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Correlations of partial and extensive methylation at the p14^{ARF} locus with reduced mRNA expression in colorectal cancer cell lines and clinicopathological features in primary tumors

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p14^{ARF} is a putative tumor suppressor gene thought to modify the levels of p53. CpG sites within the 5′-flanking region and exon 1β of p14^{ARF} are targets of aberrant methylation and transcriptional silencing in human colorectal cancer (CRC). Here we have developed methylation-specific polymerase chain reaction (MSPCR) methods to detect methylation of CpG sites in p14^{ARF} in CRC cell lines and primary CRC tumors, and correlated p14^{ARF} mRNA expression with methylation in CRC cell lines using competitive quantitative reverse transcription–polymerase chain reaction methods. Ten CRC cell lines were studied; three (DLD-1, HCT15 and SW48) showed extensive methylation and six (Colo320, SW480, HT29, Caco2, SW837 and WiDr) were unmethylated; the other cell line, LoVo, showed partial methylation that affected exon 1β but not the immediate upstream CpG sites. p14^{ARF} mRNA was expressed at extremely low levels in fully methylated cell lines and at 10^4- to 10^5-fold higher levels in unmethylated cell lines. p14^{ARF} expression in the partially methylated LoVo cell line was intermediate. Treatment of LoVo cells with 2 μM 5-aza-2′-deoxycytidine for 72 h was associated with marked (100-fold) induction of mRNA levels. Of 119 primary CRCs, 18% contained p14^{ARF} methylation, although partial methylation was the most common pattern observed (in 67% of methylated tumors). Methylation of p14*ARF* was often accompanied by p16^{INK4a} methylation; however, 50% of p14^{ARF} methylated tumors contained unmethylated p16^{INK4a}. Methylation at p14^{ARF} was associated with female gender, greater age, proximal anatomic location and poor differentiation, but not stage at diagnosis. A two-step MSPCR method for assaying p14^{ARF} methylation in human tumors is described.

Introduction

Aberrant methylation is now considered an important epigenetic alteration in colorectal cancer (CRC); examples of loci affected by methylation and transcriptional silencing include the DNA repair gene*s hMLH1* (1–3) and *MGMT* (4) and the cyclin D/cdk regulator p16^{INK4a} (5,6) on chromosome 9p21. In a previous study, we used a multiplex methylation-specific polymerase chain reaction (MSPCR) method based on primers designed by Herman *et al*. (7) to detect p16^{INK4a} methylation. We reported that 18% of a consecutive case series of sporadic CRCs contained methylation of the 5′-flanking region of p16^{INK4a} and observed associations of methylation with clinicopathological features and patient gender (8). Also residing on chromosome 9p21 is p14^{ARF} (alternative reading frame; p19^{ARF} in mice) gene, which shares a portion of the p16^{INK4a} coding region (i.e. exon 2) but has a unique promoter and first exon (exon 1β) located ~20 kb upstream of p16^{INK4a} (9,10). Mice deficient in p19^{ARF} are susceptible to a variety of spontaneous and induced neoplasms (11,12). In addition, p14^{ARF} is frequently deleted in human cancers; thus, the *ARF* locus is considered a potential human tumor suppressor (13). p19^{ARF} has recently been ascribed a role in modifying the levels of p53 by interacting with MDM2 and inhibiting MDM2-mediated p53 degradation via the ubiquitin/proteosome pathway (14). Wild-type p53 itself down-regulates p19^{ARF} expression, indicating a 'self-regulating feedback' loop between the two components. Further, p19^{ARF} has not been shown to affect p53 levels after DNA damage but is required for induction of p53 in response to hyperproliferative signals such as c-Myc, E1A and Ras (15–17). Our aim was to develop MSPCR methods to detect p14^{ARF} methylation in human tumor specimens. To maximize efficiency we explored multiplex MSPCR. During the course of these experiments we encountered an anomalous PCR result with our multiplex MSPCR method in the LoVo cell line that led to the discovery of a pattern of methylation characterized by 'partial' methylation within the untranslated region of exon 1β of p14^{ARF}. This partial methylation pattern was associated with reduced levels of p14^{ARF} mRNA and represents the predominant pattern of p14^{ARF} methylation in primary sporadic CRCs.

Abbreviations: ARF, alternative reading frame; AZA, 5-aza-2′-deoxycytidine; CRC, colorectal cancer; MSPCR, methylation-specific polymerase chain reaction; RT–PCR, reverse transcription–polymerase chain reaction.

Materials and methods

Cell lines and DNA/RNA isolation

Human CRC cell lines were obtained from the American Type Culture Collection (ATCC) and were cultured according to conditions recommended by the ATCC. DNA was isolated by standard methods including proteinase K, RNase, chloroform–isoamyl alcohol extraction and ethanol precipitation. DNA was quantified by Hoescht 33258 fluorimetry (Hoefer Scientific Instruments, San Francisco, CA). Total RNA was isolated using RNAzol B (BiotecX, Houston, TX); mRNA was then isolated using Qiagen (Valencia, CA) Oligotex spin column protocols. cDNA was prepared using MMLV reverse transcriptase and random hexamer primers (BRL, Gaithersburg, MD) according to the manufacturer's directions. A 360 bp fragment containing a distal segment of exon 1β and a portion of exon 2 (bp +173 to +533; accession no. U38945) was amplified using the following primers: sense, 5′-TTCT-TGGTGACCCTCCGGATT-3′; antisense, 5′-CAGGCATCG-

CGCACGTCCAGC-3′. All chemical reagents were HPLC grade.

