Methylation Silencing and Mutations of the $p14^{ARF}$ and $p16^{INK4a}$ Genes in Colon Cancer

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SUMMARY: The *INK4a-ARF* locus encodes two tumor suppressor proteins involved in cell-cycle regulation, $p16^{INK4a}$ and $p14^{ARF}$, whose functions are inactivated in many human cancers. The aim of this study was to evaluate $p14^{ARF}$ and $p16^{INK4a}$ gene inactivation and its association with some clinicopathological parameters in colon cancer. The mutational and methylation status of the $p14^{ARF}$ and $p16^{INK4a}$ genes was analyzed in 60 primary colon carcinomas and 8 colon cancer cell lines. We have identified the first two reported mutations affecting exon 1 β of $p14^{ARF}$ in the HCT116 cell line and in one of the primary colon carcinomas. Both mutations occur within the N-terminal region of $p14^{ARF}$, documented as important for nucleolar localization and interaction with Mdm2. Tumor-specific methylation of the $p14^{ARF}$ and $p16^{INK4a}$ genes was found in 33% and 32% of primary colon carcinomas, respectively. Methylation of the $p14^{ARF}$ was inversely correlated with p53 overexpression ($p = 0.02$). $p14^{ARF}$ and $p16^{INK4a}$ gene methylation was significantly more frequent in right-sided than in left-sided tumors ($p = 0.02$). Methylation of the $p14^{ARF}$ gene occurred more frequently in well-differentiated adenocarcinomas ($p = 0.005$), whereas the $p16^{INK4a}$ gene was more often methylated in poorly differentiated adenocarcinomas ($p = 0.002$). The present results underline the role of $p14^{ARF}$ and $p16^{INK4a}$ gene inactivation in the development of colon carcinoma. They suggest that the methylation profile of specific genes, in particular $p14^{ARF}$ and $p16^{INK4a}$, might be related to biologically distinct subsets of colon carcinomas and possibly to different tumorigenic pathways. (*Lab Invest* 2001, 81:217-229).

Both the p53 and the pRb pathways of cell-cycle control appear to be deregulated during colon carcinogenesis, contributing to uncontrolled cell proliferation. Wild-type p53 activates transcription of the cyclin-dependent kinase inhibitor $p21^{WAF1}$ gene, whose encoded protein is capable of inactivating cyclin D1-CDK4 complexes and thereby preventing cells from entering S phase (El-Deiry et al, 1993). In tumor cells, the lack of a functional p53 protein, often caused by a mutation of one p53 allele and deletion of the wild-type allele, results in reduced $p21^{WAF1}$ protein levels and consequently cell-cycle activation. A p53 gene alteration occurs in roughly 50% of colon carcinomas (Fearon and Vogelstein, 1990). The antiproliferative effect of p53 may also be abrogated by the Mdm2 protein, which inhibits the transactivating activity of p53 (Momand et al, 1992) and catalyzes p53 degradation via ubiquitination (Haupt et al, 1997). Active (hypophosphorylated) pRb protein is capable of complexing with and inactivating the E2F transcription factor, preventing exit from G1 phase. Mutations of

the *Rb* gene or phosphorylation of the pRb protein by cyclin D-CDK4/6 complexes result in the liberation and activation of E2F, which promotes entry into the S phase and activates cell proliferation (Sherr, 1996; Weinberg, 1995).

The *INK4a-ARF* locus, situated at 9p21, contains two tumor suppressor genes, $p16^{INK4a}$ (*CDKN2A*, *MTS1*) and $p14^{ARF}$ ($p19^{ARF}$ in the mouse), characterized by two distinct promoters and first exons spliced to a common exon 2 in different reading frames (Mao et al, 1995; Quelle et al, 1995; Serrano et al, 1993; Stone et al, 1995). The two encoded proteins, which are totally unrelated in terms of amino acid sequence, contribute to cell-cycle control via the pRb and the p53 pathways, respectively (Sherr, 1998). The $p16^{INK4a}$ gene product is an inhibitor of cyclin-dependent kinase 4 and 6 (CDK4/6), capable of preventing pRb phosphorylation and thereby blocking a cell in G1 (Cordon-Cardo, 1995; Serrano et al, 1993, 1996; Sherr, 1996). In the absence of a functional $p16^{INK4a}$ protein, CDK4 binds to cyclin D and phosphorylates pRb, releasing E2F, which stimulates entry into the S phase and contributes to proliferation activation. In human tumors, the $p16^{INK4a}$ gene is frequently inactivated by homozygous deletions, point mutations (Cairns et al, 1994; Kamb et al, 1994; Okamoto et al, 1994; Spruck et al, 1994), or de novo methylation of its promoter region (Gonzales-Zulueta

Received November 6, 2000.

This study was supported by the Swiss Cancer League and by the Swiss National Science Foundation (PC).

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et al, 1995; Herman et al, 1995; Merlo et al, 1995). The $p16^{\text{INK4a}}$ gene is methylated in about 40% of sporadic colon carcinomas (Toyota et al, 1999). The $p14^{\text{ARF}}$ protein inhibits cell proliferation by blocking Mdm2-mediated degradation of p53 and by neutralizing Mdm2's inhibition of the p53 transactivating activity (Kurokawa et al, 1999; Pomerantz et al, 1998; Prives, 1998; Zhang et al, 1998). Thus, $p14^{\text{ARF}}$ inactivation is a mechanism to disrupt the p53/p21 pathway, which contributes to uncontrolled proliferation in human neoplasia. Accordingly, $p19^{\text{ARF}}$ null mice develop diverse types of cancer (Kamijo et al, 1997, 1999; Serrano et al, 1996), and $p14^{\text{ARF}}$ may be silenced by promoter methylation in cancer cell lines (Robertson and Jones, 1998). However, no mutations affecting the first exon of $p14^{\text{ARF}}$ have been reported thus far.

In the present study, we have analyzed both the methylation status and the mutational status of the $p14^{\text{ARF}}$ and $p16^{\text{INK4a}}$ genes in 8 colon cancer cell lines and 60 primary colon carcinomas and evaluated their correlation with p53 expression and *hMLH1* promoter methylation, as well as with different clinicopathological parameters.

Results

Sixty primary colon carcinoma and normal colon mucosa samples and 8 colon carcinoma cell lines were analyzed for $p14^{\text{ARF}}$ and $p16^{\text{INK4a}}$ promoter methylation by using three different techniques: restriction enzyme-related PCR (REP), methylation-specific PCR (MSP), and bisulfite single-strand conformation polymorphism (BSSCP). The analyses were confirmed by bisulfite genomic sequencing (BGS). The localization of the CpG dinucleotides from the $p14^{\text{ARF}}$ - and $p16^{\text{INK4a}}$ -promoter and exon 1 regions covered by

these techniques are indicated in Figure 1. The tumor cell lines were also analyzed for $p14^{\text{ARF}}$ and $p16^{\text{INK4a}}$ mRNA expression by means of reverse transcription-polymerase chain reaction (RT-PCR) to determine the effect of methylation on gene transcription. In addition, all tissue samples and tumor cell lines were screened for $p14^{\text{ARF}}$ and $p16^{\text{INK4a}}$ gene mutations by single-strand conformation polymorphism (SSCP), and the identified mutations were confirmed by DNA sequencing. Finally, the allelic status of $p14^{\text{ARF}}$ and $p16^{\text{INK4a}}$ was determined in all primary carcinomas by analyzing two microsatellite markers at chromosome 9p21.

