SHORT COMMUNICATION

Characterization of the murine p19\(^{ARF}\) promoter CpG island and its methylation pattern in primary lymphomas

Bárbara Meléndez*, Marcos Malumbres\(^{1,8}\), Ignacio Pérez de Castro\(^2\), Javier Santos, Angel Pellicer\(^2\) and José Fernández-Piqueras\(^3\)

Laboratorio de Genética Molecular Humana, Departamento de Biología, Universidad Autónoma de Madrid, Cantoblanco, 28049 Madrid, Spain, \(^1\)Centro Nacional de Investigaciones Oncológicas Carlos III, Ctra. Majadahonda-Pozuelo, km 2, 28220 Majadahonda, Madrid and Centro Nacional de Biotecnología, CSIC, Campus de Cantoblanco, 28049 Madrid, Spain and \(^2\)Department of Pathology and Kaplan Comprehensive Cancer Center, New York University, 550 First Avenue, New York, NY 10016, USA

\(^3\)To whom correspondence should be addressed

Email: jf.piqueras@uam.es

The INK4a/ARF locus encodes two different proteins involved in cell cycle control. Both molecules, p16\(^{INK4a}\) and p19\(^{ARF}\), inhibit cell cycle progression and have been shown to act as tumor suppressors in a variety of models. Their expression is controlled by separate promoters responding to different stimuli and they therefore show independent transcriptional regulation. We have cloned and characterized a 2.5 kb region upstream of the murine p19\(^{ARF}\) gene to determine the role of DNA methylation in suppressing p19\(^{ARF}\) transcription in a wide panel of murine primary T cell lymphomas. This region contains a DNA fragment with the characteristics of a CpG island similar to those described for the murine p16\(^{INK4a}\) and p15\(^{INK4b}\) genes. Expression of p19\(^{ARF}\) is decreased in a significant number (20\%) of the murine lymphomas analyzed. Overexpression of the p19\(^{ARF}\) transcript is also frequent, suggesting alterations in molecules of the retinoblastoma or p53 pathways that are involved in p19\(^{ARF}\) regulation. Although hypermethylation of the INK4a and INK4b promoters is frequently involved in murine lymphomas, the p19\(^{ARF}\) CpG island is infrequently methylated in the murine primary lymphomas studied in this work. Since loss of p19\(^{ARF}\) expression cannot be explained as the result of homozygous deletions or hypermethylation of the ARF gene, other regulatory mechanisms seem to be altered in these malignancies.

Disruption of the pRb or p53 pathways has been shown to be involved in most tumor types, although these genes are not essential for completion of the cell division cycle. Both pathways are regulated by the products of a single genetic locus, INK4a/ARF, which encodes two potent tumor suppressor genes (reviewed in refs 1–3). The p16\(^{INK4a}\) protein (also known as MTS1, p16\(^\alpha\)) is an inhibitor of specific cyclin–CDK complexes (cyclin D–CDK4/6) which in turn regulate the phosphorylation of pRb, thus controlling progression from the G\(_1\) to S phase of the cell cycle. The p19\(^{ARF}\) protein, although sharing exons 2 and 3 of the INK4a/ARF locus, is completely different in its amino acid sequence, with no homology to other known proteins. p19\(^{ARF}\) has been shown to participate in regulation of the p53 pathway. By means of binary and/or ternary complexes together with the p53 and/or Mdm2 proteins, p19\(^{ARF}\) is involved in the control of levels of p53 (4–7). Recently, the mechanism by which p19\(^{ARF}\) activates p53 has been explained by its ability to bind and sequester Mdm2. p19\(^{ARF}\), which localizes to nucleoli, is able to form binary complexes with Mdm2, sequestering it into the nucleolus, thereby preventing negative feedback regulation of p53 by Mdm2 and leading to activation of p53 in the nucleoplasm (8–10).

