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Del Rey, B. M., O'Hagan, K., Dellett, M., Aibar, S., Colyer, H. A. A., Alonso, M. E., Díez-Campelo, M., Armstrong, R. N., Sharpe, D. J., Gutiérrez, N. C., García, J. L., De Las Rivas, J., Mills, K. I., & Hernández-Rivas, J. M. (2013). Genome-wide profiling of methylation identifies novel targets with aberrant hyper-methylation and reduced expression in low-risk myelodysplastic syndromes. *Leukemia*, *27*(3), 610-618. https://doi.org/10.1038/leu.2012.253

Published in:
Leukemia

Document Version:
Peer reviewed version

Accepted Article Preview: Published ahead of advance online publication

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Cite this article as: BM del Rey, K O'Hagan, M Dellett, S Aibar, HAA Colyer, ME Alonso, M Díez-Campelo, RN Armstrong, DJ Sharpe, NC Gutiérrez, JL García, J De Las Rivas, KI Mills, JM Hernández-Rivas, Genome-wide profiling of methylation identifies novel targets with aberrant hyper-methylation and reduced expression in low-risk myelodysplastic syndromes, *Leukemia* accepted article preview 31 August 2012; doi: 10.1038/leu.2012.253.

Received 30 May 2012; revised 22 August 2012; accepted 24 August 2012; Accepted article preview online 31 August 2012

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

Epigenetic Regulation in Low-Risk Myelodysplastic Syndromes

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Abstract

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

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

Introduction

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

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. This process is carried out in an orchestrated reaction that includes DNA methyltransferases, methyl-binding domain proteins, and histone deacetylases (4). 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 (5;6). In addition, not only the presence of epigenetic marks but also their location and density play a crucial role in regulating these processes (7;8). A close correlation between DNA hyper-methylation and transcriptional silencing has been established in many systems (9).

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 (5;7;9-11).

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 (12). Hence, epigenetic modifications in promoter and/or regulatory regions that lead to transcriptional silencing of genes and development of cancer are important events requiring to be studied in any onco-pathological state and they are attractive therapeutic targets.

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

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

Materials and Methods

Samples collection and cell separation

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

DNA and RNA isolation

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

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

Methylation CpG island amplification and microarray studies (MCAM)

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

microarray (UHN, Toronto, Canada) contains 12,192 CpG island clones. Each clone can be annotated with up to three gene symbols, depending on whether the CpG site lies upstream, downstream or within the gene. The methylation assay was done largely as described previously (17), but with the following modifications: DNA was purified after double digestion, methylated CpG amplification (MCA) reaction and labeling were done using the QIAquick PCR purification kit (Qiagen) without any indicator in the buffer PB. RMCA primers (Eurogentec, Southampton, UK) were used at ligation and MCA reaction stages. The MCA reaction was performed using 5 U HotStarTaq+ (Qiagen) and samples were aliquoted without primers before being heated to 82 °C. The primers were held at 95 °C for 1 min before adding 4 µL to each tube. Cycling conditions were: 95 °C for 10 min before 30 cycles of 95 °C for 1 min, 65 °C for 90 s, 72 °C for 2 min, and finally 72 °C for 10 min, and before holding at 4 °C. Samples were hybridized to UHN HCGI12K CpG microarrays. After hybridization, microarrays were washed as follows: 3 × 15 min at 55 °C with wash 1 in a rotating oven, then on a gyrating platform 2 × 3 min at room temperature (R.T.) 1x SSC, 2 × 3 min at R.T. Wash 2: 2 × 3 min at R.T. 0.1 × SSC, 2 × 3 min at R.T. Millipore H_2O. Finally, slides were rinsed with H_2O and centrifuged for 7 min at 370 g to dry. They were scanned with an Axon GenePix 4400A scanner (MDS Analytical Technologies, Molecular Devices, Sunnyvale, CA, USA) using Genepix Pro 7 software (MDS Analytical Technologies).

Bioinformatic analysis

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

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

Gene expression microarray studies

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

Bioinformatic analysis

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

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

Real-Time PCR

To validate the GEP results, the expression levels of four selected genes were analyzed by RT-PCR. First-strand cDNA was generated from 1 µg of total RNA using poly-dT as primers with the M-MLV reverse transcriptase (Promega). Real-time PCR was performed in triplicate. Each 20µl 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. The reactions were run on an iQ5 Real-Time PCR detection system (Bio-Rad, Hercules, CA, USA). The primers were designed for specific sequences (Supplementary Table 2) and checked with the BLAST algorithm (22). In addition, to measure miRNA-145 and miRNA-196 expression levels, TaqMan qRT-PCR miRNA assay (Applied Biosystem, Carlsbad, California) was performed. The relative expression levels normalized to RNU43 endogenous control was determined using the $2^{-\Delta Ct}$ method. Each measurement was performed in duplicate.