$p14^{\text{ARF}}$ Status in Primary Colon Cancer and in Colon Carcinoma Cell Lines

The $p14^{\text{ARF}}$ gene was methylated in 20 of 60 (33%) primary colon carcinomas and was unmethylated with all three methods in the 60 corresponding adjacent tumor-free colon mucosa samples (Table 1). Methylation was detected by REP, MSP, and BSSCP in nine cases, by REP and BSSCP in eight cases, by REP and MSP in one case, and by REP only in two cases. All cases positive for $p14^{\text{ARF}}$ methylation by BSSCP showed a characteristic additional band (Fig. 2), which was excised from the BSSCP gel and reamplified by PCR. Direct sequence analysis indicated that almost all CpG dinucleotides present in the analyzed fragment were methylated. By contrast, direct sequencing of the other BSSCP bands showed a fully unmethylated sequence. A CGG (arginine) to TGG (tryptophan) somatic mutation at codon 12 of $p14^{\text{ARF}}$ exon 1 β was detected by SSCP and confirmed by DNA sequencing in tumor 2888 (Figs. 2 and 3). There was no evidence

Figure 1.

$p14^{\text{ARF}}$ and $p16^{\text{INK4a}}$ gene promoter/exon 1 regions analyzed for methylation, derived from GenBank accession numbers AF082338 and X94154. In both diagrams, the start of the coding region is indicated by an asterisk and is defined as position +1. The bent arrows correspond to the previously identified transcription start site of $p14^{\text{ARF}}$ and to two of the putative transcription start sites of $p16^{\text{INK4a}}$. The short vertical lines correspond to the positions of the CpG dinucleotides analyzed by the different technical approaches. The positions of the restriction sites analyzed by restriction enzyme-related PCR (REP) are indicated by full circles (H, *HpaII*; K, *KspI*). The CpG dinucleotides covered by the methylation-specific PCR (MSP) primers are indicated by full arrowheads (f, reverse primer; r, reverse primer). The horizontal brackets delineate the PCR fragments amplified using the primer sets indicated in Table 4 and analyzed by bisulfite single-strand conformation polymorphism (BSSCP) and/or bisulfite genomic sequencing (BGS) (R, S, and T, fragments amplified with the R, S, and T primer sets indicated in Table 4).

Table 1. Correlation between *p14^{ARF}/p16^{INK4a}* Methylation and Clinicopathological Variables

	p14			p16		
	Meth	Unmeth	<i>p</i>	Meth	Unmeth	<i>p</i>
<i>p14</i> methylation status						
Methylated				9	11	NS
Unmethylated				10	30	
<i>p16</i> methylation status						
Methylated	9	10	NS			
Unmethylated	11	30				
p53 protein expression						
Positive	4	21	0.02	5	20	NS
Negative	15	19		14	20	
<i>hMLH1</i> methylation status						
Methylated	2	3	NS	4	1	0.03
Unmethylated	18	37		15	40	
Age						
≤ 60 yr	3	11	NS	5	9	NS
> 60 yr	17	29		14	32	
Sex						
Female	11	15	NS	9	17	NS
Male	9	25		10	24	
Differentiation						
Well	10	6	0.005	4	12	NS
Moderately or poorly	10	34		15	29	
Well or moderately	18	31	NS	11	38	0.002
Poorly	2	9		8	3	
Localization						
Right side	13	13	0.02	12	14	0.02
Left side	7	26		6	27	
Stage						
pT1 or pT2	5	5	NS	2	8	NS
pT3 or pT4	15	34		17	32	

NS, not significant.

p, values by two-tailed Fisher's exact test.

of *p14^{ARF}* exon1 β mutation by SSCP analysis in any of the remaining 59 tumors.

Of the eight colon carcinoma cell lines, four contained a methylated *p14^{ARF}* gene, as demonstrable by REP, MSP, and BSSCP and confirmed by BGS analysis. MSP and BGS analyses showed that the *p14^{ARF}* gene was homozygously methylated (*p14^{m+/+}*) in EE39c, heterozygously methylated (*p14^{m+/-}*) in HCT116, ALTI, and Isreco1 and unmethylated (*p14^{m-/-}*) in HT29, SW480, Cosut, and Colo205 (Fig. 4, Table 2). To evaluate the distribution of methylated CpG dinucleotides within the *p14^{ARF}* promoter/exon 1 β region, one of the amplified bisulfite-modified fragments analyzed by BSSCP was cloned, and individual clones were sequenced. Because the BSSCP patterns of the different PCR runs were identical, we decided to restrict cloning and sequence analysis to one of them, considering it to be representative. We observed some heterogeneity in the methylation pattern from one cell line to another, but the density of methylated CpGs in the analyzed fragments was elevated in all methylated alleles examined (Table 3). By SSCP analysis of *p14^{ARF}* exon 1 β , we found that HCT116 contained an aberrantly migrating band, suggestive of

mutation. By DNA sequencing, we identified a single base (guanidine) deletion within codon 33 generating a stop codon at codon 47. The mutation was confirmed by analyzing the sequences of independent BGS clones, which demonstrates that the mutation was present on the unmethylated allele of *p14^{ARF}* exon 1 β (Fig. 3). No evidence of *p14^{ARF}* exon 1 β mutation was observed in the other seven cell lines. Expression of *p14^{ARF}* mRNA was demonstrated by RT-PCR in the four *p14^{m-/-}* tumors and in the three *p14^{m+/-}* tumors, but was undetectable in the *p14^{m+/+}* tumor (Fig. 4, Table 2). Full-length *p14^{ARF}* cDNA from HCT116 was obtained by reverse transcription, cloned, and sequenced. The only sequence obtained corresponded to mutated cDNA, confirming silencing of the wild-type allele by methylation (data not shown).

***p16^{INK4a}* Status in Primary Colon Cancer and in Colon Carcinoma Cell Lines**

The *p16^{INK4a}* gene was methylated in 19 of 60 (32%) primary colon carcinomas and appeared unmethylated with all three methods in the 60 corresponding adjacent tumor-free colon mucosa samples (Table 1).

Figure 2.

Analysis of *p14^{ARF}* and *p16^{INK4a}* gene methylation and mutation in three primary colon carcinomas. A, *p14^{ARF}* analysis (upper panel). By MSP analysis, the *p14^{ARF}* gene is methylated in tumors 2874 and 2920 but unmethylated in tumor 2888. By BSSCP, an additional band (arrowhead) corresponding to the methylated bisulfite-modified *p14^{ARF}* sequence is observed in tumors 2874 and 2920. The position of the four bands corresponding to an unmethylated *p14^{ARF}* allele is indicated by empty arrowheads. By single-strand conformation polymorphism (SSCP) analysis of tumor 2888, the presence of an abnormal band (asterisk) corresponding to the *p14^{ARF}* point mutation illustrated in Figure 3A is determined. *p16^{INK4a}* analysis (lower panel). By MSP analysis, the *p16^{INK4a}* gene is methylated in tumor 2888 but unmethylated in tumors 2874 and 2920. By BSSCP, an additional band (arrowhead) corresponding to the methylated bisulfite-modified *p16^{INK4a}* sequence is observed in tumor 2888. The position of the four bands corresponding to an unmethylated *p16^{INK4a}* allele is indicated by empty arrowheads. B, REP analysis. Genomic DNA of tumor tissue (T) and adjacent normal colon mucosa (N) was digested with *MspI*, *KspI* and *HpaII* before PCR amplification of *p14^{ARF}* exon 1β and *p16^{INK4a}* exon 1α (see "Materials and Methods"). Undigested DNA was included as a control. Exon 1β of *p14^{ARF}* is methylated in tumors 2874 and 2920, but unmethylated in tumor 2888 as well as in the three normal colon mucosa samples (upper panel). Exon 1α of *p16^{INK4a}* is methylated in tumor 2888, but unmethylated in tumors 2874 and 2920, as well as in the three normal mucosa samples (lower panel).