Primary colorectal tumors

Tumor specimens were obtained from a consecutive case series of resected CRCs as previously described (8). Briefly, tumors were obtained from patients (aged 35–90 years) with sporadic CRC undergoing surgery at the University of Barcelona Hospital Clinic. Primary tumors were surgically dissected and immediately frozen at -80°C. Non-tumorous colon tissue was also collected. Patients provided signed informed consent; all procedures were approved by the Hospital Clinic's institutional review board. Information was collected on patient characteristics (age and gender) and tumor characteristics (stage at diagnosis, anatomical location and degree of differentiation).

MSPCR

Methylated CpG sites within the 5′ region of the $p14^{ARF}$ gene were detected using MSPCR methods. For all assays, 1.0 µg of purified DNA was diluted in 36 µl H_2O, to which was added 4 µl of 3.0 M NaOH and the DNA denatured at 37°C for 15 min. The samples were then treated with 416 µl of 3.6 M sodium bisulfite solution (pH 5.0) and 24 µl of 10 mM hydroquinone. Both bisulfite and hydroquinone solutions were prepared fresh for each analysis. Samples were incubated at 55°C for 16 h; 100 µl of mineral oil was layered on top of the solution to prevent evaporation. After incubation, the solution was cooled to –80°C for 10 min, after which the unfrozen mineral oil was removed without disturbing the bisulfite–DNA solution.

Bisulfite-modified DNA was purified with the Wizard DNA Clean-Up System and Vacuum Manifold (Promega, Madison, WI) according to the manufacturer's instructions. DNA was eluted with a total volume of 30 µl TE buffer (pH 7.8). The final step of the cytosine→uracil conversion reaction was achieved by treatment with alkali (NaOH; final concentration 0.3 M) at 37°C for 15 min followed by neutralization with ammonium acetate (pH 7.0; final concentration 3.0 M) and ethanol precipitation. Human peripheral blood lymphocyte DNA treated with methylase *Sss*I (New England BioLabs, Beverly, MA) was used as a positive control.

For each cell line and tumor specimen, both monoplex and multiplex PCR amplification of bisulfite-treated DNA was carried out with primers (BRL) specific for methylated (M-primer) and unmethylated (U-primer) CpG sites within the $p14^{ARF}$ promoter. Previous studies with $p16^{INK4a}$ showed complete concordance of multiplex and monoplex results. A region including ~150 bp upstream and downstream of the $p14^{ARF}$ transcription initiation site was chosen for assay development. For $p16^{INK4a}$ and hMLH1, primers were used as previously described (3,6).

The PCR mixture contained GeneAmp PCR buffer (Perkin-Elmer Corp., Foster City, CA), 1.5 mM $MgCl_2$, dimethylsulfoxide (DMSO; 5% final concentration), 200 µM of each deoxynucleotide triphosphate), 0.4 µM of each primer, 50 ng modified DNA templates and 2.5 U of AmpliTaq with TaqStart antibody (Clontech, Palo Alto, CA) in a total volume of 50 µl. The PCR reaction was repeated for 35 cycles on a GeneAmp 9600 thermal cycler (Perkin-Elmer Corp.) under the following conditions: preheat at 94°C for 2 min, 94°C for 30 s, 65°C for 10 s, 72°C for 30 s and a final extension at 72°C for 10 min. Aliquots (15 µl) of PCR products were loaded on to 2.5% agarose gels stained with ethidium bromide and visualized under UV light.

To assess the sensitivity of the MSPCR method, methylated and unmethylated cell line DNA samples were mixed in different ratios; a single unambiguous $p14^{ARF}$ methylated band was detectable when methylated template was present at >1:32 (3%) of the total DNA. All analyses were carried out on coded samples without knowledge of the cell lines' $p14^{ARF}$ expression status or of patients' clinical status.

DNA cloning and sequencing

After bisulfite treatments and methylation-specific amplification, PCR products were gel purified (Qiagen) and ligated into the PCR 2.1-TOPO plasmid vector using the TA cloning system (Invitrogen, Carlsbad, CA). Plasmid-transformed *Escherichia coli* were cultured overnight and plasmid DNA was isolated (Qiagen). Purified plasmid DNA containing $p14^{ARF}$ sequence was sequenced in both directions using an ABI 373 automated sequencer with dye primer chemistry and standard M13 primers.

