Hypermethylation-associated Inactivation of the *Cellular Retinol-Binding-Protein 1* Gene in Human Cancer[1]

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ABSTRACT

The effects of retinol (vitamin A) depend on its transport and binding to nuclear receptors. The cellular retinol-binding protein 1 (*CRBP1*) and the retinoic acid receptor $\beta2$ (*RARβ2*) are key components of this process. Loss of *CRBP1* expression occurs in breast tumors, but the mechanism is not known. We examined whether CpG island hypermethylation of *CRBP1* was responsible for its inactivation in cancer cell lines ($n = 36$) and primary tumors ($n = 553$) and its relation to *RARβ2* methylation. Hypermethylation of *CRBP1* was common in tumors and cancer cell lines, with the highest frequency in lymphoma and gastrointestinal carcinomas. Hypermethylation correlated with loss of *CRBP1* mRNA, and *in vitro* treatment with the demethylating agent 5-aza-2'-deoxycytidine reactivated *CRBP1* expression. *CRBP1* methylation appeared in premalignant lesions and frequently occurred with *RARβ2* hypermethylation in the same tumors. Finally, we observed that a higher dietary retinol intake was associated with reduced frequencies of methylation of both genes. Epigenetic disruption of *CRBP1* is a common event in human cancer that may have important implications for cancer prevention and treatment using retinoids.

INTRODUCTION

Retinoids, analogues of vitamin A, are known to control cellular signals involved in cell growth, differentiation, and carcinogenesis (1, 2). Retinoids suppress preneoplastic lesions and prevent the development of second primary cancers (3, 4). 9-*cis*-Retinoic acid inhibits mammary tumors induced by *N*-nitroso-*N*-methylurea (5). However, some tumors show resistance to retinoid action by mechanisms still largely unknown. Retinoid activity is mediated by nuclear hormone receptors. The RARs[3] α, β, and γ and RXRs α, β, and γ act as ligand-activated transcription factors. Disruption of RARs and RXRs results in developmental defects and neoplastic transformation (1, 2). Aberrant retinoid signaling in cancer has been found in the leukemogenic role of the dominant-negative PML-RARα fusion protein in acute promyelocytic leukemia (6) and the demonstration of down-regulation of *RARβ2* expression in solid human carcinomas (7–9) caused by CpG island promoter hypermethylation and loss of heterozygosity (10–13).

Other key components in retinoid activity are the CRBPs. The CRBPs belong to the family of fatty acid-binding proteins, and three members have been described: *CRBP2* and *CRBP3*, which show tissue-specific expression (14, 15); and *CRBP1*, which is expressed widely (16). Retinoic acid (the main active metabolite of retinol) is present in the circulation, but most tissues rely on the uptake and cytosolic metabolism of retinol to activate RARs and RXRs. CRBPs possess a high-affinity binding for retinol, possibly functioning as a chaperone-like molecule to regulate this prenuclear phase of retinol signaling (14). Furthermore, the critical role of *CRBP1* is demonstrated in the *CRBP1* knock-out mice, which are very susceptible to hypovitaminosis A syndrome (17). Recently, *CRBP1* down-regulation in breast cancer cell lines (8) and tumors (18) has been observed. The mechanism behind this inactivation has not been established. We investigated the possible role of CpG island aberrant methylation in *CRBP1* silencing and its relationship to *RARβ2* methylation and dietary retinol intake.

MATERIALS AND METHODS

Cell Lines and Tumor Samples. The cancer cell lines used in this study were MCF-7, MDA-MB-231, MDA-MB-468, T47D, Hs578, MDA-435 (breast); SW48, DLD1, RKO, SW837, HCT-116, SW480, Colo-320, HT29 (colon); H358, H1618, H249, H209, H157, H460, H69, DMS53, A549, H1299, H1752 (lung); DuPro, LnCAP, PC3, Dn145 (prostate); UMSCC1, HN12 (head and neck); D283, DaOY (glioma); Raji (lymphoma); KG1a (leukemia); HeLa (cervix); and T24 (bladder). Cell lines were maintained in appropriate medium and treated with demethylating agent 5-aza-2'-deoxycytidine as described (19). Primary malignancies were obtained at the Johns Hopkins Hospital in Baltimore, MD and the Hospitals Sant Pau and Duran i Reynals in Barcelona, Spain and have been examined for other epigenetic changes (19–21). For 131 colorectal cancer patients, dietary history was obtained according to the following cohort design. These were among the patients consecutively diagnosed and operated at the Hospital Duran i Reynals during 1996 that provided informed consent, biological specimens to extract DNA, and completed the questionnaires. They were interviewed after diagnosis, usually within 15 days before or after surgical removal of the tumor. The interview was carried out by trained personnel using a structured questionnaire to avoid potential recall biases on epidemiological factors and a dietary history based on the average food consumption 1 year previous to the disease (22). Nutrient intakes (retinol and carotene) were estimated using food composition tables. To adjust for total energy intake, nutrient densities were calculated dividing nutrient intake by total kilocalories estimated from macronutrients plus alcohol (23).