Figure 3.

Mutations of *p14^{ARF}* exon 1 β . A, Sequence analysis of *p14^{ARF}* codons 8 to 15. The arrows point the C to T substitution within codon 12, changing an arginine (CGG) into tryptophan (TGG) in tumor 2888. B, Sequence analysis of the bisulfite-modified codons 28 to 36 of *p14^{ARF}*. Presence of a single base deletion (G) within codon 33 in the unmethylated allele of the HCT116 colon cancer cell line resulting in a stop at codon 47.

Methylation was detected by REP, MSP, and BSSCP in 11 cases, by REP and MSP in 7 cases, and by REP only in 1 case (Fig. 2). In all cases that were methylation-positive by BSSCP, a characteristic additional band was observed, which was cut from the BSSCP gel and reamplified by PCR. By direct sequencing, we found that almost all CpG dinucleotides present in the analyzed fragment were methylated. All cases were analyzed for *p16^{INK4a}* point mutations by SSCP. No tumor-specific mutation of *p16^{INK4a}* exons 1, 2, or 3 was found in the 60 primary colon carcinomas.

Of the eight colon carcinoma cell lines, seven contained a methylated *p16^{INK4a}* gene, as demonstrated by REP, MSP, and BSSCP (Fig. 4) and confirmed by BGS analysis. MSP and BGS analyses showed that the *p16^{INK4a}* gene was homozygously methylated (*p16^{m+/+}*) in five cell lines (HT29, SW480, Isreco1, Cosut, and Colo205), heterozygously methylated (*p16^{m+/-}*) in two cell lines (HCT116 and ALT1), and unmethylated (*p16^{m-/-}*) in one cell line (EE39c) (Fig. 4, Table 2). In HCT116, we observed an additional band by SSCP analysis of *p16^{INK4a}* exon 1. By direct sequencing of this band, we found the single-base insertion within codon 23 previously described (Okamoto et al, 1994) and found it to be on the unmethylated *p16^{INK4a}* allele of HCT116 (Myöhänen et al, 1998). There was no evidence of *p16^{INK4a}* exon 1, 2, or 3 mutation in the seven other tumor cell lines. By RT-PCR, *p16^{INK4a}* mRNA was detected in the *p16^{m-/-}* tumor, as well as in both *p16^{m+/-}* tumors, but was undetectable in the five *p16^{m+/+}* tumors (Fig. 4, Table 2).

p14^{ARF}/p16^{INK4a} Allele Loss in Primary Colon Carcinomas

All 60 tumors and corresponding nonneoplastic mucosa samples were analyzed for allele loss at 9p21 using the D9S1748 and D9S942 microsatellite markers. Of the 57 tumors informative for at least one of the polymorphisms, 11 (19%) showed a deletion of one *p14^{ARF}/p16^{INK4a}* allele. Six of these tumors were *p16^{INK4a}* methylated, whereas none of them were *p14^{ARF}* methylated. In tumor 2888, which contains a

p14^{ARF} exon 1 β mutation, the wild-type allele was also deleted.

hMLH1 Gene Methylation in Colon Cancer

An aberrantly migrating band was observed by BSSCP analysis of the *hMLH1* promoter region in five primary colon carcinomas (Fig. 5) and in one of eight colon cancer cell lines: HT29 (Table 2). The aberrant bands were cut from BSSCP gels and reamplified by PCR. Direct sequencing showed that almost all CpG dinucleotides present in the analyzed fragment were methylated.

p53 Expression in Primary Colon Carcinomas

The expression of p53 was analyzed by immunohistochemistry in 59 primary colon carcinomas. Twenty-five carcinomas were considered as positive for p53 expression. In 18 of them, p53 staining was strongly positive in the nucleus of 70% to 100% of the tumor cells. In seven other tumors, there was a nuclear expression of p53 in 40% to 50% of the tumor cells. In the remaining 34 carcinomas, which were considered negative for p53 expression, there was no or only sparse nuclear staining for p53 (less than 1% of tumor cells).

Correlation between p14^{ARF}/p16^{INK4a} Gene Methylation, hMLH1 Gene Methylation, p53 Expression, and Clinicopathological Parameters

Methylation of the *p14^{ARF}* gene was found in 20 of 60 (33%) primary tumors and 4 of 8 (50%) colon cancer cell lines, whereas *p16^{INK4a}* was methylated in 19 of 60 (32%) primary tumor and in 7 of 8 (87%) cell lines. Simultaneous *p14^{ARF}* and *p16^{INK4a}* methylation was observed in 9 of 60 (15%) primary carcinomas and in 3 of 8 (37%) tumor cell lines. There was no statistical correlation between *p14^{ARF}* and *p16^{INK4a}* methylation (Table 1). Methylation of the *hMLH1* gene was significantly more frequent in *p16^{INK4a}*-methylated (4/19) than in *p16^{INK4a}*-unmethylated (1/41) tumors ($p = 0.03$), but it was not correlated to *p14^{ARF}* methylation.

Figure 4.

Analysis of *p14^{ARF}* and *p16^{INK4a}* mRNA expression and gene methylation in eight colon cancer cell lines. *p14^{ARF}* and *p16^{INK4a}* mRNA expression is analyzed by reverse transcription-polymerase chain reaction (RT-PCR), with *GAPDH* as a positive control. By MSP, the presence of a PCR product in the lines marked by M indicates the presence of methylated *p14^{ARF}* or *p16^{INK4a}* alleles. The presence of a PCR product in the lines marked by U indicates the presence of unmethylated *p14^{ARF}* or *p16^{INK4a}* alleles. By BSSCP, the position of the four bands corresponding to an unmethylated allele is indicated by empty arrowheads. The additional bands reflect the presence of methylated alleles with different methylation patterns (see Table 3).

Expression of p53 protein was significantly more frequent in *p14^{ARF}*-unmethylated than in *p14^{ARF}*-methylated tumors ($p = 0.02$), but was not correlated to the methylation status of the *p16^{INK4a}* gene. There was no significant correlation between *p14^{ARF}* or *p16^{INK4a}* methylation and patient's age, patient's sex, or the tumor stage. The *p14^{ARF}* gene was significantly more often methylated in well-differentiated (10/16) than in moderately or poorly differentiated (10/44) adenocarcinomas ($p = 0.005$), whereas *p16^{INK4a}* gene methylation was significantly more frequent in poorly differentiated (8/11) than in well- or moderately differ-

entiated (11/49) adenocarcinomas ($p = 0.002$). Both *p14^{ARF}* and *p16^{INK4a}* genes were more frequently methylated in right-sided than in left-sided tumors ($p = 0.02$).