Quantitative competitive PCR and $p14^{ARF}$ expression

Expression of $p14^{ARF}$ mRNA in CRC cell lines was measured by quantitative competitive PCR as previously described (18). In this method, target cDNA is amplified in the presence of a known concentration of a competitor sequence that has the same primer binding sites and the same amplification efficiency as the target sequence. By serial dilution of the competitor, the equivalence point (i.e. the point at which the ratio of target to competitor is 1:1) is determined. At the equivalence point, the amount of target cDNA can be quantified. Equal amounts of total cDNA were added to each competitive PCR reaction by normalizing cDNA with the concentration of *HPRT* cDNA; *HPRT* cDNA levels were estimated in each sample by competitive PCR as described (19). PCR conditions were identical to those for MSPCR except in this instance 5% DMSO was not added. The $p14^{ARF}$ competitor amplicon was constructed to contain primer-binding sites that span exons 1β and 2 of the $p14^{ARF}$ cDNA, and to contain an 80 bp insert to make the competitor and target amplicons distinguishable by agarose gel electrophoresis. The 80 bp fragment was inserted at the unique *Xma*I restriction site within $p14^{ARF}$ exon 2. The number of copies of $p14^{ARF}$ cDNA present at the equivalence point was calculated using the competitor DNA concentration and dilution factor at the equivalence point, and the molecular weight of the competitor. Visual inspection followed by UV densitometry was used to carry out the serial dilutions and to measure equivalence of PCR bands. For 5-aza-2′-deoxycytidine (AZA) treatments, cells were passaged and, 24 h later, treated with freshly prepared 2 µM AZA for 72 h; they were then harvested for RNA isolation.

Results

Monoplex and multiplex MSPCR

In our initial experiments, we designed two sets of methylation-sensitive primers (M-primers and U-primers) to amplify CpG-rich regions that are 5′ flanking to exon 1β and within the initial portion of exon 1β (Figure 1). The primers were designed so that the unmethylated product would share a common 5′ location but the 3′ U-primer would bind downstream of the 3′ M-primer (i.e. primers A/D and B/C) (Figure 1). This combination produces methylated and unmethylated PCR products of different sizes in multiplex reactions containing both M- and U-primers. We compared monoplex and multiplex amplifications using bisulfite-treated DNA isolated from nor-

(b)

Primer Sequences for methylation specific PCR

	Sequences
Primer A	AGGGAAGGTGGGTGTGTGTT
Primer B	GGAAGGCGGGTGCGCGTT
Primer C	CTCGACAACCGCTACGCCG
Primer D	ACCACACACACACCAAATCCA
Primer E	CGCGACGAACCGCACGCGCG

Fig. 1. (**a**) Sequence of the p14^{ARF} 5′ flanking region and exon 1β with CpG sites numbered for reference to sequencing results. ▽, Start of exon 1β of p14*ARF*. ▼, Start of translation of p14^{ARF}. Open arrows indicate the position of primers specific for unmethylated DNA and closed arrows indicate the position of primers specific for methylated DNA. (**b**) Sequences of primers A–E.

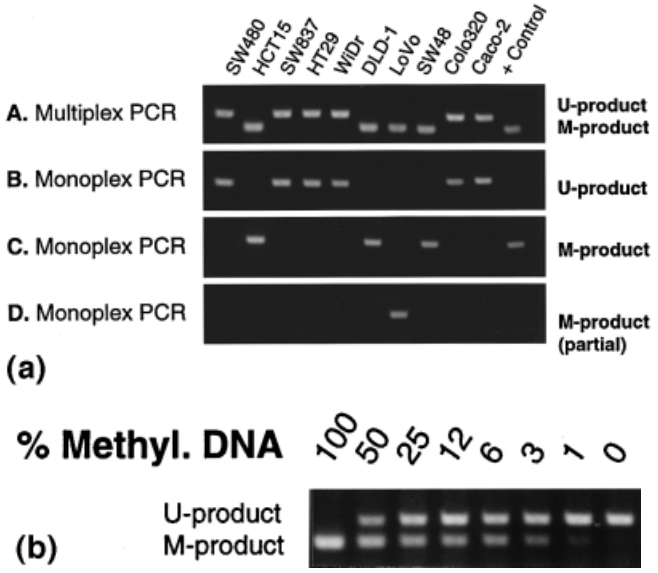

Fig. 2. (a) Multiplex and monoplex PCR of p14^{ARF} methylation in CRC cell lines. Row A: multiplex results with primers A/D and B/C; the upper band is the unmethylated product and the lower band the methylated product. Rows B and C: monoplex MSPCR reactions with primers A/D (row B) or B/C (row C). Note the concordance of results between multiplex and monoplex results for all cell lines except LoVo. Row D: result of monoplex MSPCR using partial methylation MSPCR primers A/C; these primers only amplified a product in DNA from LoVo cells. (**b**) Multiplex MSPCR amplification of serially diluted lymphocyte DNA treated with methylase *Sss*I. A methylated band was detectable at 3% methylated template. Unmethylated lymphocyte DNA was added and the total amount of template kept constant at 50 ng/reaction.

mal lymphocyte control, methylase-treated and 10 different CRC cell lines. Figure 2a shows the results of monoplex (M or U) and multiplex (M and U) reactions using primer pairs A/D (U-primers) and B/C (M-primers). For each cell line except LoVo, a single band was detected in multiplex or monoplex reactions; in SW480, SW837, HT29, WiDr, Colo320 and Caco2 the band was amplified only with U-primers, and in HCT15, DLD-1, SW48 it was amplified only with M-primers. In multiplex reactions LoVo produced a methylated product, but not in monoplex PCR reactions with M-primers only. As shown in Figure 2a, row D, monoplex PCR with the U- and M-primer pair amplified a product in LoVo but not in any other cell line. Also shown in Figure 2b is a sensitivity analysis indicating that methylation within the region can be detected when the methylated template is present at >3% of total template. The most striking result of these experiments is the discordant results when LoVo cells were examined by MSPCR in the monoplex versus multiplex format. The target sequence was amplified poorly from LoVo cells with either monoplex M- or U-primers but a very robust methylation band was produced when both sets of primers were used. Our results with monoplex PCR using a 5′ U-primer and 3′ M-primer (primers A/C; Figure 2a, row D) are consistent with the formation of a hybrid product formed in the multiplex reaction. This explanation would require that the sequence chosen for multiplex PCR amplification would span a boundary region that was unmethylated at one end and methylated at the other.