Analysis of *CRBP1* and *RARβ2* Promoter Methylation Patterns. DNA methylation patterns in the CpG island of *CRBP1* were determined by methylation-specific PCR (24). Methylation-specific PCR distinguishes unmethylated from methylated alleles in a given gene on the basis of sequence changes produced after bisulfite treatment of DNA, which converts unmethylated, but not methylated, cytosines to uracil and subsequent PCR by use of primers designed for either methylated or unmethylated DNA (24). Primer sequences of *CRBP1* for the unmethylated reaction were 5'-GTGTTGGGAATTTAGT-TGTTGTTGTTTT-3' (sense) and 5'-ACTACCAAAACAACAACTACCA-ATACTACA-3' (antisense); and for the methylated reaction, 5'-TTGG-GAATTTAGTTGTCGTCGTTTC-3' (sense) and 5'-AAACAACGACTAC-CGATACTACGCG-3' (antisense). Primers for *RARβ2* were: unmethylated reaction, 5'-TTGGGATGTTGAGAATGTGAGTGATTT-3' (sense) and 5'-CTTACTCAACCAATCCAACCAAAACAA-3' (antisense); and for the

methylated reaction, 5′-TGTCGAGAACGCGAGCGATTC-3′ (sense) and 5′-CGACCA ATCCAACCGAAACGA-3′ (antisense).

RT-PCR of *CRBP1*. RT-PCR was performed as described previously (18, 19). The PCR primers used were 5′-TTGTGGCCAAACTGGCTCCA-3′ (sense) for exon 1 and 5′-ACACTGGAGCTTGTCTCCGT-3′ (antisense) for exon 3 of *CRBP1*, which amplify a 320-bp product. Glyceraldehyde-3-phosphate dehydrogenase served as a positive control (19).

Statistical Analysis. All comparisons for statistical significance were performed by use of χ^2 or Fisher's exact test, as appropriate, with all *P*s representing two-tailed tests and statistically significant at 0.05. For the cases where dietary information was collected, binary logistic regression models adjusted for age and sex were developed. Retinol and carotene intakes were categorized into quartiles to avoid the effect of extreme values. OR and 95% confidence intervals were calculated for quartiles 2 to 4 compared with the first one. Tests for linear trend on the ORs were calculated using the categorized variable as quantitative.

RESULTS

Promoter Hypermethylation and Expression of *CRBP1* in Normal Tissues and Cancer Cell Lines. *CRBP1* is a candidate for hypermethylation-associated inactivation because a 5′-CpG island is located around the transcription start site. All normal tissues analyzed, including lymphocytes, breast, colon, bone marrow, endometrium, kidney, liver, and lung, were completely unmethylated at the *CRBP1* promoter (Fig. 1*A*). The *CRBP1* CpG island was fully methylated in 17 of 37 cancer cell lines studied: MCF-7, MDA-MB-231, T47D, Hs578, MDA-MB-435, SW48, DLD1, RKO, SW837, DuPro, LnCAP, PC3, Dn145, H358, D283, RAJI, and KG1a (examples in Fig. 1*B*). Two lung cancer cell lines were partially methylated (H1299 and H1752). The remaining 17 cell lines were unmethylated at the *CRBP1* promoter. Expression of the *CRBP1* transcript was assessed by RT-PCR in several cell lines. The cell lines MDA-MB-231, MCF-7, SW837, SW48, and Dn145, hypermethylated at the *CRBP1* promoter, did not express *CRBP1*, whereas MDA-MB-468, normal lymphocytes, and normal colon, unmethylated at the *CRBP* promoter, had strong *CRBP1* expression. The treatment of the methylated cancer cell lines with the demethylating agent 5-aza-2′-deoxycytidine restored the expression of the *CRBP1* transcript (Fig. 1*C*).

