Aberrant methylation of c-myc and c-fos protooncogenes and p53 tumor suppressor gene in myelodysplastic syndromes and acute non-lymphocytic leukemia

P.C. Papaggeli1, A.C. Kortsaris2, P.T. Matsouka3
1University of Thessaly, Department of Medicine, Larissa; 2University of Thrace, Department of Medicine, Laboratory of Biochemistry, Alexandroupolis; 3University Hospital of Patras, Hematology Division, Department of Internal Medicine, Rio, Patras, Greece

Summary

Purpose: Aberrant methylation, as an epigenetic phenomenon, may precede and regulate the expression of genes involved in transformation mechanisms that lead to leukemogenesis of hemopoietic cells. The genes involved mostly encode transcription factors and cell cycle specific inhibitors. The aim of this project was to study the DNA methylation pattern of c-myc, c-fos and p53 in myelodysplastic syndromes (MDS) and in acute non-lymphocytic leukemias (ANLL).

Patients and methods: DNA was isolated from the monocyte cell layer harvested from bone marrow or peripheral blood samples of 44 patients suffering from MDS and ANLL. Genomic DNA was digested with methylation-specific enzymes, and was electrophoresed and hybridized with probes specific for human c-myc, c-fos and p53 genes.

Results: In MDS, the c-myc gene in exons 2 and 3 was regionally hypomethylated, whereas exon 2 in ANLL was hypermethylated and exon 3 hypomethylated. The c-fos gene was hypomethylated in ANLL type 4 and presented aberrant hypomethylation in the different types of MDS. The p53 anti-oncogene appeared extensively hypomethylated in MDS.

Conclusion: Aberrant DNA methylation pattern of the c-myc, c-fos and p53 tumor suppressor gene seems to be a primary event in the transformation process from myelodysplasia to acute leukemia, affecting their expression, and, consequently, altering the proliferation, differentiation or apoptosis of hemopoietic precursor cells. The p53 hypomethylation predisposes to critical mutations that enhance the transformation process of myelodysplasia to leukemia. The recognition of altered methylation of these genes in myelodysplasia may have prognostic implications and may lead to novel therapeutic modalities.

Key words: acute nonlymphocytic leukemia, leukemogenesis, methylation, myelodysplasia, p53, protooncogenes

Introduction

Methylation is an important key-regulator mechanism of gene expression in mammals. Sites of methylation in the genome are the CpG-islands, located mainly in the promoter region of the genes [1].

DNA methylation influences the transcription activity in 3 ways. Firstly, DNA methylation interferes with the binding of specific transcription factors (e.g. c-Myc, AP1) to their respective DNA binding sites, which are located at various promoter sites. Secondly, methylation can directly induce gene silencing by suspending the binding of specific transcriptional inhibitors to methylated DNA sites. The formation of inactive chromatin structure is the third mechanism through which DNA methylation represses gene expression [2,3]. Hypomethylation enhances the transcription of genes. Hypomethylation has been characterized as an epigenetic mechanism, which contributes to tumorigenesis through the mutated or over-expressed genes, due to the increased incidence of mutations and the subsequent increased transcriptional rate [3,4].
Primary evidence regarding correlation between DNA methylation and cancer is the finding that cell lines, which were derived from tumors, were less methylated than normal tissues [2,5,6]. Another feature of neoplastic cells is the DNA methylation imbalance, which is consisted of widespread hypomethylation, regional hypermethylation and increased cellular rate for methylation [6-8].

MDS are a class of disorders characterized by ineffective hematopoiesis and excessive programmed cell death resulting in peripheral cytopenias. Since two or more hematopoietic lineages are affected, these disorders occur at the level of the pluripotent stem cell [9]. Aberrant expression of many genes or tumor suppressor genes has been found in the multistep process that evolves to leukemogenesis in MDS [10]. These genes/oncoproteins regulate proliferation, differentiation and apoptosis in primitive and differentiated hemopoietic cells, in both normal and leukemic hematopoiesis [10,11].