Bisulfite sequencing

We next amplified the putative boundary region with primers downstream of the previous M-primers (i.e. using primer A/E) (Figure 1), an area that contains 15 CpG sites and the previous M-primer annealing site of primer C. Sequencing results are shown for multiple clones of LoVo in Table I. Primers A and E in Figure 1 were used to amplify the region. CpG positions are indicated relative to the transcription start site. Nine independent clones were sequenced; CpG sites in the 5′-flanking region of p14^{ARF} were unmethylated but sites +32 and +35 were extensively methylated. These latter sites lie within the methylated primer for the multiplex reaction and occur within the untranslated region of exon 1β. These sites correspond to CpG sites 6 and 7 in Figure 1. The results confirm the MSPCR data indicating that the anomalous multiplex product arose by the U-primer annealing upstream and the M-primer annealing downstream. Also consistent with this interpretation was the fact that the reverse combination of primers [i.e. 5′ M-primer (B) and 3′ U-primer (D)] never produced a PCR product in any cell line. Extensive methylation of all 15 CpG sites in the region was confirmed by sequencing in SW48 and DLD-1 cells and in the methylase-treated positive control, whereas no CpG site was methylated in Colo320 cells (data not shown). The partial pattern of methylation was also found in another cell line, HCT116. Because the sequencing primers were designed to be sensitive to the methylation status of individual CpG sites, it should be noted that these sequencing studies and those described in primary tumors do not give an unbiased picture of the distribution of CpG sites in this region. Instead they were carried out to confirm the interpretation of the multiplex result and provide evidence for the existence of the two patterns of methylation (i.e. partial and extensive).

p14^{ARF} mRNA expression

The expression of p14^{ARF} was estimated for each cell line and the relative concentrations of p14^{ARF} cDNA are shown in

Table I. Bisulfite sequencing of cloned PCR products from the LoVo cell line

Clone	CpG position														
	1/–59	2/–54	3/–37	4/–32	5/+6	6/+32	7/+35	8/+40	9/+47	10/+53	11/+65	12/+68	13/+84	14/+102	15/+108
1	–	–	–	–	+	+	+	nd	+	+	+	+	–	+	+
2	–	–	–	–	–	+	+	nd	+	–	+	+	+	+	+
3	–	–	–	–	–	+	+	nd	–	+	+	+	+	+	+
4	–	–	–	–	–	+	+	nd	–	–	+	+	+	+	+
5	–	–	–	–	–	+	+	–	–	–	–	–	–	+	+
6	–	–	+	–	+	+	+	+	+	–	+	–	+	+	+
7	–	–	–	–	–	+	–	+	–	–	+	+	+	+	+
8	–	+	–	–	+	+	+	–	–	–	+	+	+	+	+
9	–	–	–	–	+	+	+	–	–	–	+	–	–	–	–
% methylation	0	11	11	0	44	100	89	40	33	22	89	67	67	89	89

Primers A and E in Figure 1 were used to amplify the region. CpG positions are indicated relative to the transcription start site and percentage methylation based on 9 independent clones. Note that positions +32 and +35 lie within the methylated primer for the multiplex reaction and occur within the untranslated region of exon 1β. These sites correspond to CpG sites 6 and 7 in Figure 1.and, not determined.

Table II. Methylation-specific PCR of $p14^{ARF}$, $p16^{INK4a}$, hMLH1 and $p14^{ARF}$ expression in CRC cell lines

Cell line	$p14^{ARF}$ status		Methylation status[b]	
	methylation status	mRNA expression[a]	$p16^{INK4a}$	hMLH1
DLD-1	methylated	0.0001×10^4 (1)	methylated	unmethylated
HCT 15	methylated	0.0005×10^4 (5)	methylated	unmethylated
SW 48	methylated	0.023×10^4 (230)	methylated	methylated
LoVo	methylated (partial)	0.26×10^4 (2600)	methylated	unmethylated
Colo 320	unmethylated	1.0×10^4	methylated	unmethylated
SW 480	unmethylated	3.4×10^4	methylated	unmethylated
HT 29	unmethylated	10.0×10^4	methylated	methylated/unmethylated
CaCo2	unmethylated	11.9×10^4	methylated	unmethylated
SW 837	unmethylated	12.0×10^4	methylated	unmethylated
WiDr	unmethylated	45.0×10^4	methylated	methylated/unmethylated

[a] Relative expression of mRNA normalized to the lowest expressing cell line (DLD-1) using quantitative competitive RT-PCR. See Materials and methods for details.
[b] MSPCR results indicating PCR amplification with methylated or unmethylated primers. 'Methylated/unmethylated' denotes amplification by both M and U primers.