Pyrosequencing

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

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

Integrative functional analysis of methylation and expression data

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

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

Results

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

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

Gene expression profiling distinguishes low-risk MDS from normal BM

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

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

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

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

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

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

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

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

DICER1-interacting genes are deregulated in low-risk MDS patients

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

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

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

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

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

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

Discussion

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

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

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

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

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

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

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

Apoptosis was the most widely affected function, with nine down-regulated ETS1 targets. The overall apoptosis pathway could be affected in low-risk MDS patients in two ways: (1) methylation and decreased expression of *BCL2* with the deregulation of related genes (*BCL2L11*, *MYC*, *BAX, CUX1* and *SYK*), and (2) methylation and decreased expression of the *ETS1* transcription factor with the deregulation of its apoptosis-related targets. The molecular basis of apoptosis in MDS is largely unknown and comprehensive characterization of epigenetic disruption of apoptosis-related 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

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

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

Acknowledgements

This study was partially supported by grants from the Spanish Fondo de Investigaciones Sanitarias FIS 09/01543, Proyectos de Investigación del SACYL 355/A/09, COST Action “EuGESMA” (BM0801), Northern Ireland Leukaemia Research Fund (NILRF), Northern Ireland Assembly Department of Education and Learning, Obra Social Banca Cívica (Caja Burgos), and by the "Acción Transversal del Cáncer" project, through an agreement between the Instituto de Salud Carlos III (ISCIII), Spanish Ministry of Science and Innovation, the Cancer Research Foundation of Salamanca University and the Redes de Investigación RTIIC (FIS). MdR is fully supported by an "Ayuda Predoctoral” by the Fundación Española de Hematología y Hemoterapia. We thank Irene Rodríguez, Sara González, Teresa Prieto, Mª Ángeles Ramos, Almudena Martín, Ana Díaz, Ana Simón, María del Pozo and Vanesa Gutiérrez of the Centro de Investigación del Cáncer, Salamanca, Spain and Anne Carson from the Centre for Cancer Research and Cell Biology, Belfast, UK, for their technical assistance.

Conflict of interest disclosure: The authors declare no conflict of interest.

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Figure Legends

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

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

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

(MDS: myelodysplastic syndrome)

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

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

(MDS: myelodysplastic syndrome)

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

(MDS: myelodysplastic syndrome)

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

Cellular Function	*p-Value*
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