Promoter Hypermethylation of *CRBP1* in Human Primary Tumors. To assess the prevalence of promoter hypermethylation of *CRBP1 in vivo*, we examined 553 human primary tumors. The highest incidence of *CRBP1* promoter hypermethylation was found in non-Hodgkin's lymphomas, where 39 of 65 (60%) had *CRBP1* epigenetic inactivation (examples in Fig. 2). Among different histological subtypes of lymphoma, *CRBP1* aberrant methylation was present in 24 of 39 (62%) diffuse, 5 of 9 (56%) follicular, 2 of 2 (100%) Burkitt's, and 8 of 15 (53%) lymphomas of other histologies.

Aberrant CpG island methylation of the *CRBP1* was also common in colorectal (57%; 101 of 177), gastric (42%; 8 of 19), and liver (30%; 10 of 33) tumors, as well as leukemia samples (28%; 15 of 53). Additionally, *CRBP1* hypermethylation was also observed in breast (19%; 9 of 48), bladder (18%; 5 of 27), non-small cell lung (15%; 6 of 41), glioma (14%; 3 of 21), and head and neck (7%; 1 of 15) tumors. No *CRBP1* promoter methylation was observed in endometrial (0 of 33) or ovarian (0 of 21) tumors. The data are summarized in Table 1.

Promoter Hypermethylation and Expression of *CRBP1* in Preneoplastic Lesions. To further address the relevance of *CRBP1* hypermethylation, we examined *CRBP1* methylation in preinvasive lesions of the gastrointestinal tract. *CRBP1* promoter hypermethylation was observed in 21 of 34 (62%) colorectal adenomas (Fig. 3*A*) and 13 of 21 (62%) gastric adenomas. This rate is similar to the percentage found in the invasive colorectal and gastric tumors and suggests that
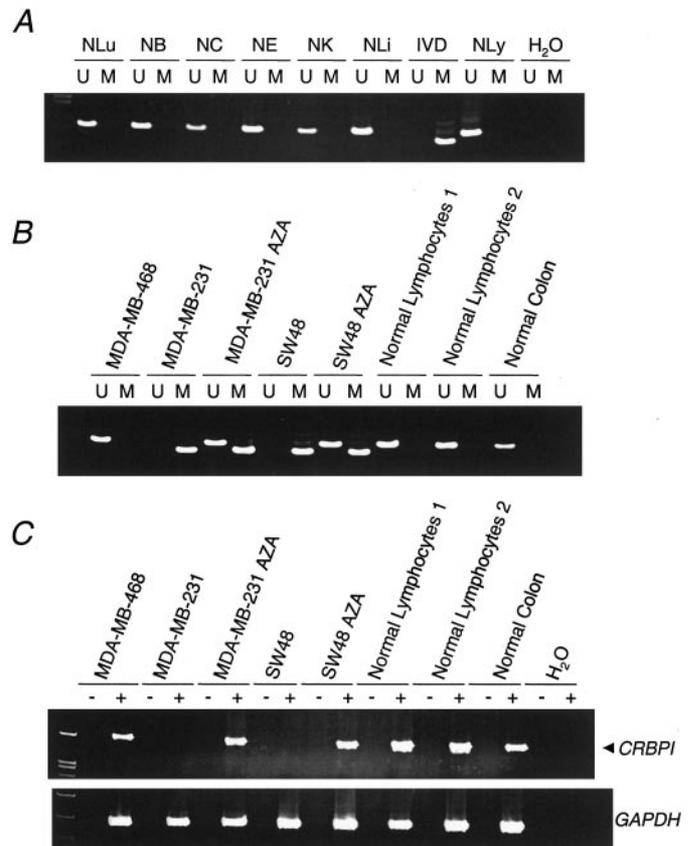