Table II and Figure 3a. Expression was normalized to the cell line with the lowest level of expression (i.e. DLD-1), which was assigned an arbitrary value of 1 unit of $p14^{ARF}$ cDNA. Methylated cell lines had very low levels of $p14^{ARF}$ expression (1–230 relative units; mean, 79), whereas unmethylated cell lines expressed an average of 1760-fold higher levels (i.e. mean = 139 000). LoVo cells expressed intermediate levels of $p14^{ARF}$, with 33-fold higher levels than in methylated cell lines and 54-fold lower levels than in unmethylated cell lines.

To confirm that the intermediate and low mRNA levels of $p14^{ARF}$ in LoVo cells were associated with CpG methylation, LoVo cells were cultured for 72 h in the presence of AZA. $p14^{ARF}$ levels were induced 100-fold (26×10^4 relative units) by AZA in LoVo cells (Figure 3b). The methylated cell line DLD-1 also showed a 10 000-fold increase in $p14^{ARF}$ transcripts after AZA treatment but still expressed much lower levels of $p14^{ARF}$ than LoVo or unmethylated cell lines (1×10^4 units). Expression of $p14^{ARF}$ in the unmethylated cell line SW837 was unaffected by the AZA treatment (10.5×10^4 units, or a 15% decrease).

Prevalence of $p14^{ARF}$ methylation in primary tumors

A total of 119 primary cancers were assayed using five different MSPCR protocols: monoplex for extensive methylation, multiplex, monoplex with 5′ U-primer and 3′ M-primer specific for the partial methylation, and monoplex with 5′ M-primer and 3′ U-primer (Figure 4). No PCR amplification was detected in any primary tumor using the 5′ M-primer and 3′ U-primer combination. Similarly, this combination of primers failed to amplify product in all CRC cell lines. Twenty-one tumors were methylated in multiplex reactions; however, monoplex reactions showed that only seven of these were extensively methylated. Most methylated primary tumors were found to contain the partial pattern of methylation (Table III). In addition, methylation was associated with increased age, female gender, proximal location and poor differentiation. In Figure 5 we present a graphical representation of these clinicopathological correlations; we calculated the Kendall correlation coefficient to test for trends in $p14^{ARF}$ methylation (i.e. unmethylated, partial methylation or extensive methylation) with each of the patient variables. The Kendall correlation is a non-parametric approach used here to test for trends in methylation with selected patient characteristics. A significant correlation implies that the numerical value of a variable in patients with partially methylated tumors is intermediate between the values for patients with unmethylated and extensively methylated tumors. As is evident from Figure 5, a significant Kendall correlation was observed for the proportion

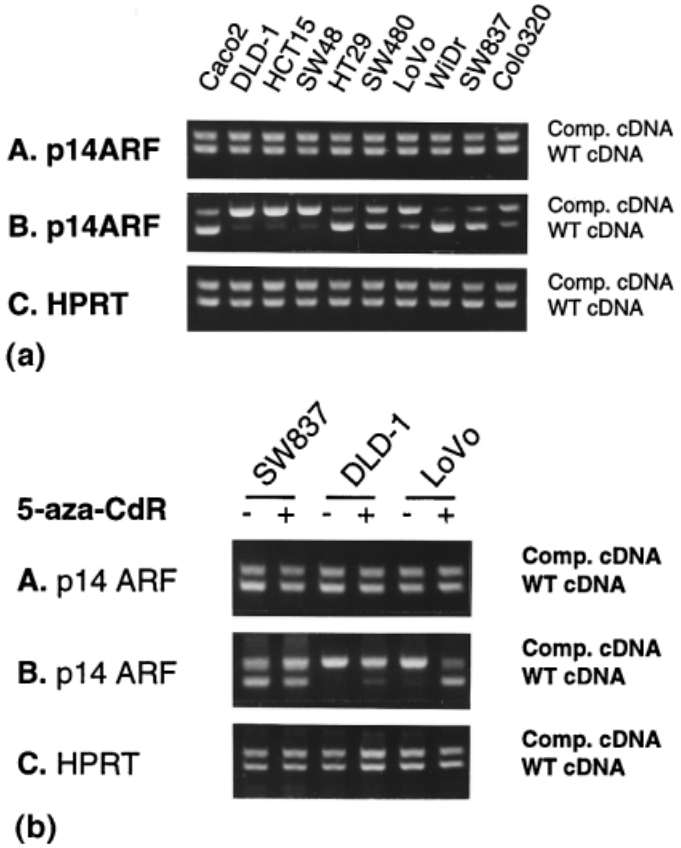

Fig. 3. (**a**) Quantitative competitive PCR analysis of p14^{ARF} expression in CRC cell lines. Competitor products contain an 80 bp insert and are seen as the upper (higher molecular weight) band. Row A: PCR results at the equivalence point, following dilution of the competitor DNA to a level where p14^{ARF} cDNA and competitor bands are of equal intensity. Row B: relative intensity of competitor and target (p14^{ARF}) before serial dilution of the competitor. Note the weak or undetectable wild-type p14^{ARF} cDNA bands in the methylated cell lines DLD-1, HCT15 and SW48 and the stronger bands in the unmethylated cell lines Caco2, HT29, SW480, WiDr and SW837. Row C: normalization of total cDNA by dilution to a standard HPRT competitor concentration. (**b**) Reactivation of p14^{ARF} after 72 h treatment with 2 μM AZA. Row A: results of competitive PCR at the equivalence point after dilution of the competitor. Row B: results of competitive PCR at equal total cDNA levels before dilution of the competitor; note the marked increase in intensity of the wild-type cDNA band after AZA treatment in LoVo cells and lack of change in the unmethylated cell line SW837. Before dilution of the competitor there appears to be no detectable increase in expression following AZA treatment in DLD-1; however, an ~10 000-fold lower dilution factor was required to achieve the equivalence point (row A), indicating a marked reactivation of p14^{ARF} in DLD-1.