The two cell cycle inhibitors, p16\(^{INK4a}\) and p19\(^{ARF}\), seem to be regulated through different mechanisms. p16\(^{INK4a}\) appears to be regulated by the retinoblastoma protein (pRb), responds to Ras oncogenic stimulus and is induced in replicative senescence (11–13). This tumor suppressor gene is frequently inactivated by homozygous deletions (in many cases also involving p15\(^{INK4b}\), which is located very close on the chromosome), point mutations in the coding sequence or hypermethylation of the CpG island in the promoter region in various types of malignancies (14–17). The expression of p19\(^{ARF}\), in contrast, has been found to be regulated by several molecules in addition to Ras, including Myc, E2F-1 and E1A, and is down-regulated by p53, although no binding sequences for p53 have been found in its promoter (18–22). Recently, the transcription factor DMP1 has been shown to play an important role in p19\(^{ARF}\) induction in response to anti-proliferative signals (23). In humans, the p19\(^{ARF}\) (also named p14\(^{ARF}\)) promoter has been cloned and sequenced (19,21) and it has been shown to be hypermethylated in some cell lines, correlating with suppression of promoter activity (21).

In order to analyze the methylation status of the p19\(^{ARF}\) promoter in primary tumors, we have cloned and sequenced the murine p19\(^{ARF}\) promoter and studied hypermethylation of this region as a model for the inactivation of p19\(^{ARF}\) expression in a set of 64 murine primary T cell lymphomas. A screen of a \(\lambda\) 129/SvJ mouse genomic library with a p16\(^{INK4a}\) exon 2 PCR probe (24) identified a genomic clone containing a 14 kb DNA fragment downstream of p15\(^{INK4b}\). The downstream DNA region p15\(^{INK4b}\) was characterized by restriction analysis and exon 1B of p19\(^{ARF}\) was localized 12 kb downstream of p15\(^{INK4b}\) by hybridization with specific probes (Figure 1). Sequencing of a 5 kb XhoI fragment was carried out with an ABI DNA Automated Sequencer 377 (Perkin Elmer) and sequencing primers designed with the program Primer3 (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). The exon 1B sequence is identical to that published by Quelle et al. (25) with the exception of a polymorphism (G→A) in position +21 from the transcriptional start site as defined by Inoue et al. (23). The estimated distance between the p19\(^{ARF}\) and p16\(^{INK4a}\) promoters is 13 kb (Figure 1).

*The first two authors contributed equally to this work.

Abbreviations: Rb, retinoblastoma; pRb, retinoblastoma protein.
The murine p19\textsuperscript{ARF} promoter shows many of the characteristics of a housekeeping gene similar to the human promotor (21), including the absence of a consensus TATA box and a sequence with homology to other initiator sequences (AAGGGGCTGGGGCGCGCTTCTCACCTCAGTTGCA; the bold G indicates the transcription initiation site as reported in ref. 23). The presence of a CpG island was analyzed using the parameters described previously (24). Computer analysis detected a 1 kb region with the characteristics of a CpG island immediately upstream of exon 1 of p19\textsuperscript{ARF} (Figure 1). The G+C content of this region is 0.69, whereas the CpG content and the CpG/GpC ratio are 12.6% and 0.98, respectively. Thus, the genomic sequences upstream of p19\textsuperscript{ARF} show the characteristics of a typical CpG island following the previously described criteria (26,27). The density of CpG dinucleotides in this DNA fragment is even higher than that described for the murine p16\textsuperscript{INK4a} and p15\textsuperscript{INK4b} genes (28), which flank the p19\textsuperscript{ARF} exon 1.

The analysis of potential transcriptional binding sites was carried out using several public computer resources, such as TFSEARCH (http://www.genome.ed.jp/SIT/TFSEARCH.html) and MatsInspector (http://www.gsf.de/cgi-bin/matssearch.pl). Numerous potential transcription factor binding sites are located upstream of the transcription initiation site and within the CpG island, including binding sites for the transcription factors Sp1, DMP1 and members of the E2F family (Figure 1). The importance of the E2F and DMP1 binding sites in human In each sample, analysis of the CDK4 gene was used as a control for the validity of the cDNA mixture and DNA genomic contamination (data not shown). Primers used for CDK4 amplification were CDK4-F (5’-TGG CTG CCA CTC GAT ATG AAC-3’) and CDK4-R (5’-CTG GTC GTC TAT ATG-3’). Cycling conditions were: 94°C for 5 min; 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min; a
island of the p19
ARF
Xho
within the most CpG-rich region of the promoter were selected. Different methylation-sensitive restriction endonucleases were carried out methylation analyses of the CpG island. Scanning and densitometry of the autoradiographs (1D Analysis and Hand Scanner Settings; Biomed Instruments, Zeineh Programs) were performed to quantify the PCR products. 

