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Advances in Brief

p14^{ARF} Silencing by Promoter Hypermethylation Mediates Abnormal Intracellular Localization of MDM2[1]

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Abstract

The *INK4a/ARF* locus encodes two distinct tumor suppressors, *p16*^{INK4a} and *p14*^{ARF}. Although the contribution of *p16*^{INK4a} to human tumorigenesis through point mutation, deletion, and hypermethylation has been widely documented, little is known about specific *p14*^{ARF} lesions and their consequences. Recent data indicate that *p14*^{ARF} suffers inactivation by promoter hypermethylation in colorectal cancer cells. Because it is known that p14^{ARF} prevents MDM2 nucleocytoplasmic shuttling and thus stabilizes p53 by attenuating MDM2-mediated degradation, we studied the relationship of *p14*^{ARF} epigenetic silencing to the expression and localization of MDM2 and p53. Cancer cell lines with an unmethylated *p14*^{ARF} promoter showed strong nuclear expression of MDM2, whereas in a colorectal cell line with *p14*^{ARF} hypermethylation-associated inactivation, MDM2 protein was also seen in the cytosol. Treatment with the demethylating agent 5-aza-2′-deoxycytidine was able to reinternalize MDM2 to the nucleus, and p53 expression was restored. No apparent changes in retinoblastoma localization were observed. We also studied the profile of *p14*^{ARF} promoter hypermethylation in an extensive collection of 559 human primary tumors of different cell types, observing that in colorectal, gastric, renal, esophageal, and endometrial neoplasms and gliomas, aberrant methylation of *p14*^{ARF} was a relatively common epigenetic event. MDM2 expression patterns revealed that lack of *p14*^{ARF} promoter hypermethylation was associated with tumors showing exclusive nuclear MDM2 staining, whereas MDM2 cytosolic staining was frequently observed in neoplasms with aberrant *p14*^{ARF} methylation. Taken together, these data support that epigenetic silencing of *p14*^{ARF} by promoter hypermethylation is a key mechanism in the disturbance of the MDM2 nuclear localization in human cancer.

Introduction

In a normal cell, microanatomical localization of a protein determines, many times, how it works. For example, the election between nuclear or cytosolic localization is tightly regulated. However, in cancer cells, this organized system often fails. The recent case of the oncoprotein MDM2 is an excellent example. MDM2 binds to and abrogates the tumor suppressor function of p53 by either targeting p53 for degradation in the cytoplasm or by repressing p53-mediated transcriptional activity in the nucleus (1). MDM2 at the same time is also under stringent regulation by the putative tumor suppressor *p14*^{ARF}. The p14^{ARF} protein is encoded by the *INK4a/ARF* locus on chromosome 9p21, a region with a high rate of loss of heterozygosity in human cancer. *p14*^{ARF} has a separate first exon (exon 1B) that splices into common exons 2 and 3, shared with the tumor suppressor gene *p16*^{INK4a} in a different reading frame, resulting in a protein bearing no amino acid sequence similarity to p16^{INK4a} (2). Showing its contribution to the tumorigenic process, the *p14*^{ARF} null mice (where only the exon 1B is lost) develop spontaneous tumors at an early age (3). p14^{ARF} physically interacts with MDM2, inducing stabilization of p53 (4–7). Furthermore, recent elegant experiments indicate that the mechanism of MDM2-mediated regulation by p14^{ARF} involves intracellular compartmentalization (8–10). p14^{ARF} would stabilize p53 by sequestering MDM2 from the cytosol and confining it to the nucleus, mainly in the nucleolus (8–10).

Despite this important function for *p14*^{ARF} in tumorigenesis, *p14*^{ARF} specific genetic lesions in human cancer are very rare. The homozygous deletions and the mutations at the exon 2 at the *INK4a/ARF* locus also affect *p16*^{INK4a}. Mutations in *p14*^{ARF} exon 2, as expected, impair the delicate nucleocytoplasmic shuttling of MDM2 (10). However, no spontaneous mutations in the unique exon 1B of *p14*^{ARF} have been described to our knowledge. An alternative and rapidly growing area of study for gene inactivation involves epigenetic mechanisms. Transcriptional silencing by promoter hypermethylation has been demonstrated affecting bona fide tumor suppressor genes and DNA repair genes including *p16*^{INK4a}, *VHL*, *BRCA1,* and *hMLH1* (11, 12). In this field, we and others (13, 14) have demonstrated that the promoter region of *p14*^{ARF} possesses a CpG island that undergoes dense hypermethylation in some colorectal cancer lines (13, 14) and primary tumors (14, 15). *In vitro* treatment with the demethylating drug 5-aza-2-deoxycytidine is able to restore p14^{ARF} expression (13, 14). The cellular functional consequences of *p14*^{ARF} epigenetic inactivation, however, remains unknown. In this study, we demonstrate that in cancer cells the methylation-mediated silencing of *p14*^{ARF} plays a central role in MDM2 localization.