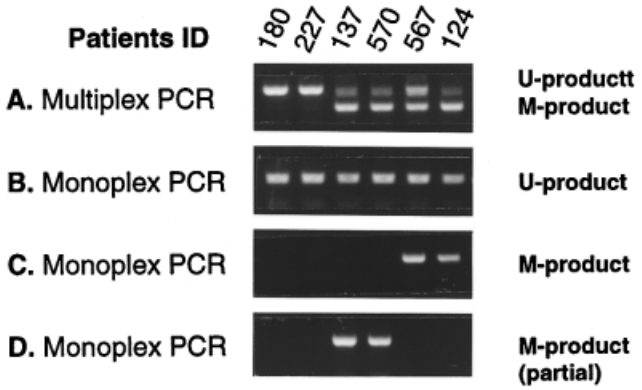

Fig. 4. MSPCR of p14^{ARF} in primary colorectal tumors. The results from six different tumors using four MSPCR protocols are shown. Row A: results of multiplex MSPCR using the A/D and B/C primer combination. Rows B and C: monoplex MSPCR reactions using primers A/D or B/C, respectively. Row D: results with the partial methylation primers A/C. Tumors 180 and 227 are unmethylated, 567 and 124 are methylated, and 137 and 570 are partially methylated. See Figure 1 for primer sequences.

of female patients, increased age and proximal location but not for stage or differentiation. Hence, these data suggest an intermediate phenotype (age, gender and anatomical location) associated with the partial pattern of methylation. It is also clear from the data that extensive but not partial methylation is associated with poor differentiation. This is reflected in the statistically significant association of extensive methylation and differentiation in Table III but a non-significant Kendall correlation in Figure 5. Several tumors demonstrating methylation and one unmethylated tumor were sequenced using the A and E sequencing primers. The partial methylation by MSPCR was confirmed by sequencing and was similar to the pattern observed in LoVo cells. All CRC tumor specimens contained unmethylated products, which probably represent non-tumorous elements in the surgical specimen. Sequencing unmethylated products yielded unmethylated CpG sites. In non-tumorous colon, p14^{ARF} methylation was never detected. We also found the partial pattern of methylation in a second CRC cell line, HCT116. Thus, LoVo is not the only cell line exhibiting this pattern of p14^{ARF} methylation.

Among the 119 primary tumors there was a significant correlation of p14^{ARF} and p16^{INK4a} methylation (χ^2 =16.8; $P < 0.001$ for any p14^{ARF} methylation); the association between p16^{INK4a} methylation and partial p14^{ARF} methylation was much less pronounced (χ^2= 4.2; $P = 0.04$). Only five (36%) of 14 tumors with partially methylated p14^{ARF} contained p16^{INK4a} methylation compared with six of seven extensively methylated tumors. Overall, 11 of 22 (50%) of p16^{INK4a} methylated tumors were also methylated at p14^{INK4a}. Conversely, 11 of 98 (11%) of p14^{ARF} unmethylated tumors contained p16^{INK4a} methylation.

Discussion

In the present study, we evaluated several MSPCR protocols for detecting CpG methylation associated with down-regulation of the p14^{ARF} gene in CRC cell lines. Our results indicate two patterns of CpG methylation within the region we examined. CRC cell lines displaying very low levels of p14^{ARF} mRNA showed extensive methylation throughout the 5′ flanking region near exon 1β, whereas LoVo cells, which display 33-fold higher levels of mRNA expression, contain a partially methylated CpG island that spans the exon 1β transcription site. Our sequencing results indicate that CpG sites immediately upstream of the transcription start site are unmethylated in partially methylated DNA from LoVo cells. MSPCR analysis using a 5′ U-primer and a 3′ M-primer readily detected this unique pattern. We never observed the opposite pattern (i.e. 5′-methylated and 3′ unmethylated) in any cell line or primary tumor. It is of interest that the two methylated CpG sites positioned within the 3′ M-primer are in the untranslated region of exon 1β; these sites were previously reported to be located within a potential binding site for members of the E2F family of transcription factors. In addition, the p14^{ARF} promoter was found to be highly responsive to E2F1 expression (20). Our results showing a 100-fold induction of p14^{ARF} mRNA levels in LoVo cells after AZA treatment are consistent with this site being a functioning promoter element in p14^{ARF}. This hypothesis will

Table III. Prevalence of p14^{ARF} methylation by patient gender and age, and anatomical location, stage and differentiation of tumor