In order to study whether the absence of p19ARF expression was due to de novo methylation of the promoter sequences, we carried out methylation analyses of the CpG island. Different methylation-sensitive restriction endonucleases within the most CpG-rich region of the promoter were selected. Thus, we carried out double digestions of tumor DNA with XhoI, Smal or EagI together with the methylation-insensitive restriction enzyme EcoRI. Twenty micrograms of genomic DNA were double digested with EcoRI and the methylation-sensitive restriction enzymes under the conditions recommended by the manufacturer (Roche Molecular Biochemicals or New England Biolabs). Digested DNA was subjected to 0.8% agarose gel electrophoresis and transferred to nylon membranes (Roche Molecular Biochemicals). Filters were then hybridized with an [α-32P]dCTP random primed-labeled PCR probe for exon 1β of p19ARF and autoradiographs were developed after 4–5 days exposure. Southern blots of 34 genomic DNAs from T cell primary lymphomas doubly digested with EcoRI and XhoI were hybridized with a p19ARF exon 1β probe. No methylation at the XhoI site was detected in any of the tumors studied (Figure 2). In addition, 15 tumors, including the ones with decreased p19ARF expression, were tested with double digestion with EcoRI and EagI, revealing only two tumors (13%) with partial methylation at the CpG island of the p19ARF promoter (Figure 2). No methylation was observed in EcoRI and Smal digests (data not shown). Our results clearly show that the number of methylated samples is low and that the ratio of methylated to non-methylated DNA is low in the positive samples. Less than 50% of the DNA is methylated at the EagI site, as detected by the presence of the 3.2 kb band (Figure 2). More significantly, none of the two methylated samples showed loss of p19ARF expression in the RT–PCR analysis, indicating that this partial methylation is not physiologically relevant. These results indicate that the p19ARF CpG island is not frequently and densely methylated in these tumors. However, although we have detected strong

hypermethylation in the p15INK4b and p16INK4a CpG islands using a similar approach (24), we cannot rule out that some partial, non-dense methylation can occur in other positions of the p19ARF promoter not analyzed in this study.

DNA methylation of regulatory regions has been accepted as a common mechanism for the inactivation of tumor suppressor genes in cancer (32). CpG islands in the promoter region of housekeeping genes are usually non-methylated in normal tissues but, in the case of some tumor suppressor genes, can suffer de novo methylation leading to transcriptional inactivation (15,16,24,32). This mechanism has been shown to be responsible for the loss of p15INK4b and p16INK4a expression in murine lymphomas induced by different treatments (24,33,34). This is specially relevant in the case of p15INK4b, a tumor suppressor gene specifically altered in hematopoietic malignancies, where hypermethylation in its regulatory sequences occurs independent of p16INK4a alterations. Although the human p19ARF promoter has been found to be methylated

Fig. 2. Analysis of p19ARF expression and methylation pattern of the CpG island in primary lymphomas. (A) Analysis by RT–PCR of the expression of p19ARF in control normal thymus (lane C) or thymic lymphomas (lanes 1–9). Some samples show null or decreased p19ARF expression (lanes 4, 7 and 9) whereas other tumors overexpress the corresponding transcript (lanes 2, 5, 6 and 8). The lower band in lanes 1, 2, 5, 6 and 8 corresponds to a smaller p19ARF product due to misannealing of one of the primers. (B) Methylation pattern of the p19ARF CpG island in XhoI or EagI digests. Whereas no methylated band (5.0 kb) is detected in EcoRI×XhoI digestions, two of the samples (lanes 9 and 11) show a partial methylation at the EagI site (represented as a 3.2 kb band; see Figure 1).
in some cell lines (21), we have found only rare cases of partial methylation at the EagI site in a proximal location to exon 1B in primary tumors. This methylation, however, does not extend to a second EagI site in the 5'-region of the CpG island (Figure 1). Similarly, methylation of the human p19ARF CpG island does not extend significantly beyond -450 relative to the transcription start site (21). Although DNA hypermethylation in the promoter region of tumor suppressor genes usually correlates with decreased expression, that is not the case for our two positive samples, where the level of methylation present is insufficient to suppress transcription, as these samples express significant amounts of p19ARF RNA.