Materials and Methods

Samples, Cell Lines, and Culture Conditions. The primary tumor samples used in this study were obtained at the time of the surgery from the Johns Hopkins Hospital and The Greenbaum Cancer Center in Baltimore and the Hospitals Sant Pau and Duran y Reynals in Barcelona as described previously (16, 17). The Ethics Committee in each institution approved specimen collection procedures. The colorectal cancer cell lines Colo 205, LoVo, SW480, HT29, and DLD1 used in this study were obtained from the American Type Culture Collection. Cell lines were maintained in appropriate medium, and LoVo cells were treated with the demethylating agent 5-aza-2′-deoxycytidine (Sigma) at a concentration of 1 μm for 3–5 days to achieve demethylation as described previously (14).

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Analysis of $p14^{ARF}$ Promoter Methylation Status. DNA methylation patterns in the CpG island of the $p14^{ARF}$ gene were determined by methylation-specific PCR (14). Methylation-specific PCR distinguishes unmethylated from methylated alleles in a given gene based on sequence changes produced after bisulfite treatment of DNA, which converts unmethylated, but not methylated, cytosines to uracil, and subsequent PCR using primers designed for either methylated or unmethylated DNA (18). The primer sequences designed for $p14^{ARF}$ spanned six CpGs within the 5′ region of the gene. Primer sequences of $p14^{ARF}$ for the unmethylated reaction were 5′-TTT TTG GTG TTA AAG GGT GGT GTA GT-3′ (sense) and 5′-CAC AAA AAC CCT CAC TCA CAA CAA-3′ (antisense), which amplify a 132-bp product; and for the methylated reaction, 5′-GTG TTA AAG GGC GGC GTA GC-3′ (sense) and 5′-AAA ACC CTC ACT CGC GAC GA-3′ (antisense), which amplify a 122-bp product. The 5′ position of the sense unmethylated and methylated primers corresponds to bp 195 and 201 of GenBank sequence no. L41934. Both antisense primers originate from bp 303 of this sequence. The annealing temperature for both the unmethylated and methylated reactions was 60°C. Placental DNA treated *in vitro* with *Sss*I methyltransferase was used as a positive control for methylated alleles. DNA from normal lymphocytes was used as negative control for methylated genes. Ten μl of each PCR reaction was directly loaded onto nondenaturing 6% polyacrylamide gels, stained with ethidium bromide, and visualized under UV illumination.

RNA and Protein Analysis. Reverse transcription (RT)-PCR was performed as described previously (14), using 3 μg of total cellular RNA to generate cDNA. One hundred ng of this cDNA were amplified by PCR with primers for exon 1β (5′-GGT TTT CGT GGT TCA CAT CCC GCG-3′) and exon 2 (5′-CAG GAA GCC CTC CCG GGC AGC-3′) of $p14^{ARF}$, which amplify a 254-bp product spanning sequence 204–437 from GenBank S78535. RT-PCR for GAPDH served as a positive control. Ten μl of each PCR reaction were directly loaded onto nondenaturing 6% polyacrylamide gels, stained with ethidium bromide, and visualized under UV illumination. Cell lysates for protein analysis were prepared and analyzed by Western blotting by using three anti-p14^{ARF} antibodies, Ab-1 (rabbit polyclonal antibody IgG; Neomarkers, Fremont, CA), Ab-2 (mouse monoclonal antibody IgG; Neomarkers), and C-18 (goat polyclonal IgG; Santa Cruz Biotechnology, Santa Cruz, CA). Equal loading was tested by reprobing with a polyclonal antibody against human topoisomerase I (Topogen, Inc., Columbus, OH). The gels were cast using the XCell SureLock Mini-Cell system (Invitrogen Corp./NOVEX, Carlsbad, CA) and developed using ECL immunodetection reagents (Amersham Pharmacia Biotech, Piscataway, NJ).