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Table 2. Integration analysis of hypo-methylated and over-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*
CDH4	4,61	**0,000**	1,22	chr20:59839134-59839709	within	**0,057**	-1,95
RAB8B	4,52	**0,000**	1,70	chr15:61235998-61237132	downStream	**0,052**	-2,58
UBE2D3	3,4	**0,004**	1,18	chr4:104009407-104010129	upStream	**0,080**	-1,43
ING1	3,19	**0,006**	1,37	chr13:110249836-110250880	upStream	**0,080**	-1,52
TBPL1	2,91	**0,011**	1,49	chr6:134258454-134259172	downStream	**0,050**	-2,96
CYB5D1	2,84	**0,012**	1,22	chr17:7702645-7702833	within	**0,043**	-1,77
FADS2	2,75	**0,015**	1,26	chr11:61394615-61395519	upStream	**0,008**	-2,09
HCN3	2,72	**0,016**	1,18	chr1:153500080-153500610	downStream	**0,006**	-4,29
H2AFJ	2,59	**0,020**	1,40	chr12:14847117-14848131	upStream	**0,049**	-1,88
RRAS2	2,57	**0,021**	1,43	chr11:14242512-14242599	downStream	**0,019**	-2,73
SYN3	2,56	**0,022**	1,22	chr22:31900470-31901416	upStream	**0,020**	-1,61
AAAS	2,53	**0,023**	1,29	chr12:52001203-52001932	within	**0,040**	-1,44
FXYD2	2,50	**0,024**	1,19	chr11:117021453-117021806	downStream	**0,064**	-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	**0,000**	0,48	chr6:144457997-144458742	downStream	**0,029**	1,91
BCL2	-4,95	**0,000**	0,74	chr18:59137439-59137855	within	**0,039**	1,37
NELL2	-4,82	**0,000**	0,39	chr12:43649424-43649603	downStream	**0,008**	2,12
DICER1	-4,70	**0,000**	0,45	chr14:94304731-94304947	upStream	**0,070**	1,46
NFE2L3	-4,46	**0,000**	0,78	chr7:25868518-25868873	upStream	**0,075**	1,62
IL27RA	-3,97	**0,001**	0,65	chr19:14046138-14046802	downStream	**0,043**	2,16
ALCAM	-3,95	**0,001**	0,57	chr3:106555109-106555818	upStream	**0,079**	1,98
OPN3	-3,88	**0,001**	0,58	chr1:239850696-239850953	within	**0,008**	2,55
IER3IP1	-3,86	**0,001**	0,67	chr18:43041077-43041115	downStream	**0,030**	3,25
BNIP2	-3,70	**0,002**	0,70	chr15:58079608-58080222	downStream	**0,093**	1,36
RPS6KA5	-3,65	**0,002**	0,58	chr14:90398496-90398580	upStream	**0,009**	1,66
SCP2	-3,60	**0,003**	0,62	chr1:53291314-53291693	downStream	**0,084**	1,42
PTPRC	-3,40	**0,004**	0,69	chr1:198277915-198278316	downStream	**0,004**	2,43
CHML	-3,33	**0,004**	0,63	chr1:239850696-239850953	upStream	**0,008**	2,55
ZNF33A	-3,27	**0,005**	0,64	chr10:38422763-38423049	downStream	**0,087**	2,22
ETS1	-3,14	**0,007**	0,69	chr11:127896681-127897162	within	**0,054**	2,29
GNS	-3,06	**0,008**	0,67	chr12:63700546-63700923	downStream	**0,098**	1,65
NPHP3	-3,05	**0,008**	0,68	chr3:134240338-134240638	downStream	**0,059**	1,94
ZNF37A	-2,91	**0,010**	0,80	chr10:38422763-38423049	upStream	**0,087**	2,22
NSMCE1	-2,74	**0,015**	0,80	chr16:27237492-27237759	downStream	**0,064**	4,37
RHOU	-2,73	**0,015**	0,59	chr1:227321939-227322103	downStream	**0,060**	1,57
CNOT6L	-2,70	**0,016**	0,69	chr4:78960590-78961293	downStream	**0,050**	2,78
RPL36AL	-2,69	**0,017**	0,86	chr14:49134842-49136086	upStream	**0,059**	7,82
KIAA1128	-2,57	**0,021**	0,78	chr10:87813066-87813308	downStream	**0,011**	1,51
ENC1	-2,49	**0,025**	0,70	chr5:73973356-73973603	downStream	**0,051**	2,31
MAP2K1	-2,48	**0,025**	0,69	chr15:64436285-64436471	upStream	**0,014**	1,60
KLHL8	-2,41	**0,029**	0,68	chr4:88375883-88376244	downStream	**0,014**	1,75
CENTD1	-2,36	**0,033**	0,63	chr4:31377103-31377307	upStream	**0,071**	2,09
PH-4	-2,34	**0,034**	0,85	chr3:48932150-48932615	upStream	**0,070**	1,49
FVT1	-2,33	**0,034**	0,80	chr18:59137439-59137855	upStream	**0,039**	1,37
CD28	-2,30	**0,037**	0,87	chr2:204053785-204053871	upStream	**0,062**	2,02
CHIT1	-2,24	**0,041**	0,41	chr1:201503249-201503687	downStream	**0,099**	1,50
C10orf11	-2,22	**0,042**	0,69	chr10:76838695-76839061	upStream	**0,013**	1,50
CTSC	-2,18	**0,046**	0,63	chr11:87548353-87548640	upStream	**0,024**	1,85
RHOQ	-2,18	**0,046**	0,72	chr2:46696964-46697947	upStream	**0,006**	3,21
AK2	-2,15	**0,049**	0,73	chr1:33319679-33319945	downStream	**0,029**	1,50