Variable	p14^{ARF} methylation status			
	no methylation	partial methylation	extensive methylation	total methylation (partial+extensive)
Gender				
male	54 (90.0)	5 (8.3)	1 (1.7)	6 (10.0)
female	44 (74.6)	9 (15.2)	6 (10.2)[a]	15 (25.4)[b]
total	98 (82.4)	14 (11.8)	7 (5.8)	21 (17.6)
Age (years)	69.9 ± 11.8	74.4 ± 10.0	76.9 ± 8.2	75.2 ± 9.3[c]
Location				
proximal	29 (69)	7 (16.7)	6 (14.3)	13 (31.0)
distal	69 (89.6)	7 (9.1)	1 (1.3)[d]	8 (10.4)[e]
Stage				
Duke's A, B	44 (86.3)	5 (9.8)	2 (3.9)	7 (13.7)
Duke's C, D	54 (79.4)	9 (13.2)	5 (7.4)	14 (20.6)
Differentiation				
poor	8 (66.7)	1 (8.3)	3 (25.0)[f]	4 (33.3)
moderate/good	83 (83.8)	12 (12.1)	4 (4.1)	16 (16.2)

The footnotes indicate statistically significant differences in univariate comparisons; the numbers in parentheses are row percentages.
[a] Fisher's exact test; $P = 0.04$.
[b] χ^2-test; $P = 0.03$.
[c] Student's *t*-test; $P = 0.05$.
[d] χ^2-test; $P = 0.005$.
[e] χ^2-test; $P = 0.005$.
[f] χ^2-test; $P = 0.03$.

require further study, however, because CpG sites downstream of the translation start signal were also heavily methylated. Generally, CpG methylation within coding regions of exons is thought to be permissive in transcriptional regulation (21). In addition, a previous study using restriction digestion and Southern blots indicated that further upstream sites in the 5′-flanking region of LoVo cells were also methylated.

It is now thought that p16^{INK4a} and p14^{ARF} are regulated independently. Our finding that all cell lines contained p16^{INK4a} methylation, but only four of the 10 were methylated at p14^{ARF} is consistent with this idea. Our data showing discordance between p16^{INK4a} and p14^{ARF} methylation in primary tumors also confirms the independence of methylation events within the respective promoters of these two genes. Our MSPCR results were also consistent with the previously reported methylated and unmethylated alleles within hMLH1 in HT29; the score methylation pattern was detected in WiDr, which is derived from HT29 (22). SW48 also showed methylation of the hMLH1. Among the unmethylated cell lines, we observed a 45-fold variation in p14^{ARF} expression levels. The significance of this variation is unknown. The unmethylated cell lines showing the lowest p14*ARF* expression were Colo320, SW480 and HT29. Other researchers have shown that c-*myc* is amplified 35-, 4- and 1.8-fold in Colo320, SW480 and HT29, respectively (23). No other cell lines in our series are known to contain *c*-myc amplification. Expression of p14^{ARF} may be reduced in cell lines containing c-*myc* amplification through the action of *trans*-acting factors. For example, down-regulation of p14^{ARF} by the transcriptional repressor *bmi-1* has recently been shown to be involved in cell immortalization by c-*myc* (24). Further investigation of the variations in p14^{ARF} expression we have observed may provide clues concerning methylation-independent mechanisms modifying p14^{ARF} expression.

To examine whether the two patterns of methylation found in established CRC cell lines were detectable in primary human tumors, we applied MSPCR methods to a series of 119 primary sporadic CRCs. About 18% of CRCs showed methylation using multiplex MSPCR. Of the methylated tumors, however, the majority of tumors (14/21; 67%) showed the partial methylation pattern observed in the LoVo cell line. Interestingly, we observed significant trends between the three patterns of methylation (unmethylated, partially methylated, extensively methylated) and female gender, proximal tumor location and older age. These data indicate that partial methylation, which showed intermediate *in vitro* mRNA expression in cell lines, may be associated with an intermediate clinical phenotype *in vivo*. In contrast, the degree of tumor differentiation was inversely associated with extensive but not partial methylation. This latter result suggests that the more complete silencing of p14^{ARF} expression by extensive methylation compared with partial methylation may be required to promote the poorly differentiated tumor growth pattern.

The associations of p14^{ARF} methylation with female gender and proximal tumor location are similar to our previous studies of p16^{INK4a} in this series of patients (8). The clustering of both of these methylation events within CRCs arising in the proximal colon is consistent with the hypothesis that there exists a hypermethylation phenotype for a subset of CRCs. Our data, however, also show that the methylation status of 50% of CRCs were discordant at the two loci. Thus, our findings emphasize that even among CRC exhibiting hypermethylation, different genetic pathways can be targeted in individual cases. Further research is needed to elucidate the mechanisms that give rise to the specific constellation of genes affected by aberrant methylation. The significance of these differences in methylation patterns with respect to the etiology of CRC, or their potential to improve diagnosis or prognosis, also remains to be determined.

It is evident that extensive methylation of p14^{ARF} is not common in sporadic CRC. Similarly, p14^{ARF} is seldom extensively methylated in bladder cancer (25). However, the partial methylation pattern we found associated with relatively low levels of p14^{ARF} in the LoVo cell line appears relatively