Immunostaining. Immunoperoxidase staining of deparaffinized sections was performed as described previously (19). Briefly, deparaffinized sections were treated with 1% H_2O_2 to block endogenous peroxidase activity, subsequently immersed in boiling 0.01% citric acid in a microwave oven to enhance antigen retrieval, allowed to cool, and incubated with 10% normal horse serum to block nonspecific tissue immunoreactivities. Primary antibodies were incubated overnight at 4°C. Biotinylated horse antimouse IgG antibodies (Vector Laboratories, Burlingame, CA; 1:500 dilution) were applied for 30 min, followed by avidin-biotin-peroxidase complexes (Vector Laboratories; dilution, 1:25). Diaminobenzidine was used as the final chromogen. Cytospins of the cells were prepared using a Cytospin 2 (Shandon, Cheshire, United Kingdom) at 1400 rpm for 4 min and fixed in acetone:methanol for 1 min and stained using a similar protocol as described above. A panel of well-characterized antibodies were used, which included mouse monoclonal antibodies to MDM2 (clone 2A10, to the human MDM2 product; kindly supplied by Dr. A. Levine, Rockefeller University, New York, NY; dilution, 1:500), p53 (clone DO-7, to the human p53 product; DAKO Corp., Carpinteria, CA), and Rb[3] (clone 1F8, to the human Rb product; Neomarkers). Staining was evaluated in coded slides by three authors (C.C-C., K. S. P., and D. N. W.), who had no knowledge of the results of the molecular analyses.

Results

Methylation and Expression Status of $p14^{ARF}$ and Localization of MDM2 in Cultured Tumors. To address first how $p14^{ARF}$ aberrant methylation affects the patterns of MDM2 expression, we analyzed the $p14^{ARF}$ methylation and expression status of several colorectal cell lines and the corresponding MDM2 protein localization. Methylation-specific PCR analysis showed that the colorectal cancer cell lines Colo205, SW837, and SW480 were unmethylated at the $p14^{ARF}$ CpG island, whereas LoVo was fully methylated at this locus (Fig. 1). Concordant with this analysis, $p14^{ARF}$ expression at the mRNA level was abundant in the unmethylated cell lines Colo205, SW837, and SW480, but no $p14^{ARF}$ transcript was detected in LoVo, a *p14ARF* hypermethylated cell line, as described previously (data not shown; Ref. 14). Western blot analysis corroborated this data showing undetectable p14^{ARF} protein in LoVo cells, whereas the unmethylated cell line SW480 demonstrated abundant p14^{ARF} expression (Fig. 1). When LoVo cells were treated with the demethylating agent 5-aza-2-deoxycytidine, reexpression of $p14^{ARF}$ was observed at the transcript and protein levels (Fig. 1), coincident with the appearance of unmethylated alleles (Fig. 1).

These cell lines were then cytospinned, fixed in histological slides, and studied for MDM2 protein status by immunostaining. The $p14^{ARF}$ unmethylated and expressing cell lines Colo205, SW837, and SW480 demonstrated exclusive nuclear staining as shown in Fig. 2*a*. However, LoVo cells ($p14^{ARF}$ hypermethylated and nonexpressor), in addition to the nuclear staining, showed strong cytosolic staining (Fig. 2*a*). This would be the pattern expected if $p14^{ARF}$ function was impaired; MDM2 cannot be sequestered in the nucleus and may "leak" to the cytosol. A careful examination of the MDM2 protein staining revealed that the nuclear staining in the $p14^{ARF}$ unmethylated cell lines is compatible with a nucleolar localization. Interestingly, the nuclear staining that remained in the methylated cell line LoVo is