The cumulative nature of genetic abnormalities on hemopoietic stem cells or in myeloid and lymphoid progenitors are characteristic findings in the different types of MDS and particularly in the types which represent the transition state to leukemia [11]. Deletions, mutations and loss of expression of different genes may be the consequence of abnormal methylation in critical regions of the protooncogenes or tumor suppressor genes involved [12].

The network of events responsible for the leukemic transformation in the different types of MDS involves aberrant proliferation, block of differentiation and increased apoptosis occurring in the stem cell progenitors of hemopoietic cells [10]. Apoptosis is triggered by a variety of extracellular and intracellular signals, which use different pathways in order to activate transcription factors, which regulate cell proliferation and programmed cell death [11].

The c-myc and c-fos protooncogenes are transcription factors that respond to intra or extracellular signals via specific sequences located mainly in the promoter region. C-Myc and c-Fos proteins control proliferation, differentiation or apoptosis in various cell types [13,14]. On the other hand the p53 gene, as an anti-oncogene, regulates the entrance of cell to the cell cycle if the DNA is normal. In case of severe damage in the structure of DNA, p53 leads the cell to apoptosis, whereas in case of less important damage, p53 inhibits the progression of the cell cycle and initiates the repair of the damaged DNA [15].

We studied the methylation profile of CpG sites of the human protooncogenes c-myc and c-fos and that of the tumor suppressor gene p53 as well. The methylation status of DNA of hemopoietic cells harvested from normal individuals and from patients suffering from MDS of all types (refractory anemia (RA), refractory anemia with ring sideroblasts (RARS), refractory anemia with excess of blasts (RAEB), and chronic myelo-monocytic leukemia (CMMML)) and ANLL of M1, M2, M3, M4 types according to the FAB classification was analysed.

Patients and methods

Patients

Samples of 44 patients were analysed in this study. We studied 19 patients with MDS and 25 patients with ANLL, types M1, M2, M3, M4, according to the FAB classification. The myelodysplastic group included 4 patients with RA, 4 patients with RARS, 8 with RAEB and 3 with CMMML, according to the FAB classification.

Bone marrow and peripheral blood cells were harvested at initial diagnosis, after the informed consent of the patients at the Hematology Division, University Hospital, Patras. Peripheral blood from 12 healthy volunteers was harvested after informed consent.

It should be pointed out that the methylation pattern of the digested and hybridized DNA of the samples under investigation, with the different probes of the protooncogenes c-myc, c-fos and the antioncogene p53, was compared to a normal sample, electrophoresed and hybridized under the same experimental conditions.

DNA digestion

Bone marrow and peripheral blood cells were collected in sodium heparin diluted in RPMI 1:10. Cells were washed in RPMI and PBS 1% (Sigma, St. Louis, MO, USA). Mononuclear cell layers from bone marrow cells or peripheral blood were isolated by centrifugation on a Ficoll-Hypaque density gradient (Sigma, St. Louis, MO, USA) [16]. Genomic DNA was subsequently extracted using the standard organic extraction procedure [17]. In samples with low number of cells, the extraction of DNA was performed using isopropanol-fractionation with concentrated sodium iodide and SDS [18]. The amount of the extracted DNA was calculated in the Hitachi U-1100 fluorometer, and DNA from each sample was divided into equal aliquots. 10μg of purified DNA were digested with EcoRI restriction enzyme (12u/μl, Promega, Madison, WI, USA) at 37° C for 4h. Then, the digested DNA was further digested with the me-
thylation insensitive restriction enzyme MspI (10u/μl, Promega, Madison, WI, USA) and the other half of the sample with the methylation sensitive restriction enzyme HpaII (10u/μl, Promega, Madison, WI, USA). MspI restriction enzyme recognizes and digests the CCGG sequence, whereas, when cytosine is methylated, it does not digest the following sequences: mC-CGG, GGCmCGGm. HpaII restriction enzyme recognizes and digests the CCGG sequence, though, when cytosine is double-methylated, it cannot digest the same sequence CmCGG, mCmCGG [19]. In general, the restriction enzyme MspI cuts unmethylated or methylated DNA, whereas HpaII cuts only unmethylated DNA. Digestion was performed at 37° C for 4h, and 20u of the restriction enzyme per μg of DNA were used. All the experiments were performed using an excess of enzyme quantities to avoid partial digestion’s artifacts.