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

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

774

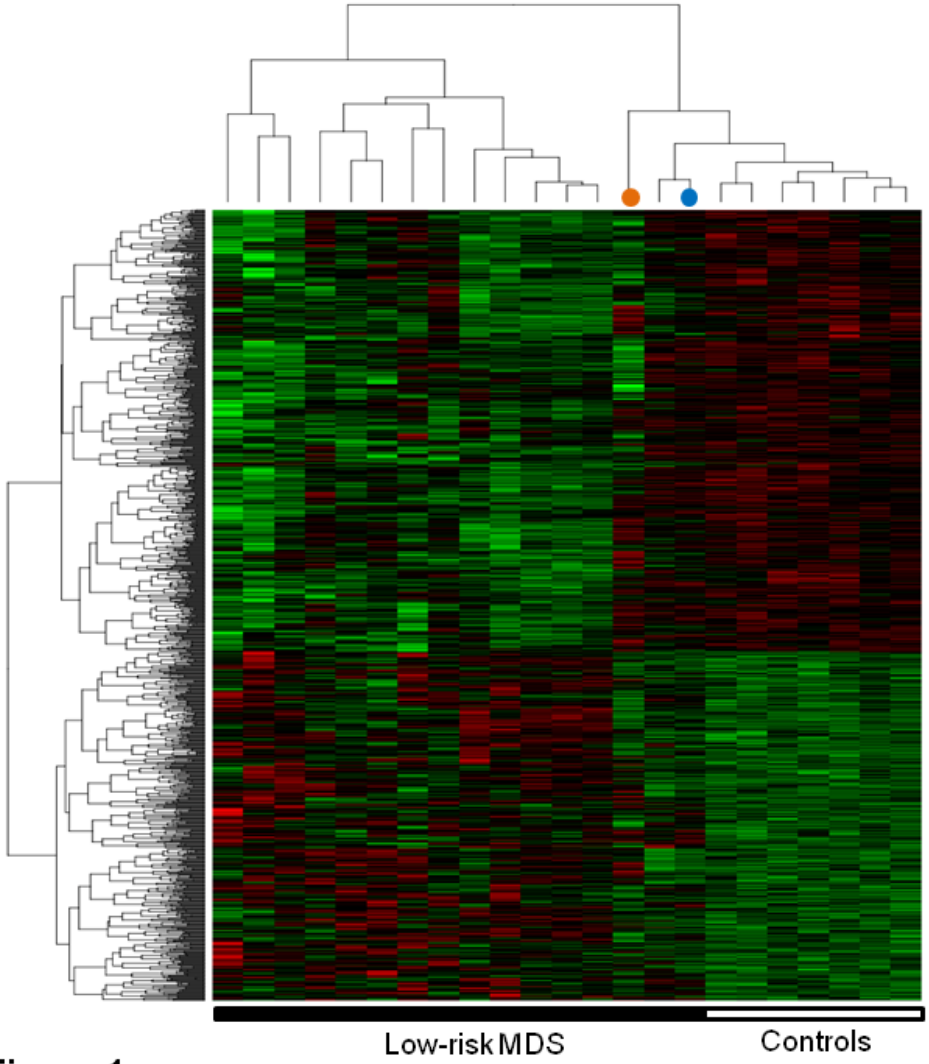
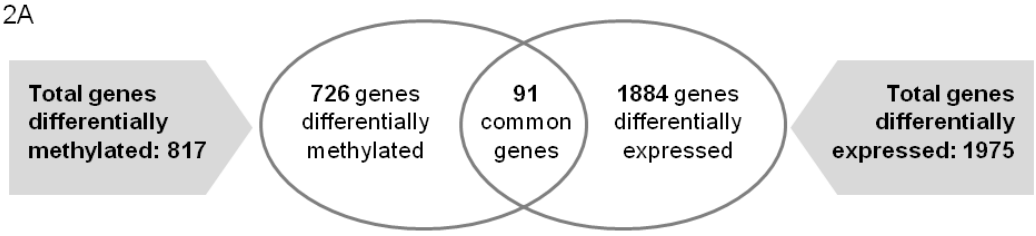