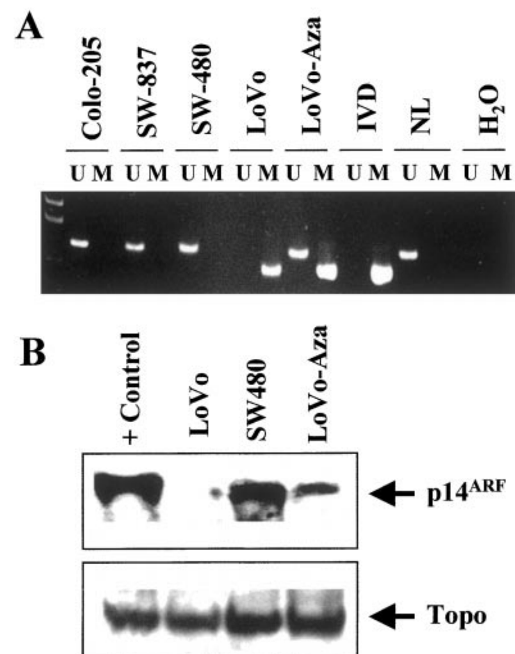

Fig. 1. *A*, methylation-specific PCR of the $p14^{ARF}$ promoter in colorectal cancer cell lines. The presence of a visible PCR product in those lanes marked *U* indicates the presence of unmethylated genes of $p14^{ARF}$; the presence of product in those lanes marked *M* indicates the presence of methylated genes. *In vitro* methylated DNA (*IVD*) was used as positive control for $p14^{ARF}$ promoter hypermethylation and normal lymphocytes (*NL*) as negative control for methylation. Water controls for PCR reactions are also shown. Colo205, SW837, and SW480 are unmethylated at the $p14^{ARF}$ promoter, whereas LoVo cells demonstrate fully $p14^{ARF}$ promoter hypermethylation. After the use of the demethylating drug 5-aza-2′-deoxycytidine, the appearance of $p14^{ARF}$ unmethylated alleles is evident. *B*, analysis of p14^{ARF} protein expression by Western blot. A positive control for the p14^{ARF} protein is shown in the first lane. The unmethylated cell line SW480 expresses abundant levels of the p14^{ARF} protein, whereas the fully methylated cell line LoVo demonstrates absence of p14^{ARF} expression. Treatment of the LoVo cell line with 5-aza-2′-deoxycytidine restores p14^{ARF} expression.

[3] The abbreviation used is: Rb, retinoblastoma.

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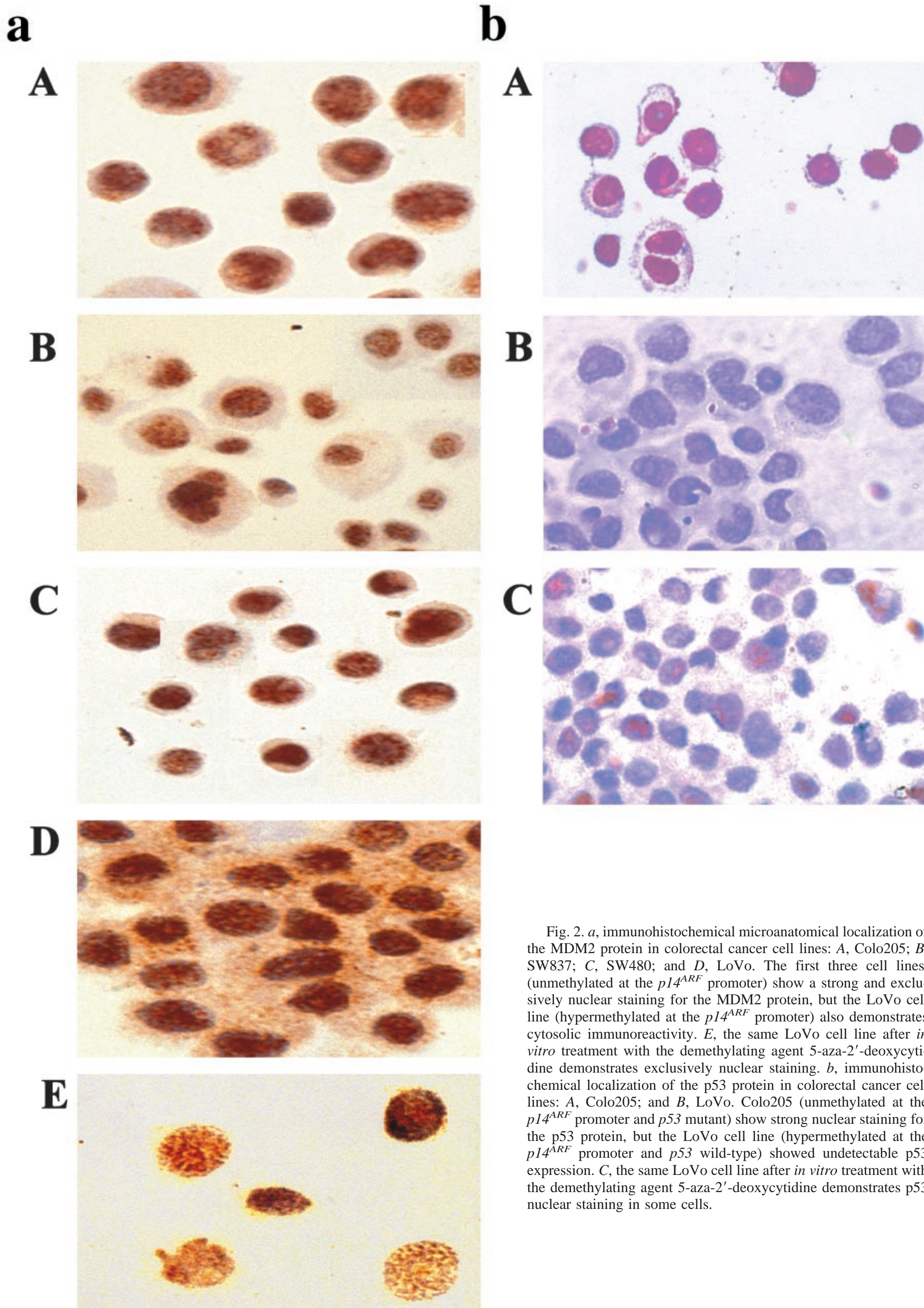

