6:144457997-144458742	downStream	<b>0,029</b>	1,91
BCL2	-4,95	<b>0,000</b>	0,74	chr18:59137439-59137855	within	<b>0,039</b>	1,37
NELL2	-4,82	<b>0,000</b>	0,39	chr12:43649424-43649603	downStream	<b>0,008</b>	2,12
DICER1	-4,70	<b>0,000</b>	0,45	chr14:94304731-94304947	upStream	<b>0,070</b>	1,46
NFE2L3	-4,46	<b>0,000</b>	0,78	chr7:25868518-25868873	upStream	<b>0,075</b>	1,62
IL27RA	-3,97	<b>0,001</b>	0,65	chr19:14046138-14046802	downStream	<b>0,043</b>	2,16
ALCAM	-3,95	<b>0,001</b>	0,57	chr3:106555109-106555818	upStream	<b>0,079</b>	1,98
OPN3	-3,88	<b>0,001</b>	0,58	chr1:239850696-239850953	within	<b>0,008</b>	2,55
IER3IP1	-3,86	<b>0,001</b>	0,67	chr18:43041077-43041115	downStream	<b>0,030</b>	3,25
BNIP2	-3,70	<b>0,002</b>	0,70	chr15:58079608-58080222	downStream	<b>0,093</b>	1,36
RPS6KA5	-3,65	<b>0,002</b>	0,58	chr14:90398496-90398580	upStream	<b>0,009</b>	1,66
SCP2	-3,60	<b>0,003</b>	0,62	chr1:53291314-53291693	downStream	<b>0,084</b>	1,42
PTPRC	-3,40	<b>0,004</b>	0,69	chr1:198277915-198278316	downStream	<b>0,004</b>	2,43
CHML	-3,33	<b>0,004</b>	0,63	chr1:239850696-239850953	upStream	<b>0,008</b>	2,55
ZNF33A	-3,27	<b>0,005</b>	0,64	chr10:38422763-38423049	downStream	<b>0,087</b>	2,22
ETS1	-3,14	<b>0,007</b>	0,69	chr11:127896681-127897162	within	<b>0,054</b>	2,29
GNS	-3,06	<b>0,008</b>	0,67	chr12:63700546-63700923	downStream	<b>0,098</b>	1,65
NPHP3	-3,05	<b>0,008</b>	0,68	chr3:134240338-134240638	downStream	<b>0,059</b>	1,94
ZNF37A	-2,91	<b>0,010</b>	0,80	chr10:38422763-38423049	upStream	<b>0,087</b>	2,22
NSMCE1	-2,74	<b>0,015</b>	0,80	chr16:27237492-27237759	downStream	<b>0,064</b>	4,37
RHOA	-2,73	<b>0,015</b>	0,59	chr1:227321939-227322103	downStream	<b>0,060</b>	1,57
CNOT6L	-2,70	<b>0,016</b>	0,69	chr4:78960590-78961293	downStream	<b>0,050</b>	2,78
RPL36AL	-2,69	<b>0,017</b>	0,86	chr14:49134842-49136086	upStream	<b>0,059</b>	7,82
KIAA1128	-2,57	<b>0,021</b>	0,78	chr10:87813066-87813308	downStream	<b>0,011</b>	1,51
ENC1	-2,49	<b>0,025</b>	0,70	chr5:73973356-73973603	downStream	<b>0,051</b>	2,31
MAP2K1	-2,48	<b>0,025</b>	0,69	chr15:64436285-64436471	upStream	<b>0,014</b>	1,60
KLHL8	-2,41	<b>0,029</b>	0,68	chr4:88375883-88376244	downStream	<b>0,014</b>	1,75
CENTD1	-2,36	<b>0,033</b>	0,63	chr4:31377103-31377307	upStream	<b>0,071</b>	2,09
PH-4	-2,34	<b>0,034</b>	0,85	chr3:48932150-48932615	upStream	<b>0,070</b>	1,49
FVT1	-2,33	<b>0,034</b>	0,80	chr18:59137439-59137855	upStream	<b>0,039</b>	1,37
CD28	-2,30	<b>0,037</b>	0,87	chr2:204053785-204053871	upStream	<b>0,062</b>	2,02
CHIT1	-2,24	<b>0,041</b>	0,41	chr1:201503249-201503687	downStream	<b>0,099</b>	1,50
C10orf11	-2,22	<b>0,042</b>	0,69	chr10:76838695-76839061	upStream	<b>0,013</b>	1,50
CTSC	-2,18	<b>0,046</b>	0,63	chr11:87548353-87548640	upStream	<b>0,024</b>	1,85
RHOQ	-2,18	<b>0,046</b>	0,72	chr2:46696964-46697947	upStream	<b>0,006</b>	3,21
AK2	-2,15	<b>0,049</b>	0,73	chr1:33319679-33319945	downStream	<b>0,029</b>	1,50