Fig. 1. Analysis of *CRBP1* methylation and expression in normal tissues and cancer cell lines. The presence of a visible PCR product in *Lanes U* indicates the presence of unmethylated genes of *CRBP1*; the presence of product in *Lanes M* indicates the presence of methylated genes. Placental DNA treated *in vitro* with *Sss*I methylase (*IVD*) was used as a positive control for *CRBP1* promoter hypermethylation, and normal lymphocytes (*NL*) were used as negative control for methylation. Water controls for PCR reactions are also shown. *A,* methylation-specific PCR of *CRBP1* in normal tissues: lung (*NLu*), breast (*NB*), colon (*NC*), endometrium (*NE*), kidney (*NK*), and liver (*NLi*). *B,* methylation-specific PCR of *CRBP1* in cancer cell lines. MDA-MB-468 is fully unmethylated at *CRBP1,* and MDA-MB-231 and SW48 are fully methylated. The reappearance of unmethylated alleles is observed in those cell lines treated with the demethylating agent 5-aza-2′-deoxycytidine (MDA-MB-231 AZA and SW 48 AZA). In *C,* the pattern of expression was determined by RT-PCR of the *CRBP1* transcript in cancer cell lines before and after 5-aza-2′-deoxycytidine treatment and in normal lymphocytes and colon. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) expression demonstrates equal amounts of initial mRNA.

methylation-associated inactivation of *CRBP1* is an early event in human tumorigenesis. To confirm that the methylation of this 5′ region of the *CRBP1* gene was functionally relevant, we examined the expression of *CRBP1* using RT-PCR in 8 colorectal adenomas. Five colorectal adenomas without *CRBP1* methylation expressed high levels of *CRBP1* mRNA, whereas all 3 adenomas with *CRBP1* aberrant methylation had undetectable *CRBP1* transcript (Fig. 3*B*).

Relationship of *CRBP1* Aberrant Methylation to *RARβ2* Promoter Hypermethylation. Because alteration of the *RARβ2* gene is common in human malignancies (10–13), we wondered whether any relationship between methylation of *RARβ2* and *CRBP1* existed. Similar to *CRBP1*, we found that hypermethylation of the *RARβ2* promoter was also a common event in colon cancer (54%; 93 of 172) and non-Hodgkin's lymphoma (26%; 11 of 42). The most common group observed was those tumors where both genes were concomitantly methylated, 39% (84 of 214), but closely followed for the category where both were unmethylated, 30% (65 of 214). In 20% (43 of 214) of cases, *CRBP1* was hypermethylated alone, and only in a minority of cases, 10% (22/214), *RARβ2* was hypermethylated alone. No significant differences between colon tumors and lymphomas

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Fig. 2. *CRBP1* promoter hypermethylation in human cancer using methylation-specific PCR. *A*, non-Hodgkin's lymphoma samples (*Ly1* to *Ly 6*). *B*, colorectal carcinomas (*C1* to *C7*). *C*, leukemia samples (*Le1* to *Le6*). *D*, gastric carcinomas (*G1* to *G7*). *In vitro* methylated DNA (*IVD*) was used as a positive control for *CRBP1* promoter hypermethylation, and normal lymphocytes (*NL*) were used as a negative control for methylation. Water controls for PCR reactions are also shown.
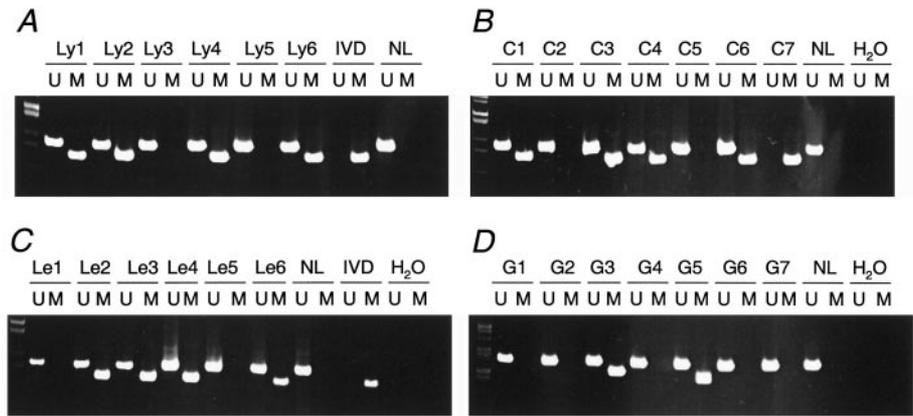
Table 1 *Patterns of CRBPI promoter hypermethylation in human cancer according to the prevalence of the alteration*

Lymphoma	60% (39/65)
Colon	57% (101/177)
Gastric	42% (8/19)
Liver	30% (10/33)
Leukemia	28% (15/53)
Breast	19% (9/48)
Bladder	18% (5/27)
Lung	15% (6/41)
Glioma	14% (3/21)
Head and neck	7% (1/15)
Ovary	0% (0/21)
Endometrial	0% (0/33)
Total: 553 primary tumors	

were observed. Thus, both genes were simultaneously hypermethylated more often than expected by chance (Kappa statistic $P < 0.0001$), although *RARβ2* was methylated more often than *CRBP1* (Exact McNemar test, $P = 0.013$).