Fig. 2. *a*, immunohistochemical microanatomical localization of the MDM2 protein in colorectal cancer cell lines: *A*, Colo205; *B*, SW837; *C*, SW480; and *D*, LoVo. The first three cell lines, (unmethylated at the $p14^{ARF}$ promoter) show a strong and exclusively nuclear staining for the MDM2 protein, but the LoVo cell line (hypermethylated at the $p14^{ARF}$ promoter) also demonstrates cytosolic immunoreactivity. *E*, the same LoVo cell line after *in vitro* treatment with the demethylating agent 5-aza-2′-deoxycytidine demonstrates exclusively nuclear staining. *b*, immunohistochemical localization of the p53 protein in colorectal cancer cell lines: *A*, Colo205; and *B*, LoVo. Colo205 (unmethylated at the $p14^{ARF}$ promoter and *p53* mutant) show strong nuclear staining for the p53 protein, but the LoVo cell line (hypermethylated at the $p14^{ARF}$ promoter and *p53* wild-type) showed undetectable p53 expression. *C*, the same LoVo cell line after *in vitro* treatment with the demethylating agent 5-aza-2′-deoxycytidine demonstrates p53 nuclear staining in some cells.

more homogeneously distributed in the nucleus, suggesting a nucleoplasmic nonnucleolar localization.

Functional Consequences of Restoring $p14^{ARF}$ Expression. Unlike mutational inactivation, epigenetic changes offer the possibility of reversibility. For other genes, such as the mismatch repair gene *hMLH1* and the apoptotic gene *DAPK*, cell treatment with demethylating agents not only restores gene expression but also gene function in those respective cases where DNA repair activity and IFN-γ induced apoptosis (20, 21). We examined the MDM2 pattern of expression in the LoVo cell line after 5-aza-2-deoxycytidine treat-

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ment. LoVo cells treated with a demethylating agent resulted in MDM2 cytosolic staining no longer being observed, and the only demonstrable MDM2 staining was limited to the nucleus (Fig. 2*a*). Furthermore, its pattern is clearly suggestive of a nucleolar localization. Thus, these data support that the reexpression of $p14^{ARF}$ was able to reinternalize the "leaked" cytosolic MDM2 to the nucleus.

We next examined whether the reexpression of $p14^{ARF}$ and the resultant return of MDM2 to the nucleus affect targets downstream in this pathway. We studied the expression and localization of two described MDM2 interactors, p53 and Rb (22, 23). We observed recently that in cancer cell lines with a mutant p53, the $p14^{ARF}$ promoter remains unmethylated (14), following the reasoning that only one "hit" in the same pathway is necessary. Thus, in the cell lines SW480, Colo205, and SW837, which all harbor a mutant *p53* gene, a strong nuclear p53 expression was observed (Fig. 2*b*). In addition, in LoVo, a cell line with wild-type *p53* and a methylated $p14^{ARF}$ promoter, p53 expression was not detectable (Fig. 2*b*). However, after $p14^{ARF}$ expression was induced by the treatment with the demethylating drug, not only MDM2 was now normally confined in the nucleus as described above, and some nuclear p53 expression was detectable, consistent with the $p14^{ARF}$ function of triggering p53 stabilization (6, 24, 25). In contrast, no difference in Rb expression or localization was observed in these cell lines independently of their $p14^{ARF}$ methylation status, and no change was observed upon reexpression of $p14^{ARF}$ after the treatment with 5-aza-2′-deoxycytidine (data not shown).