In order to monitor the complete digestion of the DNA, parallel digestion of lambda phage DNA was performed under the same experimental conditions. Southern blot analysis of DNA
The digested DNA was subjected to electrophoresis in 0.8% agarose gel, in 1× TBE running buffer (10× TBE: 106 g/l Tris, 55 g/l boric acid, 9.3 g/l EDTA), at 30V (overnight running). λ phage DNA digested with the restriction enzyme BglII (36u/μl, Promega, Madison, WI, USA), was used as a molecular weight marker.

The fractionated DNA was transferred to Hybond-N membranes (Amerham, Germany) in 10× SSC buffer (20× SSC: 175.3 g/l NaCl, pH 7 and 88.3 g/l sodium citrate) for 18h, according to the Southern protocol [20]. The membranes, protected with Whatmann 3mm paper, were air-dried and baked at 80° C for 2h.

Hybridization of DNA
The specific probes used in these experiments were: (1) the first exon of human c-myc gene (pMyc 6514-1); (2) the second exon of c-myc gene (pMyc 6514-2); (3) the third exon of c-myc gene probe (pMyc 6514-R3); (4) the human c-fos gene probe [pc-fos (human)-1]; and (5) the p53 gene probe (pSP65). The Japanese Cancer Research Resources Bank (JCRB) provided the cDNAs of the abovementioned probes (Table 1). For the hybridization of DNA, membranes were labeled with high probe specificity (at least 1×106 cpm/μg DNA) with 32P dCTP (5.000 Ci/mmol, Amersham, Germany) following the nick translation method [21]. Hybridization was performed at 65° C for at least 18h. Subsequent washings were carried out according to the protocol of Sambrook et al. [17]. Hybridized membranes were exposed to Kodak X-OMAT AR (or Agfa Curix XP) film at –70° C for a varying period of time.

Results

1. c-myc

While all 3 exons of the c-myc gene are transcribed, only exon 2 and exon 3 encode the Myc protein. The gene has 2 major promoters (P1 and P2), which are located in exon 1 (Figure 1). The predominant promoter is P1. There are 2 other promoters: P0 (located in 5’ region), and P3 (located in intron 1). P3 promoter is activated in cases of translocation of the gene [13].

We used 3 cDNA probes for the study of c-myc methylation. Probe 1 covers the region of exon 1, probe 2 covers exon 2 and the 3’ region of intron 1 and probe 3 covers exon 3 and a segment of 3’ region of the gene. We studied 20 CCGG sites (sites M1-M20) of the c-myc gene, which are spread throughout the gene. Sites M1-M7 can be studied with probe 1, sites M12-M19 can be studied using probe 2, and M20 and the 3’ region of the gene are studied with probe 3 (Figure 2).