Dietary Intake of Retinoids and Its Relation to *CRBP1* and *RARβ2* Methylation Patterns and Colorectal Cancer Risk. For 131 colorectal cancer patients, a dietary history was available as described in "Materials and Methods." Median daily retinol intake in these patients was 155 μg (interquartile range, 102 to 239). These values were used as cutpoints to define quartiles. We found that cases in the highest quartile of retinol intake were more likely to have *CRBP1* and *RARβ2* unmethylated. The ORs were 1.9 (P for linear trend = 0.069) for *CRBP1* and 2.5 (P for linear trend = 0.039) for

RARβ2. Other nutrients analyzed and found unrelated to the methylation status of the genes were vitamin C, E, lycopene, lutein, α-carotene, and β-carotene. The methylation status of *CRBP1* was also unrelated to age, sex, or stage at diagnosis in this sample of subjects.

DISCUSSION

Our data demonstrate that epigenetic disruption of *CRPBI* is a common event in human cancer. The first described alteration in the retinoid pathway was the leukemogenic role of the PML-RARα fusion protein (6). Later, evidence supported the role of *RARβ2* as a tumor suppressor gene: the induction of *RARβ2* related to the chemopreventive effects of retinoids (25), the loss of *RARβ2* expression in human neoplasms (7, 9), frequent chromosomal losses at 3p21–3p24 where *RARb2* is located (26, 27), and the methylation-mediated silencing of *RARβ2* (10, 13). We now provide another piece of this puzzle; the epigenetic silencing of *CRBP1* is also a common alteration in human cancer.

What are the biological consequences of the methylation-mediated silencing of *CRBP1*? The loss of *CRBP1* may compromise retinoic acid metabolism by diminishing retinol transport and blocking the formation of retinyl esters (14, 28). Interestingly, it has also been demonstrated that the lack of retinoic acid function may increase the activity of the β-catenin-LEF/T-cell factor signaling pathway (29), a central element in malignant cell transformation. Our data also show the relatively common simultaneous inactivation of *CRBP1* and *RARβ2*. One explanation is that *CRBP1* may have a function independent of its retinol-binding ability. In this regard, *CRBP1* also functions in mammary epithelial cell inhibition of the phosphatidylinositol 3-kinase/Akt survival pathway and suppresses anchorage-independent growth (30).

In addition, aberrant methylation of *CRBP1* may have predictive value. Until now, the use of retinoids to prevent or treat human cancer has achieved only modest success (31). The disruption of *CRBP1* and *RARβ2* by promoter hypermethylation in many of the human neoplasms may in part explain this resistance. However, in colorectal tumors, ~30% of malignancies did not present any apparent lesion in the retinoid pathway. This subset of tumors may be extremely sensitive to treatment with retinoids.
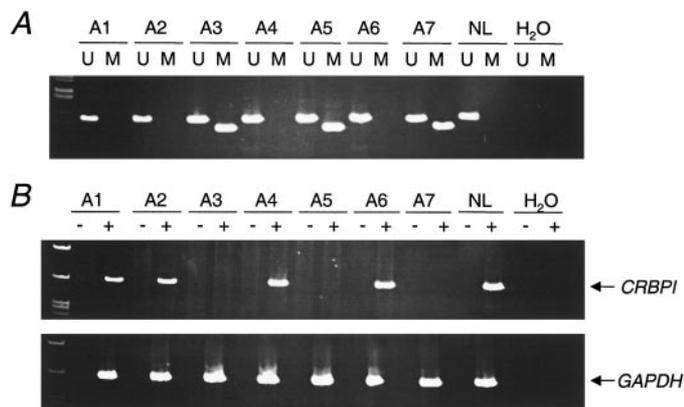

Fig. 3. Analysis of *CRBP1* methylation and expression in premalignant lesions. *A*, methylation-specific PCR of *CRBP1* in colorectal adenomas (*A1* to *A7*). *B*, corresponding pattern of expression of *CRBP1* by RT-PCR in the adenomas shown above. The colorectal adenomas A3, A5, and A7 with *CRBP1* aberrant methylation show loss of expression of the *CRBP1*. Water controls for PCR reaction are also shown.

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