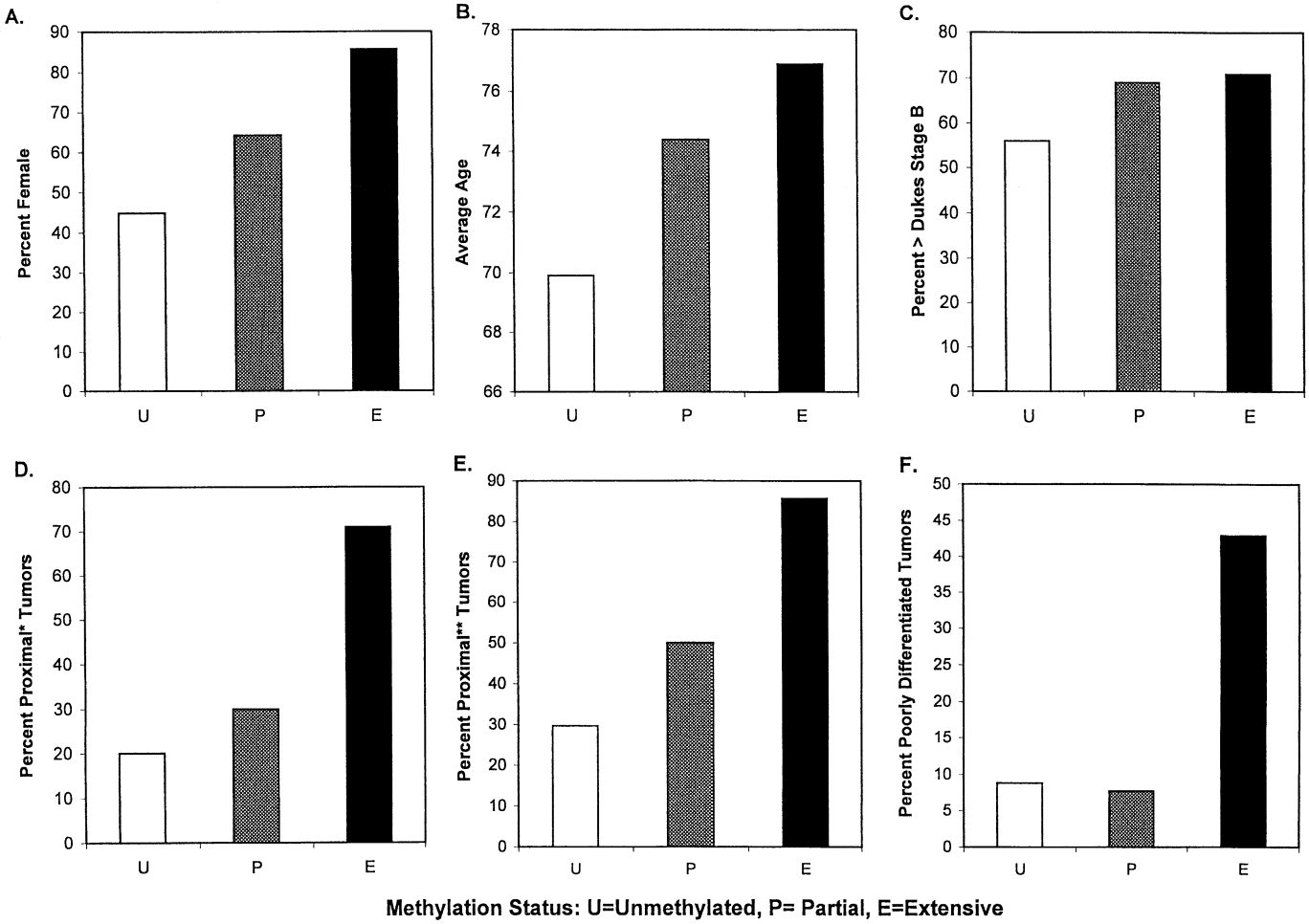

Fig. 5. Selected patient characteristics displayed according to the methylation status of the patient's tumor (unmethylated, partially methylated or extensively methylated). Kendall's correlation was used to test for trends between methylation status and patient characteristics. (**A**) Significant correlation of methylation status with female gender (τ coefficient 0.21; $P = 0.02$); (**B**) marginally significant association with age at diagnosis (τ coefficient 0.14; $P = 0.06$); (**C**) non-significant association with advanced stage cancer (i.e. greater than Duke's stage B) (τ coefficient 0.08; $P = 0.34$); (**D**) significant association with proximal anatomic location (τ coefficient 0.27; $P = 0.003$) in which proximal includes the cecum and the ascending and transverse segments of the colon; (**E**) significant association of proximal anatomic location (τ coefficient 0.25; $P = 0.006$) in which proximal includes the cecum and the ascending, transverse and descending segments of the colon; (**F**) non-significant trend for methylation and percentage of tumors displaying poor differentiation. Analyses included 119 patients except for differentiation, which only included 111, because of missing data.

common in CRC (i.e. 12%). Both extensive and partial methylation can be detected by three PCR reactions; for example, a multiplex MSPCR to identify methylated tumors, followed by monoplex PCR reactions on methylated tumors using the 5′ U-/3′ M-primer or 5′ M-/3′ M-primer combinations to confirm the presence of the partial and extensive methylation products, respectively. Because p14^{ARF} is not methylated in most tumors, screening with this approach involves about half the number of PCR reactions compared with a monoplex scheme.

LoVo cells, which show partial CpG methylation and intermediate expression of p14^{ARF}, carry a wild-type p53 gene, as do the more heavily methylated SW48 and DLD-1. All of the unmethylated cell lines studied contain mutant p53. Only HCT15 is methylated and has been reported to carry a p53 mutation (i.e. codon 241 TCC→TTC transition) (26). It is not known whether the intermediate expression of p14^{ARF} observed in LoVo cells or the related partially methylated p14^{ARF} in primary CRCs affects the levels or function of p53.

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