Figure 1

775

2A



2B

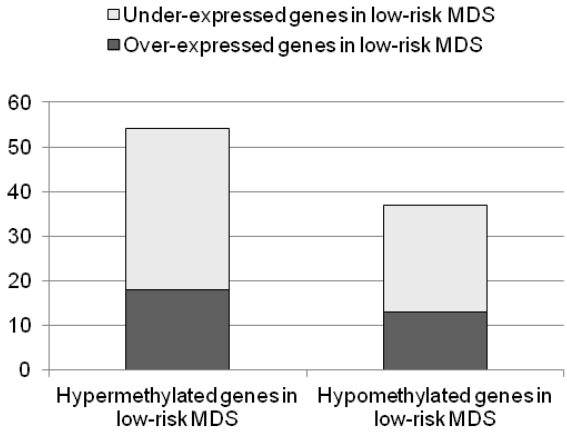


Figure 2

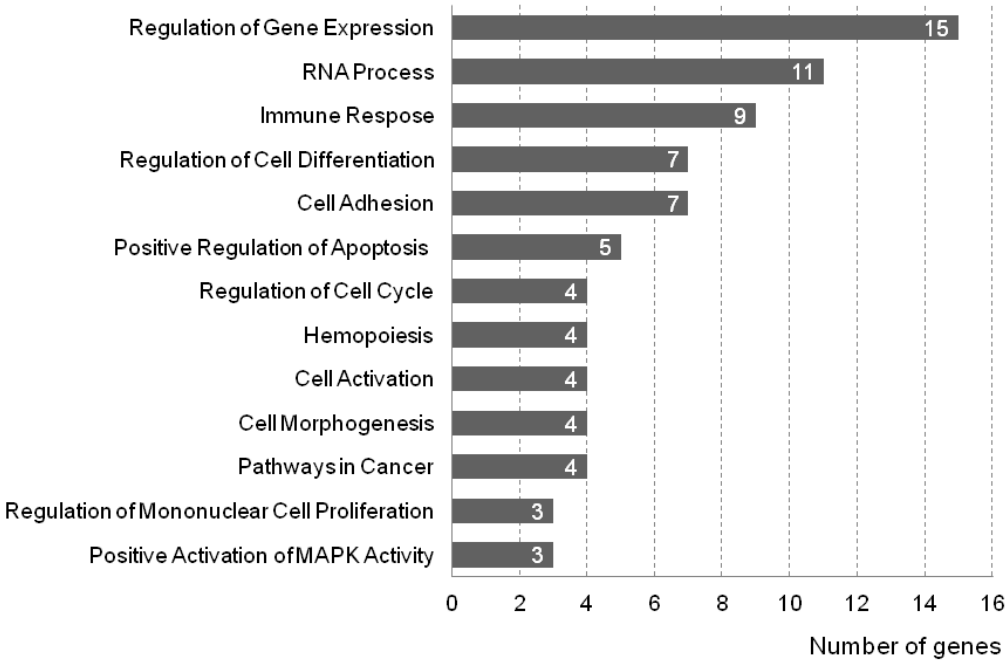


Figure 3

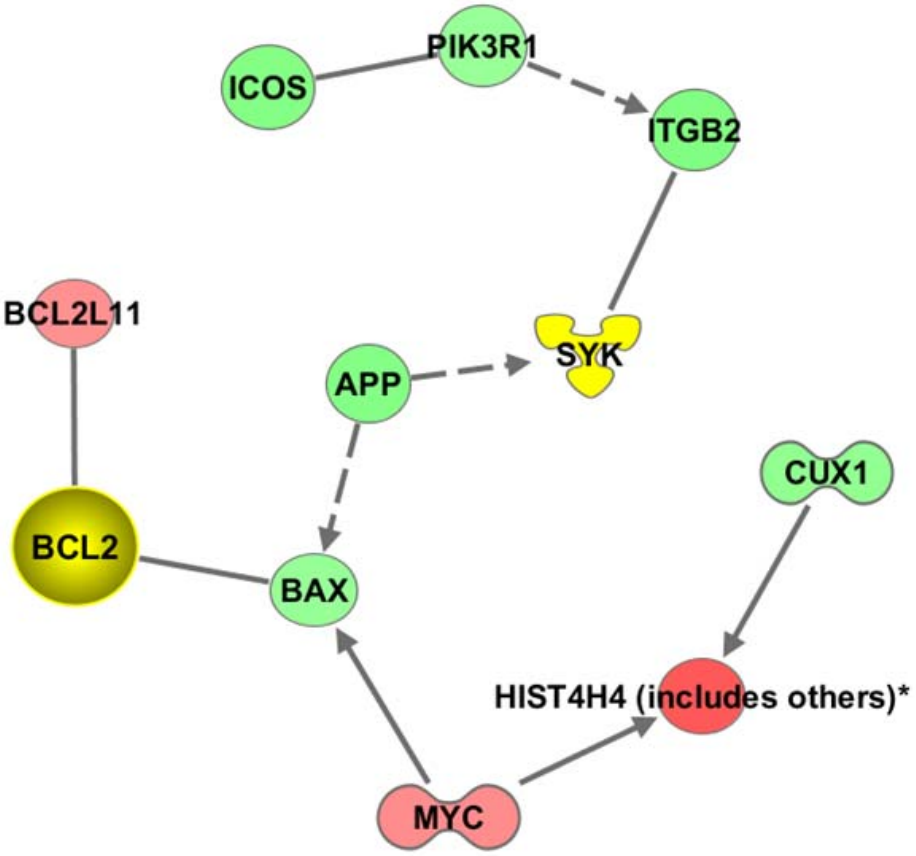


Figure 4