Discussion

The *p14^{ARF}* tumor suppressor gene product activates and stabilizes p53 by interacting directly with Mdm2, resulting in cell-cycle arrest (Sherr, 1998). It has been demonstrated that the amino-terminal portion of *p14^{ARF}*, comprising the 64 amino-acids encoded by

Table 2. Status of *p14^{ARF}* and *p16^{INK4a}* in Colon Cancer Cell Lines

Cell line	<i>p14</i>			<i>p16</i>			<i>hMLH1</i>
	Meth status	Mut	mRNA expr	Meth status	Mut	mRNA expr	Meth status
HT29	U/U	—	+	M/M	—	—	M/U
HCT116	M/U	+	+	M/U	+	+	U/U
SW480	U/U	—	+	M/M	—	—	U/U
ALTI	M/U	—	+	M/U	—	+	U/U
Isreco1	M/U	—	+	M/M	—	—	U/U
EE39c	M/M	—	—	U/U	—	+	U/U
Cosut	U/U	—	+	M/M	—	—	U/U
Colo205	U/U	—	+	M/M	—	—	U/U

M, methylated; U, unmethylated; +, presence of expression or mutation; —, absence of expression or mutation. The methylation status of both *p14^{ARF}*, both *p16^{INK4a}*, and both *hMLH1* alleles is indicated.

exon 1 β , is sufficient to induce cell-cycle arrest (Quelle et al, 1997; Zhang et al, 1998). In human tumors, however, no tumor-specific point mutations of *p14^{ARF}* exon 1 β have been reported thus far (Robertson and Jones, 1998; Ruas and Peters, 1998; Sherr, 1998).

In this study, we describe the two first-point mutations affecting the first exon of the *p14^{ARF}* gene. In the HCT116 colon cancer cell line, we identified a single base deletion within codon 33 resulting in a 47 amino acid truncated protein composed of the correct first 32 amino acids from *p14^{ARF}* and of 15 incorrect amino acids. A second mutation, an arginine to tryptophan substitution at codon 12 of *p14^{ARF}* exon 1 β , was identified in a primary colon carcinoma (Case 2888), indicating that point mutations in this region do not simply represent in vitro cell culture artifacts. *p14^{ARF}* localizes to the nucleolus via a C-terminal domain, and mutations of *p14^{ARF}* exon 2 disrupt *p14^{ARF}*'s nucleolar localization and its ability to stabilize p53 (Zhang and Xiong, 1999). Therefore, it is likely that the former mutation, which amputates two thirds of the C-terminal part of *p14^{ARF}*, compromises both nucleolar localization and *p14^{ARF}*-dependent growth arrest. On the other hand, given that one of the Mdm2 binding sites is localized within the 15 N-terminal amino acids of *p14^{ARF}* (Midgley et al, 2000; Weber et al, 2000), it is likely that the codon 12 mutation, which changes a positively charged arginine into a hydrophobe tryptophan in a conserved region, abrogates Mdm2 binding and cell-cycle arrest. We are currently investigating how these mutations affect the activity of *p14^{ARF}* on cell-cycle regulation. It should be emphasized that for both identified *p14^{ARF}* mutations the wild-type *p14^{ARF}* allele was inactivated, by methylation in HCT116 and by deletion (loss of heterozygosity) in tumor 2888, which supports the hypothesis that these mutations are of functional relevance. A similar observation of mutation on one allele and methylation of the wild-type allele has been reported (Myöhänen et al, 1998) for the *p16^{INK4a}* gene in the HCT116 colon cancer cell line. It would be interesting to determine if the *p14^{ARF}*-mutated allele is cis or trans to the *p16^{INK4a}*-mutated allele.

Using three different technical approaches, we observed that methylation of the *p14^{ARF}* and *p16^{INK4a}* genes occurs in 33% and 32% of sporadic colon

carcinomas, respectively. Methylation of these genes is clearly tumor-specific because it was not detected in any of the 60 normal colon mucosa adjacent to the tumors. By comparing the methylation status and mRNA expression of the *p14^{ARF}* and *p16^{INK4a}* genes in eight colon carcinoma cell lines, we observed that for both genes, methylation of both alleles was required to completely silence transcription. Similar observations have been reported recently on colon cancer cell lines (Robertson and Jones, 1998) and in primary colon carcinomas (Esteller et al, 2000). Altogether, the present and previously reported data strongly suggest that methylation silencing of the *p14^{ARF}* gene, as for the *p16^{INK4a}* gene, is frequently involved in the pathogenesis of colon carcinoma. In accordance with Esteller et al (Esteller et al, 2000), we found no correlation between *p14^{ARF}* and *p16^{INK4a}* methylation in colon cancer, which is intriguing given the proximity of the two genes. We previously observed that one third of human B-cell lymphomas contained methylated *p16^{INK4a}* genes, whereas the *p14^{ARF}* gene was never methylated in these tumors (Baur et al, 1999). These observations suggest that de novo methylation during tumorigenesis results from independent and possibly sequence-specific mechanisms. In addition, these observations are in agreement with the hypothesis that the methylation profile of different genes or CpG islands might be a tumor type-specific phenomenon (Liang et al, 1998; Melki et al, 1999). Using BGS, we found that several *p14^{ARF}* methylation patterns may coexist from one allele to another in the same colon cancer cell line. This has already been reported in different tumor types for other genes, such as *p16^{INK4a}* (Baur et al, 1999; Melki et al, 1999), *Rb* (Stirzaker et al, 1997), *p15^{INK4b}* (Melki et al, 1999), and *E-cadherin* (Melki et al, 1999), but remains unexplained. Methylation of both *p14^{ARF}* and *p16^{INK4a}* genes was significantly more frequent in right-sided than in left-sided colon carcinomas ($p = 0.02$), as observed for *hMLH1* methylation (Toyota et al, 1999), suggesting that general de novo methylation might be related to localization in the colon. The fact that the *p14^{ARF}* gene was significantly more often methylated in well-differentiated adenocarcinomas ($p = 0.005$) and, conversely, that *p16^{INK4a}* methylation was more

Table 3. Methylation Patterns of *p14^{ARF}* Analyzed by BGS in 7 Colon Cancer Cell Lines

		CpG*										H										K									
Cell line	Clone	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27			
		ooo																													
HT29	a	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
	b	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
	c	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
	d	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
HCT116	a	-	+	+	+	-	-	+	-	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+		
	b	-	-	+	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+	-	+		
	c	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
	e	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		
SW480	f	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
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	i	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+			
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	a	-	+	+	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+			
	b	-	+	+	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+			
	c	+	+	-	-	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+			
	d	+	+	-	-	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+			
	e	-	+	+	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+			
	f	-	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-			
	g	-	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	-			
Cosut	h	-	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-	+			
	a	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-				
	b	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			

+, methylated; -, unmethylated; H, HpaII site analyzed by restriction enzyme-related PCR (REP); K, KspI site analyzed by REP.

* A bisulfite-modified 255-bp fragment from *p14^{ARF}* promoter/exon 1 β region containing 27 CpG dinucleotides (numbered 1–27) was amplified by PCR, cloned, and sequenced. The CpG dinucleotides inside the *p14^{ARF}* coding sequence are indicated in bold. The CpG dinucleotide covered by the forward primer in methylation-specific PCR (MSP)-analysis is underlined by small circles. The methylation status of each CpG is indicated.

frequent in poorly differentiated carcinomas ($p = 0.002$), suggests that a given methylation profile might not only reflect the histogenesis of a tumor but might also define biologically distinct subsets and possibly different tumorigenic pathways within a given tumor category.