Table 1. Specific probes used in hybridization

<table>
<thead>
<tr>
<th>Probe</th>
<th>Source</th>
<th>Size (bp)</th>
<th>Cloning site</th>
<th>Vector</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myc-exon 1</td>
<td>Human</td>
<td>600</td>
<td>KpnI - BamHI</td>
<td>pUC18, Amp'</td>
</tr>
<tr>
<td>Myc-exon 2</td>
<td>Human</td>
<td>1530</td>
<td>SacI - SacI</td>
<td>pUC19</td>
</tr>
<tr>
<td>Myc-exon 3</td>
<td>Human</td>
<td>1400</td>
<td>Clal - EcoRI</td>
<td>pSPT18, Amp'</td>
</tr>
<tr>
<td>c-fos</td>
<td>Human</td>
<td>8800</td>
<td>EcoRI</td>
<td>pBR322</td>
</tr>
<tr>
<td>p53</td>
<td>Human</td>
<td>1985</td>
<td>EcoRI - BamHI</td>
<td>SP65</td>
</tr>
</tbody>
</table>

Figure 1. Localization of the promoters in c-myc gene. c-myc gene has 4 promoters (P0-P3). P1 is the predominant promoter. Promoters P1 and P2, located in exon 1 of the gene are the major promoters. P0 promoter is located in 5’ flanking region, while P3 is located within intron 1. The fact that a promoter is located within a non-coding region of the gene is rare, but in c-myc gene, this promoter P3 is activated during translocation of the gene, an event occurring often in leukemias.
a) Sites of methylation of exon 1 in c-myc gene

Following digestion with the enzymes MspI and HpaII, 2 distinct bands of 1.5 and 2.1 Kbp size were observed, both in normal and in RAEB samples. These bands were recovered from digestion of the CCGG sites M5 and M7 in exon 1 (Figure 2, lanes 1, 2, 5, 6, 7, 8; panel A). RARS samples showed only one band in 1.5 Kbp, suggesting digestion in the M7 CCGG site of exon 1 (Figure 2, lanes 5, 6; panel A). ANLL samples gave a 2.1 Kbp band resulting from the M5 CCGG site digestion (Figure 2, lanes 9, 10; panel A). A different pattern was obtained after the digestion of RA samples. These samples gave a 2.6 Kbp band, which became obvious after the digestion of the M1 site at the 5’ end of exon 1 (Figure 2, lanes 3, 4; panel A). These results indicate that in the 5’ region of c-myc gene – including exon 1 – the sites of hypomethylation in RA are different from RARS and ANLL, and only the RAEB sample gives a pattern similar to the normal one.

In exon 1, 2 promoters of the gene are located, and this hypomethylated region may enhance the expression of the gene in RAEB. The samples of ANLL, RARS and RA give only one band, of different molecular weights and they seem to be regionally methylated (or hypermethylated) in exon 1.

b) Sites of methylation of exon 2 in c-myc gene

Using probe 2, which covers exon 2 and the 3’ flanking region in intron 1 of the c-myc gene, we studied the methylation pattern of exon 2. The 3 CCGG sites (M17 – M19) in exon 2 and 5 CCGG sites (M12 – M16) in intron 1 (Figure 3, panel B) were uncovered by probe 2. In double digestion of genomic DNA (EcoRI-HpaII enzymes) from RAEB samples, 3 bands of approximately 4.3, 2.4 and 1.9 Kbp were detected. Double digestion with EcoRI-MspI of the same RAEB sample revealed 2 bands sized 1.9 and 1.5 Kbp. The bands 2.4 and 1.9 Kbp indicate that CCGG sites M17 (exon 2) and M15 (intron 1) are hypomethylated in RAEB samples as well as in normal controls. The difference between normal and RAEB samples appears in a 4.3 Kbp band that has resulted from the digestion of CCGG site M7 (exon 1) and M20 (exon 3) (Figure 3, lane 6; panel A). This finding indicates that CCGG site M7 (exon 1) is hypomethylated.
lated in RAEB samples, but not in normal samples (Figure 3, lanes 1, 2, 5, 6; panel A).