Methylation Status of $p14^{ARF}$ and Localization of MDM2 in Primary Human Tumors. The above data indicating that epigenetic silencing of $p14^{ARF}$ disrupts *MDM2* function impelled us to examine the prevalence of the $p14^{ARF}$ promoter hypermethylation alteration in human cancer. Previously, this question has only been addressed in primary colorectal and gastric tumors (14, 15). The study of an extensive panel of 559 human primary tumors revealed that $p14^{ARF}$ aberrant methylation has a tumor type-specific pattern. $p14^{ARF}$ promoter hypermethylation was particularly common in gastrointestinal tumors, being found in 28% of colorectal, 26% of gastric, and 22% of esophageal tumors (Table 1). It was also found in 15% of endometrial and 13% of renal tumors. However, other tumor types, including lung and ovary, show that *p14ARF* methylation in <5% of cases and other tumor types such as breast, pancreas, or liver are devoid of this epigenetic lesion. No evidence of $p14^{ARF}$ methylation was found in any normal tissue analyzed. Table 1 summarizes all of the data obtained in primary tumors, and Fig. 3 illustrates several examples of the tumors analyzed for $p14^{ARF}$ methylation.

Similar to the study developed in the cell lines, we wondered about the putative relation between $p14^{ARF}$ promoter hypermethylation and MDM2 intracellular distribution in primary neoplasms. Thus, we analyzed, blinded to molecular data concerning the $p14^{ARF}$ methylation status, the localization of the MDM2 protein in histological sections of 45 primary tumors (33 colorectal and 12 endometrial), where the $p14^{ARF}$ methylation status had been studied. Exclusive nuclear staining was observed in the vast majority, 81% (22 of 27) of the tumors unmethylated at $p14^{ARF}$, whereas only 19% (5 of 27) presented both nuclear and cytosolic positivity. In comparison, the tumors with hypermethylation at $p14^{ARF}$ demonstrated a higher rate of cytosolic staining, being present in a 44% of the cases (8 of 18). A representative example of the methylation analysis and the two patterns of MDM2 protein localization observed are illustrated in Fig. 4. Thus, the trend between $p14^{ARF}$ epigenetic inactivation and MDM2 cytosolic localization was present but without the exclusivity demonstrated in the cell lines. We observed a similar phenomena previously when comparing $p14^{ARF}$ methylation and *p53* mutational status. *In vitro*, all of the cell lines hypermethylated at $p14^{ARF}$ are *p53* wild type; in primary tumors, only a trend toward this relation was observed (14).

Table 1 *Patterns of $p14^{ARF}$ promoter hypermethylation in human cancer (n = 559)*

Tumor type	Samples with aberrant methylation
Colorectal	28% (37/132[a])
Gastric	26% (31/118)
Esophageal	22% (8/37)
Endometrial	16% (4/25)
Renal	13% (5/38)
Gliomas	9% (2/22)
Ovarian	5% (1/20)
Lung	5% (1/20)
Leukemia	5% (1/20)
Bladder	5% (1/20)
Head and neck	4% (1/25)
Breast	0% (0/20)
Pancreas	0% (0/20)
Liver	0% (0/20)
Lymphomas	0% (0/22)

[a] 110 of these samples were previously described in Ref. 14.

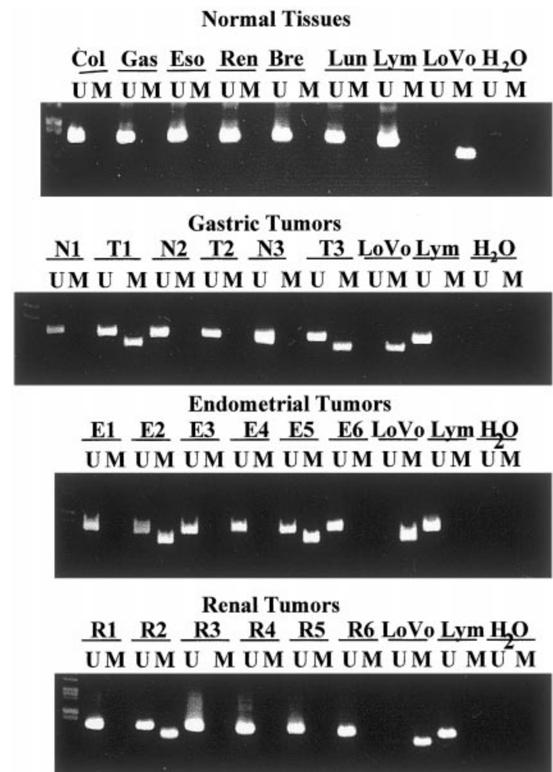

Fig. 3. Methylation-specific PCR of the $p14^{ARF}$ promoter in normal tissues and human primary tumors. The presence of a visible PCR product in those lanes marked *U* indicates the presence of unmethylated genes of $p14^{ARF}$; the presence of product in those lanes marked *M* indicates the presence of methylated genes. *In vitro* methylated DNA (*IVD*) was used as positive control for $p14^{ARF}$ promoter hypermethylation and normal lymphocytes (*NL*) as negative control for methylation. Water controls for PCR reactions are also shown.