The specificity of DNA methylation in the different CpG islands of the INK4a–INK4d/ARF loci is an intriguing finding. Although these sequences are located in close proximity on the chromosome (Figure 1) there is an interesting specificity in their methylation patterns in mouse lymphomas. These differences can be explained as a result of the specific role of each protein in different tissues since different transcription factors can be involved in their transcriptional regulation and can interfere in de novo methylation. In fact, binding of Sp1 molecules to its binding sites can protect against methylation in some promoters (29,35,36).

p19ARF expression is lost or reduced in a significant percentage of these tumors with neither deletion of the INK4a/ARF locus (unpublished results) nor methylation in their promoter sequences. Alternative mechanisms must therefore account for the loss of p19ARF expression. These could include small deletions or mutations in the promoter, alterations in the regulatory proteins involved in p19ARF expression or stability of its transcript. Some sequences in the human ARF promoter that are important for p19ARF expression show no obvious consensus sites for known transcription factors (21). Thus, further explanations for the loss of p19ARF expression in tumors could arise once the regulatory mechanisms for p19ARF expression are clarified.

Overexpression of the ARF transcript in almost 50% of the tumors analyzed should be more easily explained as a function of its transcriptional regulation. For instance, ARF levels are increased in some p53-deficient cell lines (25,37) and this effect could depend on a lack of repression by p53 of the ARF promoter (21). We have therefore studied the status of p53 in our lymphomas by western analysis and sequencing. As described previously (38), p53 is infrequently inactivated in induced murine lymphomas and, in our hands, only two out of 30 tumors presented a p53 mutation (data not shown). Alternatively, the inactivation of p53 could be due to overexpression of mdm2 and we are currently analyzing the expression of this p53 regulator in our panel of tumors. In addition to deregulation of the p53 pathway, overexpression of p19ARF could occur in response to deregulation of the retinoblastoma (Rb) pathway. The ARF promoter is highly responsive to overexpression of the transcription factors of the E2F family (21) and any increase in pRb phosphorylation by activation of CDK2, 4 or 6 or deletions and mutations in pRb would result in E2F activation. We have analyzed the expression of pRb, cyclin D1 and the p15INK4b and p16INK4a inhibitors in a wide panel of mouse primary lymphomas induced by irradiation (24,33,39). Deregulation of the Rb pathway is found in more than 75% of the tumors analyzed and most tumors with increased p19ARF expression show an alteration in at least one of the Rb regulators. Any of these alterations, loss of pRb, overexpression of cyclin D1 or p15INK4b and p16INK4a inactivation, would produce increased levels of active cellular E2F proteins, giving rise to p19ARF overexpression. Therefore, the increased levels of p19ARF in some tumors is likely to be due to additional alterations in other members of the p53 and/or Rb pathways.

In summary, we have described here the genomic sequence upstream of the mouse p19ARF gene, showing the presence of a CpG island susceptible to de novo methylation and corresponding transcriptional down-regulation. A recent report on human tumors has shown that the human p19ARF gene is infrequently methylated in human B and T cell lymphomas (40). In our hands, p19ARF expression is frequently lost or decreased in γ-irradiation-induced mouse primary thymic lymphomas (up to 20% of the tumors), although this alteration cannot be explained as a result of p19ARF hypermethylation at the promoter sequences.

Acknowledgements

I.P.C. received a fellowship from the Ministerio de Educación y Cultura (Madrid, Spain). This work was supported by grants PM 96/001 (Ministerio de Educación y Cultura, Spain) and 08/0009/1997 (Comunidad Autónoma de Madrid) and the Fundación Ramón Areces (Spain) to J.F.-F., grant BIOMED2 (BMH4-98-3426) to J.S. and grant CA 36327 (NIH, USA) to A.P.

References


Received July 23, 1999; revised November 4, 1999; accepted November 7, 1999