In RA samples 2 bands of approximate size 4.8 and 4.6 Kbp were detected upon double digestion with EcoRI - Hpa II (Figure 3, lane 3, 4; panel A). The 1.5 Kbp band was derived from the Eco RI - Msp I cleavage. This 1.5 Kbp band was the result of digestion in the CCGG sites M10 and M19 within intron 1 and exon 2, respectively. The 4.8 Kbp and 4.6 Kbp bands may result from the cleavage of CCGG sites M8 and M10 within intron 1 (Figure 3, lanes 3, 4; panel A). The data above indicate that there are different sites of hypomethylation in the RA and RAEB samples regarding the second exon of the c-myc gene, that is, the CCGG site M17 in RA and M19 in RAEB samples (Figure 3, lanes 3, 4, 5, 6; panel A). It was observed that in intron 1 and exon 2, the CCGG sites M8, M10 and M15 were also hypomethylated in RA and RAEB samples. Normal samples seemed to be hypomethylated only in the CCGG sites M15 (intron 1) and M17 (exon 2) (Figure 3, lanes 1, 2; panel A).

ANLL gave the same pattern of methylation as in normal controls (Figure 3, lanes 1, 2, 7, 8; panel A). The density of the band (1.9 Kb) was stronger in both pairs of enzymes used for digestion in ANLL samples (Figure 3, lanes 7, 8; panel A), compared to the normal samples.

c) Sites of methylation of exon 3 in c-myc gene

Probe 3 covers the sequence of exon 3 and detects the methylation of the CCGG site M20 of exon 3 and the 3 region of the gene, where 4 critical CCGG sites (M21 – M24) are located. Analysis of the methylation pattern obtained from the double digestion of the DNA with the enzymes EcoRI-MspI in all MDS (RA, RARS, RAEB) and ANLL samples revealed mainly 4 bands of approximate sizes 8.6, 6.0, 4.7 and 1.0 Kbp (Figure 4, lanes 3-12; panel A). Double EcoRI-HpaII digestion gave rise to the following bands: 8.6, 6.0, 4.7, 3.6 and 1 Kbp, in all types of MDS samples. According to the genetic map of c-myc gene, the 8.6 and 6.0 Kbp bands found in these experiments resulted from the digestion of M20 site (exon 3) and the CCGG sites M21 and M23 (in the 3 region of the gene), respectively, which are hypomethylated in the same experiments. A different band of 3.1 Kbp mol. weight was found in normal samples after double digestion with EcoRI-HpaII enzymes (Figure 4, lanes 1, 2; panel A). This result suggests that exon 3 is methylated in normal samples, whereas the MDS samples reveal an extensive hypomethylation pattern. RA samples present 2 more bands of hypomethylation: 2.1 and 1.0 Kb, compared to RAEB and ANLL samples. ANLL and RAEB samples were found less hypomethylated compared to RA and RARS samples (Figure 4, lanes 7-12; panel A). The MDS samples in the CCGG site M21 and M23 in the 3 region are hypomethylated and the CCGG sites M10 and M19 (in intron 1 and exon 2) were found hypomethylated by hybridization with probes 2 and 3.

In summary, intron 1, exon 2 and exon 3 methylation sites of the c-myc gene are hypomethylated in MDS, but the rate of methylation is increased in RAEB and ANLL, albeit c-myc appears to be less methylated compared to the normal samples (Table 2).
2. Sites of methylation in c-fos gene

The probe used for the study of the methylation status of c-fos protooncogene, covers the whole gene sequence. C-fos gene has a plethora of CpG sites found across the entire sequence of the gene, an excess of which is located near the 5’ region of the gene [22]. In our experiments, dense bands of methylation were observed in all of the samples examined, as well as a smear of multiple bands. The pattern of DNA methylation in myelodysplasia gave regions of hypomethylated and hypermethylated DNA (Figure 5, panel A).