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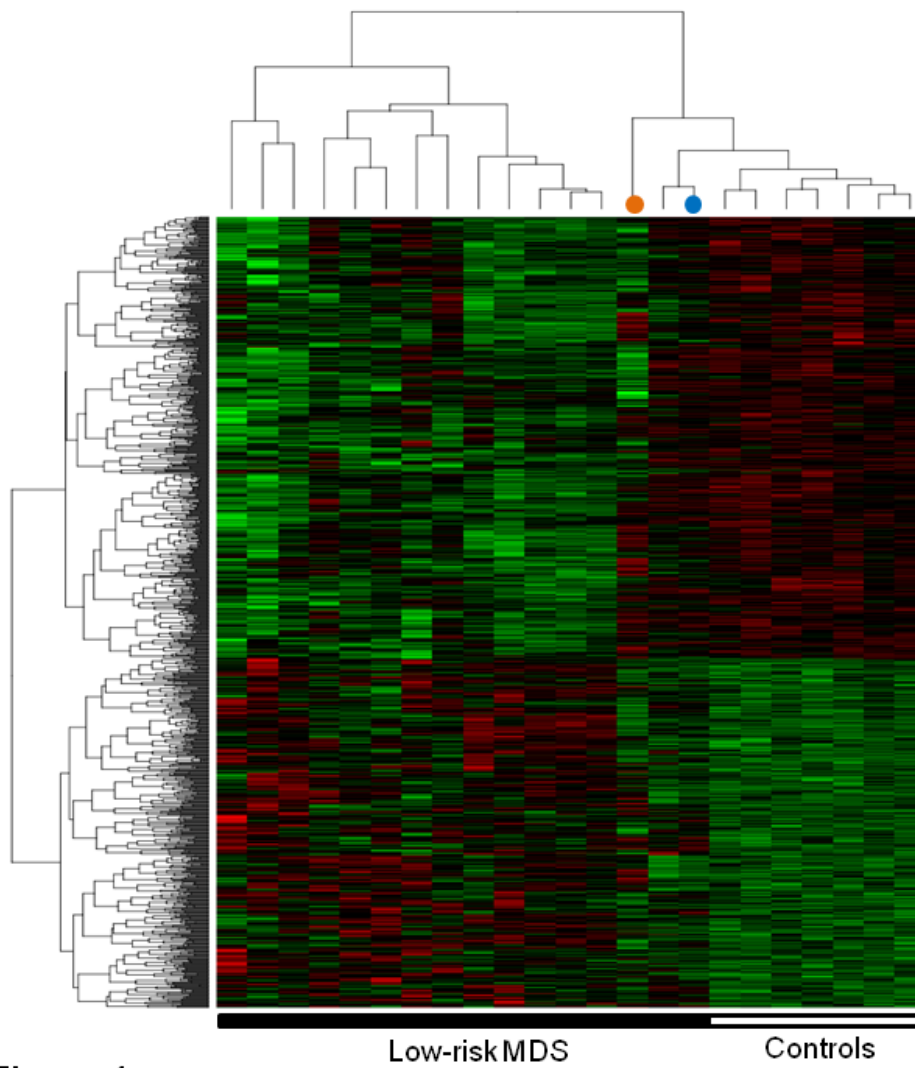
**Table 4. ETS1 under-expressed targets involved in deregulated pathways in low-risk MDS patients.**