It has been suggested that in colon carcinoma, virtually all cancer-specific methylation events (called type C methylation) occur in a distinct subset of tumors, thereby defining a new phenotype, the CpG island methylator phenotype (CIMP) (Toyota et al, 1999). In the proposed concept, the CIMP+ colon carcinomas would be characterized by the simultaneous methylation of multiple tumor-suppressor genes such as *p16^{INK4a}* and by the induction of mismatch repair deficiency through *hMLH1* gene inactivation (Toyota et al, 1999). The remaining non-

tumor-specific methylation events (called type A, for aging-specific methylation) detected in colon cancer would be related to incremental hypermethylation in the normal colon as an age-related phenomenon (Toyota et al, 1999). In agreement with the CIMP concept, we found that *p16^{INK4a}* gene methylation (a) occurred in about one third of primary colon carcinomas, more often in right-sided tumors, (b) was not age-dependent, and (c) was statistically correlated to *hMLH1* methylation ($p = 0.03$). As with *p16^{INK4a}* methylation, *p14^{ARF}* methylation fulfilled the criteria of type C, but not type A, methylation, in that it was not detectable in normal colon mucosa and was not associated with patient's age. In addition, contrary to *p16^{INK4a}* methylation, *p14^{ARF}* methylation was not associated with *hMLH1* methylation. These observations suggest that *p14^{ARF}* and *p16^{INK4a}* may be meth-

Figure 5.

Methylation of *hMLH1* promoter analyzed by BSSCP. The position of the four bands corresponding to an unmethylated allele is indicated by empty arrowheads. Two additional bands indicated by arrowheads reflect the presence of a methylated allele in tumor 2890.

ylated through distinct mechanisms and that there are at least two cancer-specific methylation pathways in colon cancer. As a consequence, the notion resulting from the CIMP concept that all tumor-specific methylation events result from a unique pathway and occur in parallel in colon cancer is not consistent with the present findings.

The interaction between *p14^{ARF}* and Mdm2 blocks Mdm2-mediated degradation of p53, and neutralizes Mdm2's inhibition of p53 transactivating activity (Kurokawa et al, 1999; Pomerantz et al, 1998; Prives, 1998; Zhang et al, 1998). In addition, the *p14^{ARF}* protein interacts with p53 on chromatin, but there is no direct evidence that *p14^{ARF}* plays a physiological role as a p53 coactivator (Kamijo et al, 1998; Sherr, 1998). In human tumor cell lines, there is an inverse correlation between *p14^{ARF}* expression and p53 function (Stott et al, 1998). In agreement with this observation, we found an inverse statistical correlation between p53 immunohistochemical expression, which generally reflects p53 mutation (Baas et al, 1994; Costa et al, 1995), and *p14^{ARF}* methylation. Esteller et al (2000) observed that *p14^{ARF}* methylation was slightly more frequently observed in colon carcinomas with wild-type p53 compared with p53-mutated tumors, although this difference was not statistically significant. It is well established that p53 protein overexpression by immunohistochemistry may be found in p53 wild-type tumors as a result of alternative mechanisms leading to p53 stabilization (Baas et al, 1994; Costa et al, 1995). One of the factors contributing to p53 stabilization in p53 wild-type tumors might be stabilization of p53 via expression of *p14^{ARF}*. This would explain why we observed a statistically significant association between the presence of unmethylated *p14^{ARF}* and p53 overexpression and why comparing the presence of unmethylated *p14^{ARF}* and p53 mutations did not result in a statistically significant correlation (Esteller et al, 2000). In addition to the fact that *p14^{ARF}* activates p53, it has been suggested that, inversely, p53 might negatively regulate *p14^{ARF}* expression in a feedback loop (Stott et al, 1998). Given that wild-type p53 down-regulates the activity of some CpG-rich promoters by binding Sp1 consensus sites and that p53 mutants lose this repressive effect (Bargonetti et al, 1997), it is possible that p53 contributes

to *p14^{ARF}* inactivation (methylation) by interacting with the Sp1 sites of the *p14^{ARF}* promoter (Robertson and Jones, 1998). Because occupied Sp1 sites within CpG islands protect them from de novo methylation (Brandeis et al, 1994; Macleod et al, 1994), the putative binding of wild-type p53 within the promoter of *p14^{ARF}* might abolish the protective effect of Sp1-bound elements, leading to methylation. This hypothesis should be verified by determining whether or not p53 interacts with the *p14^{ARF}* promoter and whether this interaction influences *p14^{ARF}* methylation.

In conclusion, this study shows that (a) rare tumor-specific mutations within *p14^{ARF}* exon 1 β may occur in colon cancer, (b) the *p14^{ARF}* and *p16^{INK4a}* genes are frequently but independently methylated in colon cancer, which underlines the complexity of the mechanisms leading to de novo methylation in cancer, and (c) the methylation profile of the *p14^{ARF}* and *p16^{INK4a}* genes might define biologically distinct subsets of colon carcinoma.

Materials and Methods

Primary Tumor Samples and Colon Cancer Cell Lines

Primary Tumors. Samples from 60 primary colorectal adenocarcinomas and corresponding adjacent normal colon mucosa were obtained from surgical resections of colon cancers patients consecutively operated on between 1997 and 1998 at the Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland. The average age of the patients was 69.9 years (range, 37–89 yr). There were 26 females and 34 males. The tumor was localized in the right colon (caecum, ascending) in 26 cases, in the transverse colon in 1 case, and in the left colon (descending, sigmoid, rectum) in 33 cases. Tumors were graded and staged on routinely processed, formalin-fixed and paraffin-embedded tissue samples. According to the tumor node metastasis (TNM) staging system, there were 3 pT1, 7 pT2, 32 pT3, and 17 pT4 tumors. One case could not be staged because of inadequate sampling. Sixteen tumors were well differentiated, 33 moderately differentiated, and 11 poorly differentiated adenocarcinomas. The immunohistochemical expression of p53 was examined on formalin-fixed, paraffin-embedded sections on 2 different tumor samples in 59 cases. Tumor and nontumor tissue samples were immediately frozen in liquid nitrogen and stored at -70°C until DNA extraction. Hematoxylin- and eosin-stained (H&E) sections of the frozen specimens were examined to verify representativity.

Colon Cancer Cell Lines. Frozen pellets from 8 colon carcinoma cell lines, HT29, HCT116, SW480, ALTI, Isreco1, EE39c, Cosut, and Colo205, were kindly provided by the laboratory of Dr. Bernard Sordat (Institut Suisse de Recherche Expérimentale sur le Cancer, Epalinges, Switzerland).

Nucleic Acid Isolation

Genomic DNA was extracted from ten 25- μm -thick tumor and normal tissue sections and from the cell

lines by means of the Elu-quick DNA Purification Kit (Schleicher & Schuell, Dassel, Germany), according to the manufacturer's protocol. Total RNA was isolated from the cell lines by the single-step guanidium thiocyanate method (Chomezynski and Sacchi, 1987).

Reverse Transcription-Polymerase Chain Reaction

cDNA synthesis was performed on total RNA samples extracted from the colon cancer cell lines as described previously (Baur et al, 1999). A 207-bp fragment from exon 1 to exon 2 of *p14^{ARF}* and a 210-bp fragment from exon 1 to exon 2 of *p16^{INK4a}* were amplified by polymerase chain reaction (PCR) using the primer set and conditions indicated in Table 4 (A and B). A 238-bp fragment from *Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* cDNA was amplified as a positive control (Table 4, C). A reliable semiquantitative assessment of *p14^{ARF}* and *p16^{INK4a}* mRNA expression was not feasible in our primary tumor/normal mucosa samples because of the low amounts and poor quality of RNA extracted from our samples and also because of the contamination of tumor cells by relatively large numbers of nontumor cells (eg, mesenchymal and inflammatory cells) in our tumor samples.