After double digestion with EcoRI-MspI, the MDS samples (RARS and RAEB) showed distinct bands of 6.5, 5.0 and 4.3 Kb in high molecular weights and other bands in low molecular weights (1.2 and 1.0 Kbp). When MDS samples were analysed with the double digestion EcoRI-HpaII, smear and intense bands of approximate sizes 6.5, 5.0 and 4.3 Kbp were observed (Figure 5, lanes 3-6; panel A). The CMML samples showed a different pattern compared to the rest of the MDS subtypes, forming an intense distinct band of hypomethylation in high molecular weight (6.5 Kbp), after both double digestions (Figure 5, lanes 7, 8; panel A). The normal samples (Figure 5, lanes 1, 2; panel A) revealed smears of bands without distinct bands of methylated DNA independently of the enzymes used (MspI, HpaII). ANLL samples gave a smear of bands which was more intense in the M4 subtype of ANLL, compared to the M2 subtype of ANLL, suggesting broad hypomethylation in the M4 samples (Figure 5, lanes 9-12; panel A). The hypomethylated sites compared to the normal samples were numerous.

In conclusion, c-fos in RARS and RAEB was found hypomethylated in more sites than in normal controls. In CMML, the c-fos gene was generally hypermethylated with one distinct region of hypomethylation. The M2 subtype of ANLL was regionally hypomethylated and the M4 subtype was extensively hypomethylated (Table 2).

3. Sites of methylation in p53 gene

Digestion of RARS and RAEB samples with MspI and HpaII enzymes showed multiple bands of hypomethylation in the p53 sequence (Figure 6, lanes 3-6; panel A). Normal DNA appeared to be methylated and gave only 2 bands (Figure 6, lane 2; panel A).

As a result, the normal samples digested with EcoRI/MspI exhibited 2 bands of 5.4 and 1.8 Kb of hypomethylation in the whole DNA sequence of the p53 gene. The RARS and RAEB samples digested with EcoRI/MspI and EcoRI/HpaII exhibited multiple hypomethylated sites in high (8.4, 6.3, 5.4, 4.5

Table 2. Schematic presentation of the methylation profile of the studied genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Normal</th>
<th>RA</th>
<th>RARS</th>
<th>RAEB</th>
<th>CMML</th>
<th>AML</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-myc exon 1</td>
<td>+</td>
<td>n.s.</td>
<td>+</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>c-myc exon 2</td>
<td>+</td>
<td>n.s.</td>
<td>+</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>c-myc exon 3</td>
<td>+</td>
<td>n.s.</td>
<td>+</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>c-fos</td>
<td>n.s.</td>
<td>n.s.</td>
<td>+</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>p53</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

*Regionally hypomethylated gene; *regionally hypermethylated gene; ^the methylation status of the genes was not studied.

For abbreviations, see text

Figure 5. DNA methylation pattern of c-fos gene.
Panel A: Methylated fragments of DNA observed in the different molecular weights with the use of restriction enzymes MspI (M) and HpaII (H). Normal (lane 1, 2), RARS (lane 3, 4), RAEB (lane 5, 6), CMML (lane 7, 8), ANLL type M2 (lane 9, 10) and ANLL type M4 (lane 11, 12).
Panel B: CCGG methylation sites of c-fos DNA sequence. Notice the clustering of CCGG sites in the regions of the 1st intron and 1st exon of the gene where the promoter and the SRE element is located.
Kbp) and low molecular weight regions of the gene (2.0, 1.8, 1.3 and 1.2 Kb). More hypomethylated CCGG sites were observed in the RARS samples than in the RAEB samples. In conclusion, p53 is extensively hypomethylated in RARS and less hypomethylated in RAEB samples (Figure 6, lanes 5, 6; panel A) compared to normal samples (Table 2).

**Discussion**

The methylation status of DNA in different genes, and particularly the methylation of CpG sites located within the promoter region or other critical regions of DNA, are inversely related to gene expression [3].

Methylation in CpG-rich genes may serve as a locking-off mechanism, or precede other events that turn a gene off [22]. There is strong evidence that hypomethylation, areas of hypermethylation and increased DNA methyltransferase activity are components of methylation imbalance in the genome, contributing to neoplastic transformation and tumor progression [7,23].