<i>Category</i>	<i>p- Value</i>	<i>Number of Genes</i>	<i>Molecules</i>
<b>Cell Death</b>	7,62E-03 - 4,13E-02	9	IL7R, ITGAL, LCK, MAP4K1, PAK2, PTAFR, TNFSF13, TOPBP1, TRADD
<b>Cellular Function and Maintenance</b>	9,03E-05 - 4,62E-02	6	IL7R, ITGAL, LCK, MAP4K1, ZAP70, FOXP1
<b>Hematological System Development and Function</b>	1,45E-04 - 4,62E-02	6	IL7R, ITGAL, LCK, MAP4K1, ZAP70, TNFSF13
<b>Cellular Development</b>	1,47E-03 - 4,62E-02	6	IL7R, ITGAL, LCK, MAP4K1, ZAP70, LPP
<b>Hematopoiesis</b>	1,47E-03 - 4,62E-02	5	IL7R, ITGAL, LCK, MAP4K1, ZAP70
<b>Cell-To-Cell Signaling and Interaction</b>	9,03E-05 - 3,46E-02	4	FOXP1, ITGAL, ZAP70, LCK
<b>Cell-mediated Immune Response</b>	1,45E-04 - 4,62E-02	4	IL7R, LCK, MAP4K1, ZAP70
<b>Genetic Disorder</b>	5,43E-03 - 2,12E-02	4	ATXN1, ITGAL, MAP4K1, TRADD
<b>Molecular Transport</b>	7,01E-03 - 3,65E-02	4	LCK, TRAT1, ZAP70, PATAFR
<b>Gene Expression</b>	2,89E-04 - 4,99E-02	3	LCK, ZAP70, LEF1
<b>Cellular Growth and Proliferation</b>	6,63E-03 - 1,49E-02	3	IL7R, TNFSF13, ZAP70
<b>Cell Morphology</b>	7,01E-03 - 4,81E-02	3	LCK, LPP, ZAP70
<b>Cellular Assembly and Organization</b>	7,01E-03 - 4,81E-02	3	LCK, PTAFR, ZAP70
<b>Cancer</b>	8,50E-03	2	LCK, ZAP70
<b>Cell Cycle</b>	7,01E-03	1	PTAFR

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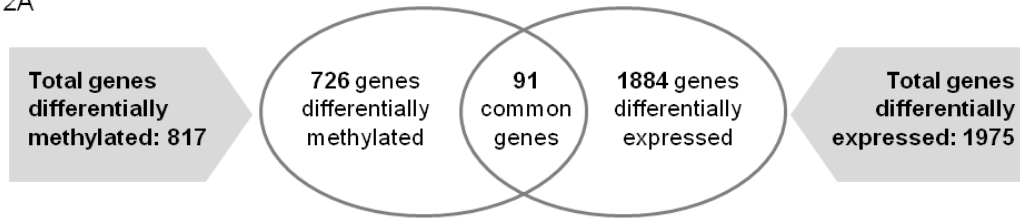
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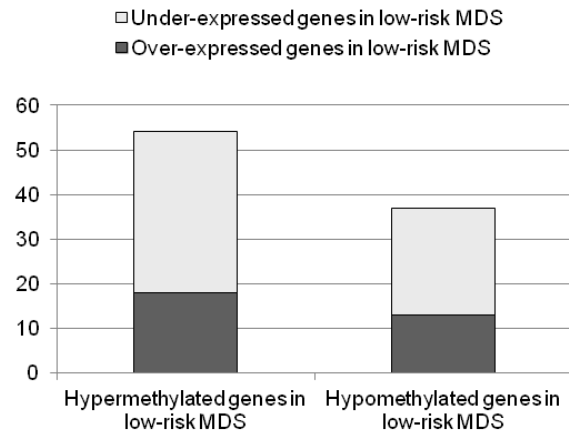
**Figure 1**