Mutation Screening for *p14^{ARF}* and *p16^{INK4a}* Genes

The first exon of the *p14^{ARF}* gene and the three exons of the *p16^{INK4a}* gene were screened for mutation by PCR SSCP as described previously (Chaubert et al, 1993). The primers and annealing conditions used for PCR are given in Table 4 (D–I).

Determination of 9p21 Allelic Loss

DNA of the 60 primary tumors and corresponding normal colon mucosa was analyzed for *p14^{ARF}*/*p16^{INK4a}* allele loss using two microsatellite markers mapping at chromosome 9p21: D9S1748 and D9S942. PCR conditions, primer sequences, and annealing temperatures are indicated in Table 4 (J and K). PCR products were run on denaturing polyacrylamide/urea gels, as described previously (Chaubert et al, 1997). Allelic loss was scored in informative cases when the intensity of the signal for a tumor allele was significantly lower than that of the matched normal allele.

Methylation Analysis of the *p14^{ARF}*, *p16^{INK4a}*, and *hMLH1* Genes

The methylation status of the *p14^{ARF}* and *p16^{INK4a}* genes was determined in all 8 tumor cell lines and in the 60 primary tumor and normal colon samples by three different techniques: restriction enzyme related PCR (REP), methylation-specific PCR (MSP) (Herman et al, 1996), and bisulfite single-strand conformation polymorphism (BSSCP) (Burri and Chaubert, 1999). REP analysis of the *p14^{ARF}* and *p16^{INK4a}* genes was performed as described previously (Baur et al, 1999). After digestion with two methyl-sensitive (KspI and

HpaII) and one non-methyl-sensitive (*MspI*) restriction enzymes, genomic DNA was amplified by PCR using primers specific for *p14^{ARF}* and *p16^{INK4a}*. The primers and PCR conditions used for REP analysis are indicated in Table 4 (L and M). For MSP and BSSCP, genomic DNA was chemically modified by sodium bisulfite as described (Baur et al, 1999; Clark et al, 1994). This treatment changes the unmethylated, but not the methylated, cytosines into uracil residues. In MSP, the bisulfite-treated DNA is subjected to PCR amplification using primers designed to anneal specifically to the methylated, as opposed to the unmethylated, bisulfite-modified DNA within a given gene. Thus, a PCR product is obtained only when the sequence covered by the primers is methylated, as opposed to unmethylated. For both *p14^{ARF}* and *p16^{INK4a}* MSP analyses, one primer set specific for the methylated and one primer set specific for the unmethylated *p14^{ARF}* or *p16^{INK4a}* sequence was used (Table 4, N–Q). In BSSCP, bisulfite-treated DNA was amplified by PCR using methyl-independent primers designed to recognize both the methylated and the unmethylated bisulfite-modified sequence of interest and then loaded on a SSCP gel (Burri and Chaubert, 1999). By this approach, one can distinguish the different methylation patterns present in a cell population (Burri and Chaubert, 1999). This method was applied to determine the methylation status of the *p14^{ARF}* and the *p16^{INK4a}* genes, as well as that of the *hMLH1* gene. Two fragments from the bisulfite-modified sequence of *p14^{ARF}*, one from the bisulfite-modified sequence of *p16^{INK4a}*, and one from the bisulfite-modified sequence of *hMLH1* were amplified by PCR using the primers and conditions indicated in Table 4 (R–U). The CpG dinucleotides from *p14^{ARF}* and *p16^{INK4a}* promoter/exon 1 regions analyzed by the three methods are indicated in Figure 1. It is of importance to emphasize that the three methods described above differ in terms of sensitivity and specificity, which requires the following remarks. (a) In contrast to BSSCP, both MSP and REP are highly sensitive techniques, allowing the detection of CpG dinucleotides that are infrequently methylated within a tumor; therefore, one can expect to find cases methylation-positive by REP and/or MSP, but negative by BSSCP, particularly by studying primary tumors in which methylation at specific sites is often heterogeneous (Baur et al, 1999). (b) Because both the forward and the reverse methyl-specific primers used for *p14^{ARF}* MSP contain a methyl-specific base at the 3' end (Table 4, N), both CpG dinucleotides covered by these primers must be methylated to allow PCR amplification. For this reason and given the heterogeneous distribution of methylated CpG dinucleotides, one can expect to find tumors in which *p14^{ARF}* methylation is undetectable by our MSP approach. Conversely, in our MSP approach for *p16^{INK4a}* methylation analysis, only the forward primer has a methyl-specific 3' end, which makes false negative results less probable.

The results obtained by BSSCP were confirmed by BGS (Frommer et al, 1992), which precisely maps the

Table 4. Primers Used for RT-PCR, PCR-SSCP, LOH, REP, MSP, BGS, and BSSCP Analyses

Primer set	Application ^a and specificity ^b	Template DNA ^c	Forward primer (5'→3')	Reverse primer (5'→3')	Product size	Anneal temp
RT-PCR						
A	p14	cDNA	GTGGCCCTCGTGTGATG	AGCACCACGCGGTGTC	207 bp	58° C
B	p16	cDNA	GGTGGGGGCTGCTGGA	AGCACCACGCGGTGTC	210 bp	58° C
C	GAPDH	cDNA	TTGTCAAGCTCATTTCCTG	AGGCCCTCCCTCTTC	238 bp	55° C
PCR-SSCP						
D	p14 exon 1	G	GCCTGGGGGGGGAGAT	GGGGCTGCTGCCCTAGA	316 bp	58° C
E	p16 exon 1	G	GGGAGCAGCATGGAGCCG	AGTCGCCGCCATCCCT	203 bp	58° C
F	p16 exon 2A	G	CTGGCTCTGACCATCTGT	AGCACCACGCGGTGTC	171 bp	58° C
G	p16 exon 2B	G	GACCCGGCCACTCTCACC	AGGTACCGTGGACATCGC	170 bp	58° C
H	p16 exon 2C	G	GATGCTGGGGCGCTCT	CAGGGTACAAATTCAGAT	169 bp	55° C
I	p16 exon 3	G	GTAGGGACGGCAAGAGA	ACCTTCGGTACTGATG	159 bp	55° C
Polymorphism						
J	D9S1748	G	CCTCAGAAGTCAGTGAGT	CTTGAAATACACCTTTCC	130–150 bp	55° C
K	D9S942	G	GCAAGATTCCAAACAGTA	CTCATCTGCGGAACCAT	100–130 bp	55° C
REP						
L	p14 exon 1	G, RE+	GCCTGGGGGGGGAGAT	GGGGCTGCTGCCCTAGA	316 bp ^d	58° C
M	p16 exon 1	G, RE+	GGGAGCAGCATGGAGCCG	CTGGATCGGCTCCGACCGTA	159 bp ^e	57° C
MSP						
N	p14 exon 1, M	G, Bis +	TAGGTTTTGGTGATTTTC	ACCTAATCTCTTAAAAACG	187 bp	48° C
O	p14 exon 1, U	G, Bis +	TAGGTTTTGGTGATTTTC	ACCTAATCTCTTAAAAACG	187 bp	48° C
P	p16 exon 1, M	G, Bis +	GGGGAGTAGTATGGAGTC	AATCGAAAGGCTACCTAA	253 bp	51° C
Q	p16 exon 1, U	G, Bis +	GGGGAGTAGTATGGAGTC	AAATCAAAACACTACCTAA	254 bp	51° C
BGS and BSSCP						
R	p14 exon 1, M/U	G, Bis +	GGGTTTATGTTTGTAGTT	CCTCAATAACATCAACAC	255 bp	48° C
S	p14 exon 1, M/U	G, Bis +	GTAGGTTTTGGTGATTTTC	AAAACCTTCTACCTAATCT	200 bp	52° C
T	p16 promoter, M/U	G, Bis +	GGGGAGATTAAATTGG	AAAACCTCATACTACTCC	312 bp	50° C
U	hMLH1, M/U	G, Bis +	TTAGGAGTGAAGGAG	ACCTATACCTAATCTAT	173 bp	48° C

^a PCR-SSCP, polymerase chain reaction-single-strand conformation polymorphism; REP, restriction enzyme-related PCR; MSP, methylation-specific PCR; BGS, bisulfite genomic sequencing; BSSCP, bisulfite single-strand conformation polymorphism.