In the relevant literature there are multiple paradigms of gene activation or silencing, function of which is directly related to leukemogenesis or tumorigenesis in lymphomas, chronic and acute leukemias, as well as in solid tumors [4-6, 24,25].

Hypermethylation of p15-ink4B tumor suppressor gene has been found in 50% of the high risk group of MDS (RAEB and RAEB-t) compared to 8% of hypermethylation found in low risk MDS (RA and RARS) [26]. The percentage of cases carrying hypermethylated inhibitor of cyclin kinase (p15ink) increases to 78% in leukemia secondary to MDS [27]. In addition, 75% of ANLL patients had hypermethylation of multiple genes related to cell cycle regulation, and 95% of them had hypermethylation at least of one gene. These genes were mainly cell cycle regulators and cancer type-specific [8, 28].

c-myc, c-fos and p53 genes, transcription factors that are directly involved in hemopoiesis were studied and aberrant patterns of methylation in the different syndromes of leukemic transformation were found.

The c-myc protooncogene regulates proliferation and programmed cell death of normal cells in response to endogenous and extracellular signals [9,13, 29]. Since 1984 it is known that altered methylation, mainly hypomethylation, of the c-myc gene is found in cancer cell lines, in tumor cells from solid cancers and in multiple myeloma cells [1-5, 30-32]. In CMML and in ANLL secondary to myelodysplasia, c-myc was found to be hypomethylated in the 3′ region of the gene [6].

Our study revealed more CCGG sites of hypomethylation, particularly in exon 2 and 3 of the gene and intron 1 in MDS of RA, RARS and RAEB type. Hypomethylated regions were found in exon 3 in ANLL cases, but they were less than in the MDS cases.

The c-myc gene has several primers (P0-P3, Figure 1), even though primer P1 is the dominant one. Meanwhile, we have to notice that primer P3, located in intron 1, is activated in cases of translocation observed in cases of lymphomas. It is known that exon 1 or exon 2 possibly encode serum response elements [13,29]. Our data showed that CCGG sites M5, M7 (and in some cases M1) of exon 1 appeared unmethylated in MDS cases. The fact that MDS are transition stages to leukemic transformation may explain why MDS methylation patterns resemble to normal in the region of exon 1 of c-myc gene. Ohtsuki et al. [33] studying the methylation status of the c-myc gene in human myeloma cell lines found that exon 1 regions were hypomethylated. Because sequences nearby and within exon 1 act as transcription in-
hilitors [13], changes of methylation status of this region may influence the c-myc transcription rate.

The CpG islands in the region of exon 2 seem to be hypomethylated in MDS as other studies also have shown [1,5,6]. The hypomethylated regions of exon 2 of c-myc may be prone to translocations, affecting the function of the gene and promoting leukemogenesis [34].

In exon 3, one CCGG site (M20) and M21-M24 sites of the 3' region appear to be hypomethylated in all MDS subtypes. Several studies have shown similar results in human myeloma cell lines, leukemias and liver cancers [5,6,30,35]. In exon 3, there are sequences, which regulate the addition of polyA tails in RNA transcripts. Furthermore, exon 3 encodes for the domain of c-Myc protein, which is responsible for the nuclear localization of the protein [13]. The fact that exon 3 is hypomethylated in MDS may indicate that c-myc is not only actively transcribed, but also that the c-myc mRNA is stable, the protein translocates to the nucleus and all these facts may promote programmed cell death or proliferation of hemopoietic precursor cells.

We conclude that hypomethylation of c-myc in multiple sites is an early event in leukemogenesis. It is more prominent in low risk MDS where its expression is high, participating in increased apoptosis of hemopoietic cells [11]. The gene is only regionally hypomethylated in high risk MDS and in ANLL, resulting in a decline of its expression and of the rate of apoptosis [11,36,37]. It is also probable that the regional restricted hypomethylation in exon 3 of c-myc in ANLL permits growth advantage to the leukemic clone.