Discussion

In human cancer, the frequency that the *INK4a/ARF* locus is disrupted is almost as prevalent as *p53* mutations (26). Loss of heterozygosity, homozygous deletions, and point mutations occur at this site, but a major mechanism for inactivation of both genes is epigenetic silencing by promoter hypermethylation (12, 14). Aberrant methylation of the cell cycle inhibitor $p16^{INK4a}$ in cancer cells has been described extensively, and recently the other occupant of the *INKa/ARF* locus, $p14^{ARF}$, has also been shown to undergo hypermethylation-associated inactivation in cultured cancer cells and primary tumors (13–15). This last alteration is particularly intriguing in view of

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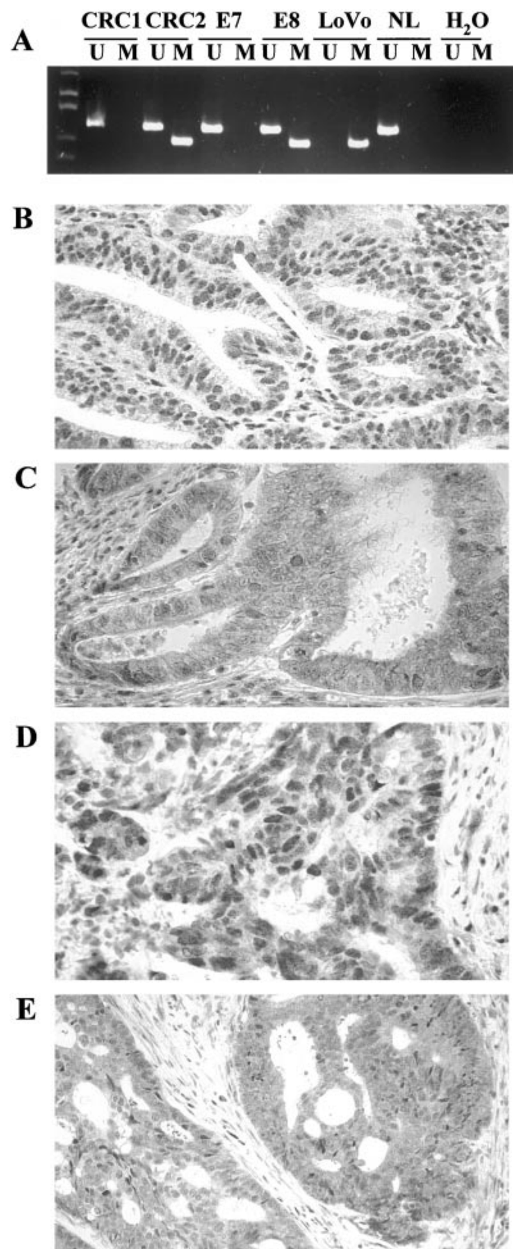

Fig. 4. *A*, methylation-specific PCR of the $p14^{ARF}$ promoter in colorectal and endometrial tumors. The tumors CRC2 (colorectal) and E8 (endometrial) are hypermethylated at the $p14^{ARF}$ promoter, whereas CRC1 (colorectal) and E7 (endometrial) remain unmethylated at this locus. *B–E*, immunostaining of the MDM2 protein in primary tumors; the presence of a positive signal is restricted to the nucleus in the unmethylated tumors CRC1 (*B*) and E7 (*D*), whereas the tumors with aberrant $p14^{ARF}$ methylation show preferentially a cytosolic MDM2 staining, as shown for CRC2 (*C*) and E8 (*E*).

the recently recognized interplay between $p14^{ARF}$ and *MDM2* and because aberrant methylation of $p14^{ARF}$ can have important consequences in the *p53* pathway. Supporting this postulate, we now provide data that the transcriptional inactivation of $p14^{ARF}$ by the methylation of its CpG island has important effects for the compartmentalization of the MDM2 protein in cancer cells.