^b M, specific for methylated bisulfite modified-DNA; U, specific for unmethylated bisulfite-modified-DNA.

^c G, genomic DNA; RE+, restriction enzyme-digested DNA; Bis+, bisulfite-treated DNA.

^d Fragment containing one HpaII/MspI site and one KspI site.

^e Fragment containing two HpaII/MspI sites and one KspI site.

methylated CpG dinucleotides present in a given DNA fragment. Aberrant bands indicative of methylation, as well as bands corresponding to normal patterns, were excised from the BSSCP gels, reamplified by PCR using the same methyl-independent primers as for BSSCP (Table 4, R–U), and directly sequenced. In addition, to assess the proportion of methylated and unmethylated alleles in tumor cell lines, the PCR products analyzed by BSSCP were cloned into a pGEM-T vector according to the manufacturer's protocol (Promega, Madison Wisconsin). Ten to twenty independent clones were reamplified and screened by BSSCP (Burri and Chaubert, 1999). At least two clones of each observed BSSCP pattern were sequenced.

Immunohistochemical Analysis of p53 Expression

The expression of p53 was examined by immunohistochemistry using the peroxidase-antiperoxidase (PAP) technique described previously (Chaubert et al, 1994). The anti-p53 monoclonal antibody used (Clone DO7, 1:500; Dako Diagnostics, Zug, Switzerland) recognizes both wild-type and mutated forms of p53. Tumors containing more than 40% p53 immunostained nuclei were considered to be positive for p53 protein expression.

Statistical Analysis

The association between $p14^{ARF}$ and $p16^{INK4a}$ methylation and the correlation between $p14^{ARF}/p16^{INK4a}$ methylation and different parameters was evaluated by Fisher's exact test. A p value < 0.05 was considered as significant.

Acknowledgments

The authors thank M. M. Bertholet, V. Buchillier, S. Burki, M. Correvon, S. Laurini, J. Maillardet, and V. Noguet for technical assistance.

References

Baas IO, Mulder JWR, Offerhaus GJA, Vogelstein B, and Hamilton SR (1994). An evaluation of six antibodies for immunohistochemistry of mutant p53 gene product in archival colorectal neoplasms. *J Pathol* 172:5–12.

Bargonetti J, Chicas A, White D, and Prives C (1997). p53 represses Sp1 DNA binding and HIV-LTR directed transcription. *Cell Mol Biol* 43:937–949.

Baur AS, Shaw P, Burri N, Delacretaz F, Bosman FT, and Chaubert P (1999). Frequent silencing of $p15^{INK4b}$ (MTS2) and $p16^{INK4a}$ (MTS1) in B-cell and T-cell lymphomas. *Blood* 94: 1773–1781.

Brandeis M, Frank D, Keshet I, Siegfried Z, Mendelson M, Nemes A, Temper V, Razin A, and Cedar H (1994). Sp1 methylation protects a CpG island from de novo methylation. *Nature* 371:435–438.

Burri N and Chaubert P (1999). Complex methylation patterns analyzed by single-strand conformation polymorphism. *Biotechniques* 26:232–234.

Cairns P, Mao L, Merlo A, Lee DJ, Schwab D, Eby Y, Tokino K, Van der Riet P, Blaugrund JE, and Sidransky D (1994). Rates of $p16$ (MTS1) mutation in primary tumors with 9p loss. *Science* 265:415–417.

Chaubert P, Bautista D, and Benhattar J (1993). An improved method for rapid screening of DNA mutation by nonradioactive single-strand conformation polymorphism procedure. *Biotechniques* 15:586.

Chaubert P, Benhattar J, Saraga E, and Costa J (1994). K-ras mutations and p53 alterations in neoplastic and nonneoplastic lesions associated with longstanding ulcerative colitis. *Am J Pathol* 144:767–775.

Chaubert P, Guillou L, Kurt AM, Bertholet MM, Mettetz G, Leisinger HJ, Bosman F, and Shaw P (1997). Frequent $p16^{INK4}$ (MTS1) gene inactivation in testicular germ cell tumors. *Am J Pathol* 151:859–865.

Chomezynski P and Sacchi N (1987). Single step method of RNA isolation by acid guanidium thiocyanate-phenol-chlorophorm extraction. *Anal Biochem* 162:156–159.

Clark SJ, Harrison J, Paul CL, and Frommer M (1994). High sensitivity mapping of methylated cytosines. *Nucleic Acids Res* 22:2990–2997.

Cordon-Cardo C (1995). Mutation of cell cycle regulators. Biological and clinical implications for human neoplasia. *Am J Pathol* 147:545–560.

Costa A, Marasca R, Valentini B, Savarino M, Faranda A, Silvestrini R, and Torelli G (1995). p53 gene point mutations in relation to p53 nuclear protein accumulation in colorectal cancers. *J Pathol* 176:45–53.

El-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW, and Vogelstein B (1993). WAF1, a potential mediator of p53 tumor suppression. *Cell* 69:817–825.

Esteller M, Tortola S, Toyota M, Capella G, Peinado MA, Baylin SB, and Herman JG (2000). Hypermethylation-associated inactivation of $p14^{ARF}$ is independent of $p16^{INK4a}$ methylation and p53 mutational status. *Cancer Res* 60:129–133.

Fearon ER and Vogelstein B (1990). A genetic model for colorectal tumorigenesis. *Cell* 61:759–767.

Frommer M, McDonald LE, Millar DS, Collis CM, Watt F, Grigg GW, Molloy PL, and Paul CL (1992). A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. *Proc Natl Acad Sci USA* 89:1827–1831.

Gonzales-Zulueta M, Bender CM, Yang AS, Nguyen T, Beart RW, Van Tornout JM, and Jones PA (1995). Methylation of the 5' CpG island of the $p16/CDKN2$ tumor suppressor gene in normal and transformed tissues correlates with gene silencing. *Cancer Res* 55:4531–4535.

Haupt Y, Maya R, Kazaz A, and Oren M (1997). Mdm2 promotes the rapid degradation of p53. *Nature* 387:296–299.

Herman JG, Graff JR, Myöhänen S, Nelkin BD, and Baylin SB (1996). Methylation-specific PCR: A novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci USA* 93:9821–9826.

Herman JG, Merlo A, Mao L, Lapidus RG, Issa JP, Davidson NE, Sidransky D, and Baylin SB (1995). Inactivation of the $CDKN2/p16/MTS1$ gene is frequently associated with aberrant methylation in all common human cancers. *Cancer Res* 55:4525–4530.