The c-fos protooncogene is a member of the AP-1 transcription factor complex, and the constitutive expression of the c-fos protein regulates the homeostasis of monocytic and myeloid lineage through differentiation and programmed cell death [14]. The serum response element of c-fos responds to extracellular stimuli for growth or apoptosis. C-fos activation is the earliest response of cells to genotoxic agents for DNA by inducing programmed cell death of the damaged cells [14].

Recently Bakin et al. have found that c-fos may transform cells through alterations in DNA methylation and histone acetylation [38]. Alterations in DNA methylation of c-fos gene have been described in gliomas, compared to normal tissues, both in the promoter region and in the encoding sequence of the c-fos protein [39].

In our study, in low risk MDS (RA, RARS) there are regions of extensive hypomethylation throughout the gene, with a different distribution of methylation sites compared to normal c-fos. The pattern of hypomethylation in low risk MDS (RA, RARS) may indicate inhibition of differentiation and accelerated apoptosis of mature hemopoietic cells, particularly of the monocytoid lineage. The gene is generally methylated in CMML, with a focus of restricted hypomethylation in the promoter region. It could be hypothesized that hypomethylated promoter of c-fos in CMML leukemic cells could respond to extracellular or intracellular signals for uncontrolled proliferation or inhibition of apoptosis, resulting in accumulation of the monocytic leukemic clone [14]. This pattern of CMML methylation is consistent with the high expression of c-fos gene that was found in CMML compared to RA, RARS and RAEB (data not shown). In the ANLL samples, and particularly the M4 subtype, where the c-fos gene appears extensively hypomethylated, the c-fos protein is probably the key protein for the transformation process, inducing accelerated proliferation of the monocytic leukemic clone.

The p53 tumor suppressor gene is considered as the “gatekeeper” of the cell cycle in normal cells, inducing apoptosis when DNA damage is recognized [40-42]. In the literature it is recognized that the p53 mutant protein is oncogenic and in leukemias and lymphomas p53 is highly mutated [42,43].

Our findings support that p53 gene is hypomethylated in different regions in both low (RARS) and high risk (RAEB) MDS, compared to normal samples where it appears methylated.

The widespread hypomethylation of the p53 gene found in our experiments may predispose to mutations in critical regions of the gene. The hypomethylated state of the p53 gene confers eligibility to mutations and to leukemic transformation of the mutant gene-carrying cell [44].

Another mechanism that the mutated p53 protein may gain oncogenic functions is by interfering with the p53-dependent apoptosis and cell cycle arrest [45]. The extended hypomethylation of p53 found in our experiments may confer loss of normal p53 function on hemopoietic progenitor cells, enhancing apoptosis in low risk MDS (RARS).

From our study and from the results of other investigators it is obvious that a preceding event in leukemic transformation is an “instability” of the methylation in the DNA of regulatory genes, with regional hypo- or hypermethylated sequences [46]. This unstable methylation probably affects the expression and function of genes that regulate cell cycle, differ-
entiation and apoptosis of hemopoietic cells. The functional activity of tumor suppressor genes or antioncogenes is abolished in leukemia and lymphoma by hypermethylation of their encoding DNA [47].

Changes of the methylation pattern of these cell cycle regulatory genes probably act as a preceding event in a series of methylation changes in the genome during leukemic transformation [47-49]. The number of cases studied in each group of MDS is small, but the methylation pattern of the genes under investigation was identical in each experiment compared to normal controls.

There is a perspective that the methylation profile of many genes in MDS, leukemias and lymphomas, may have prognostic value [8, 49] and methylation modifiers may be beneficial for the treatment of hematological diseases.

Acknowledgements

We thank Dr. C. Giannakenas for his invaluable technical assistance, Prof. Dimitriadis for helpful discussions, and E. Karligiotou for her valued help in proofreading the manuscript.

References