The tumor suppressor function of $p14^{ARF}$ is thought to be related to binding to MDM2, thereby blocking p53 degradation by inhibiting the E3 ubiquitin-ligase activity associated with MDM2 (27) and preventing nuclear export of MDM2 (8, 10). A proposed step in this process is that a fully functional $p14^{ARF}$ should sequester MDM2 in the nucleolus. $p14^{ARF}$ has nucleolar localization signals and is predominantly localized in nucleoli but also can be noted in the nucleoplasm (28). Consistent with these data, our results show that in colorectal cancer cells with an intact and unmethylated $p14^{ARF}$ gene, MDM2 is confined to the nucleus with a pattern compatible with nucleolar localization. However, if $p14^{ARF}$ expression is shut-down by promoter hypermethylation, MDM2 became predominantly nucleoplasmic and even cytosolic. These data agree with several studies (9, 10, 29) that also show that in the absence of $p14^{ARF}$, MDM2 was localized mainly in the nucleoplasm of the transfected cells, with clear evidence of nucleolar exclusion. In our case, because $p14^{ARF}$ was only silent but genetically intact, and taking advantage of the well-recognized capacity of releasing the epigenetic silencing by the use of the demethylating agent 5-aza-2′-deoxycytidine, we were able to "rescue" $p14^{ARF}$ function. The induced reexpression of $p14^{ARF}$ now relocalized MDM2 to the nucleus in a pattern that closely resembled that observed in the unmethylated cancer cell lines. Similar experiments restoring the function of a gene silenced by methylation has also been done in the past with the mismatch repair gene *hMLH1* and the apoptotic gene *DAPK* (20, 21). Furthermore, the reactivation of $p14^{ARF}$, coincident with the redistribution of MDM2, was able to induce nuclear p53 expression. Supporting our data, the transient transfection with a cDNA encoding $p14^{ARF}$ also causes a nuclear p53 accumulation (30). We did not observe p53 expression in all cells treated with 5-aza-2′-deoxycytidine, most likely because of incomplete demethylation of $p14^{ARF}$ (Fig. 1).

The relation between the $p14^{ARF}$ methylation status and MDM2 localization is not a phenomenon restricted to cancer cell lines. After a screening of more than 500 primary human tumors of different cell types, $p14^{ARF}$ promoter hypermethylation was found as a relatively common event in several neoplasms, including colorectal, gastric, and uterine tumors. The patterns of MDM2 expression in a set of these tumors showed a trend between the presence of $p14^{ARF}$ epigenetic silencing and abnormal localization of MDM2. This association between both events was not as exclusive as we found with the cell lines, but it agrees with other studies that show that $p14^{ARF}$ loss of function, either by epigenetic (14) or genetic (31) mechanisms, is not either restricted to primary tumors with wild-type *p53*. Mouse models also provide similar examples of overlapping between $p14^{ARF}$, *MDM2,* and *p53* defects, such as the presence of some *p53* mutations in the tumors arising in the $p19^{ARF}$ knock-out (3). These emergent data may illustrate the fact that the relation of $p14^{ARF}$, *MDM2,* and *p53* is not always linear. Each component may have other potential partners such as Rb, p300, E2F1, and other p53 family members for MDM2 (26); each protein may receive cross-talk from other cell networks, *i.e.,* Ras regulates *MDM2* and $p19^{ARF}$ (32); and each gene may suffer a wide spectrum of concomitant lesions (epigenetic silencing, gene amplification, homozygous deletion, and point mutation) in a primary tumor, that is more heterogeneous than in a cell line. For example, a primary tumor with $p14^{ARF}$ methylation may also harbor a p53 mutation for the $p14^{ARF}$ functions unrelated to its binding to MDM2 or the MDM2 functions unrelated to its binding to p53. Finally, there are complex feed-back loops, *i.e.,* p53 has been shown to repress the $p14^{ARF}$ promoter (13), MDM2 is itself a p53-responsive gene (33), and MDM2-p53 levels oscillate in a tightly regulated manner (34). Nevertheless, considering the vast amount of literature generated in recent years about the presence of altered expression of MDM2 in a wide variety of human tumors, the loss of $p14^{ARF}$ function by aberrant methylation seems to be a central factor behind the observed abnormalities.

In summary, the data compiled in this current work support an important role for the putative tumor suppressor gene $p14^{ARF}$ in delimiting where the oncoprotein MDM2 is localized in the cell. Furthermore, it shows how the epigenetic silencing of $p14^{ARF}$ by promoter hypermethylation has profound consequences in that proc-

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ess, allowing an aberrant MDM2 compartmentalization in the transformed cell.

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p14ARF Silencing by Promoter Hypermethylation Mediates Abnormal Intracellular Localization of MDM2

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