- Kamb A, Gruis NA, Weaver-Feldhaus J, Liu Q, Harshman K, Tavtigian SV, Stockert E, Day RS 3rd, Johnson BE, and Skolnick MH (1994). A cell cycle regulator potentially involved in genesis of many tumor types. *Science* 246:436–440.
- Kamijo T, Bodner S, Van de Kamp E, Randle DH, and Sherr C (1999). Tumor spectrum in ARF-deficient mice. *Cancer Res* 59:2217–2222.
- Kamijo T, Weber JD, Zambetti G, Zindy F, Roussel MF, and Sherr CJ (1998). Functional and physical interactions of the ARF tumor suppressor with p53 and Mdm2. *Proc Natl Acad Sci USA* 95:8292–8297.
- Kamijo T, Zindy F, Roussel MF, Quelle DE, Sowning JR, Asmun RA, Grosveld G, and Sherr CJ (1997). Tumor suppression at the mouse *INK4a* locus mediated by the alternative reading frame product p19 ARF. *Cell* 91:649–659.
- Kurokawa K, Tanaka T, and Kato JY (1999). p19^{ARF} prevents G1 cyclin-dependent kinase activation by interacting with MDM2 and activating p53 in mouse fibroblasts. *Oncogene* 18:2718–2727.
- Liang G, Salem CE, Yu MC, Nguyen HD, Gonzales FA, Nguyen TT, Nichols PW, and Jones PA (1998). DNA methylation differences associated with tumor tissues identified by genome scanning analysis. *Genomics* 53:260–268.
- Macleod D, Charlton J, Mullins J, and Bird AP (1994). Sp1 sites in the mouse *aprt* gene promoter are required to prevent methylation of the CpG island. *Genes Dev* 8:2282–2292.
- Mao L, Merlo A, Bedi G, Shapiro IG, Edwards CD, Rollins BJ, and Sidransky D (1995). A novel p16^{INK4A} transcript. *Cancer Res* 55:2995–2997.
- Melki JR, Vincent PC, and Clark SJ (1999). Concurrent DNA hypermethylation of multiple genes in acute myeloid leukemia. *Cancer Res* 59:3730–3740.
- Merlo A, Herman JG, Mao L, Lee DJ, Gabrielson E, Burger PC, and Sidransky D (1995). 5' CpG island methylation is associated with transcriptional silencing of the tumor suppressor p16/CDKN2/MTS1 in human cancer. *Nat Med* 1:686–692.
- Midgley CA, Desterro JMP, Saville MK, Howard S, Sparks A, Hay RT, and Lane DP (2000). An N-terminal p14^{ARF} peptide blocks Mdm2-dependent ubiquitination in vitro and can activate p53 in vivo. *Oncogene* 19:2312–2323.
- Momand J, Zambetti GP, Olson DC, Georges D, and Levine AJ (1992). The *mdm-2* oncogene product forms a complex with the p53 protein and inhibits p53 mediated transactivation. *Cell* 69:1237–1245.
- Myöhänen SK, Baylin SB, and Herman JG (1998). Hypermethylation can selectively silence individual p16^{ink4A} alleles in neoplasia. *Cancer Res* 58:591–593.
- Okamoto A, Demetrick DJ, Spillare EA, Hagiwara K, Hussain SP, Bennett WP, Forrester K, Gerwin B, Serrano M, Beach DH, and Harris CC (1994). Mutation and altered expression of p16/INK4 in human cancer. *Proc Natl Acad Sci USA* 91:11045–11049.
- Pomerantz J, Schreiber-Agus N, Liegeois NJ, Silverman A, Alland L, Chin L, Potes J, Chen K, Orlow I, Lee HW, Cordon-Cardo C, and DePinho RA (1998). The *INK4a* tumor suppressor gene product, p19^{ARF}, interacts with MDM2 and neutralizes MDM2's inhibition of p53. *Cell* 92:713–723.
- Prives C (1998). Signaling to p53: Breaking the MDM2-p53 circuit. *Cell* 95:5–8.
- Quelle DE, Cheng M, Ashmun RA, and Sherr CJ (1997). Cancer associated mutations at the *INK4a* locus cancel cell cycle arrest by p16 INK4a but not by the alternative reading frame protein p19ARF. *Proc Natl Acad Sci USA* 94:669–673.
- Quelle DE, Zindy F, Ashmun RA, and Sherr CJ (1995). Alternative reading frames of the *INK4a* tumor suppressor gene encode two unrelated proteins capable of inducing cell cycle arrest. *Cell* 83:993–1000.
- Robertson KD and Jones PA (1998). The human *ARF* cell cycle regulatory gene promoter is a CpG island which can be silenced by DNA methylation and down-regulated by wild-type p53. *Mol Cell Biol* 18:6457–6473.
- Ruas M and Peters G (1998). The p16INK4a/CDKN2A tumor suppressor and its relatives. *Biochim Biophys Acta* 1378:115–177.
- Serrano M, Hannon GJ, and Beach D (1993). A new regulatory motif in cell cycle control causing specific inhibition of cyclin D/CDK4. *Nature* 366:704–707.
- Serrano M, Lee HW, Chin L, Cordon-Cardo C, Beach D, and DePinho RA (1996). Role of the *INK4a* locus in tumor suppression and cell mortality. *Cell* 85:27–37.
- Sherr CJ (1996). Cancer cell cycles. *Science* 274:1672–1677.
- Sherr CJ (1998). Tumor surveillance via the ARF-p53 pathway. *Genes Dev* 12:2984–2991.
- Spruck CH 3rd, Gonzales-Zulueta M, Shibata A, Simoneau AR, Lin MF, Gonzales F, Tsai YC, and Jones PA (1994). p16 gene in uncultured tumors. *Nature* 370:183–184.
- Stirzaker C, Millar DS, Paul CL, Warnecke PM, Harrison J, Vincent PC, Frommer M, and Clark SJ (1997). Extensive methylation spanning the *Rb* promoter in retinoblastoma tumors. *Cancer Res* 57:229–237.
- Stone S, Jiang P, Dayananth P, Tavtigian SV, Katcher H, Parry D, Peters G, and Kamb A (1995). Complex structure and regulation of the p16 (*MST1*) locus. *Cancer Res* 55:2988–2994.
- Stott FJ, Bates S, James MC, McConnell BB, Starborg M, Brookes S, Palmero I, Ryan K, Hara E, Vousden KH, and Peters G (1998). The alternative product from the human *CDKN2A* locus, p14^{ARF}, participates in a regulatory feedback loop with p53 and MDM2. *EMBO J* 17:5001–5014.
- Toyota M, Ahuja N, Ohe-Toyota M, Herman JG, Baylin SB, and Issa JP (1999). CpG island methylator phenotype in colorectal cancer. *Proc Natl Acad Sci USA* 96:8681–8686.
- Weber JD, Kuo ML, Bothner B, DiGiammarino EL, Kriwacki RW, Roussel MF, and Sherr CJ (2000). Cooperative signals governing ARF-Mdm2 interaction and nucleolar localization of the complex. *Mol Cell Biol* 20:2517–2528.
- Weinberg RA (1995). The retinoblastoma protein and cell cycle control. *Cell* 81:323–330.
- Zhang Y and Xiong Y (1999). Mutations in human *ARF* exon 2 disrupt its nucleolar localization and impair its ability to block nuclear export of MDM2 and p53. *Mol Cell* 3:579–591.
- Zhang Y, Xiong Y, and Yarbrough WG (1998). *ARF-INK4a* locus deletion impairs both the RB and p53 tumor suppression pathways. *Cell* 92:725–734.