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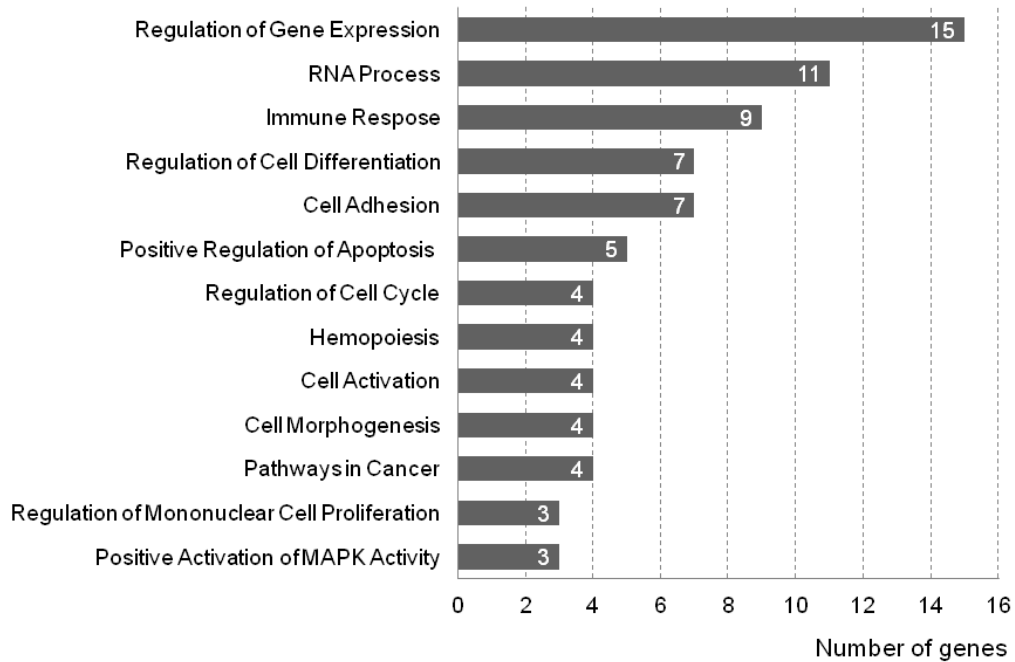


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Figure 2



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Figure 3

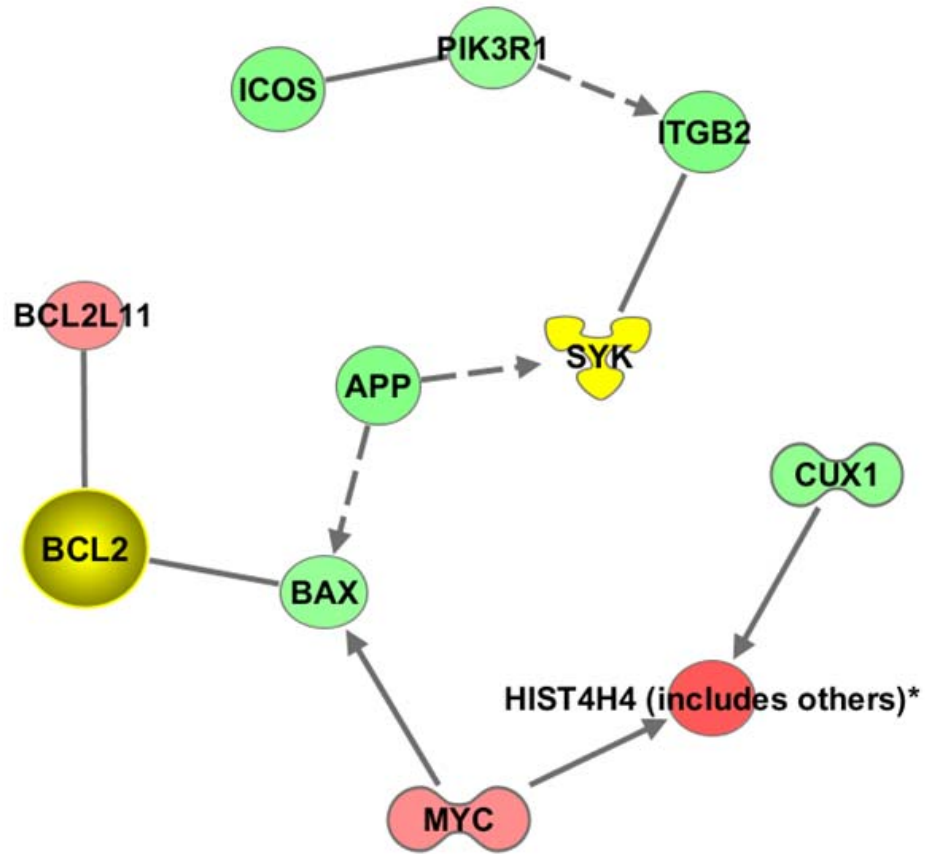


Figure 4

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