

# Aberrant cellular retinol binding protein 1 (CRBP1) gene expression and promoter methylation in prostate cancer

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**Aims:** Retinoids are involved in cell growth, differentiation, and carcinogenesis. Their effects depend on cytosolic transport and binding to nuclear receptors. CRBP1 encodes a protein involved in this process. Because altered CRBP1 expression and promoter hypermethylation occur in several tumours, these changes were investigated in prostate tumorigenesis.

**Methods:** The CRBP1 promoter was assessed by methylation specific polymerase chain reaction on tissue samples from 36 radical prostatectomy specimens (paired normal tissue, adenocarcinoma, and high grade prostatic intraepithelial neoplasia (HGPIN)), 32 benign prostatic hyperplasias (BPHs), and 13 normal prostate tissue samples from cystoprostatectomies. Methylation of DNA extracted from microdissected tissue was examined blindly. CRBP1 expression was assessed by immunohistochemistry on formalin fixed, paraffin wax embedded tissue.

**Results:** Loss of CRBP1 expression was seen in 15 of 36 adenocarcinomas and 18 of 36 HGPINs. Fifteen adenocarcinomas and nine HGPINs showed overexpression, whereas the remainder showed normal expression. BPH displayed normal expression. No significant associations were found between CRBP1 expression and Gleason score or stage. CRBP1 promoter hypermethylation was found in 17 of 36 adenocarcinomas, three of 35 HGPINs, one of 36 normal prostate tissues from the same patients, none of 32 BPHs, and none of 13 normal prostate tissues from cystoprostatectomies. Loss of expression and hypermethylation of CRBP1 were not significantly associated.

**Conclusions:** Altered CRBP1 expression and hypermethylation are common in prostate carcinoma, although CRBP1 hypermethylation is not an early event in tumorigenesis. Moreover, both adenocarcinoma and HGPIN show frequent CRBP1 overexpression. The molecular mechanisms underlying altered CRBP1 expression in prostate cancer deserve further study.

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Retinoids are structural and functional analogues of vitamin A known to mediate cellular signals promoting differentiation and cell arrest at G1 phase.<sup>1</sup> They are inhibitors of tumorigenesis, can reverse preneoplastic lesions, and may prevent second primary tumours of the upper aerodigestive tract.<sup>2–5</sup> Furthermore, the use of 9-cis-retinoic acid inhibits mammary tumours induced by *N*-nitroso-*N*-methylurea, although other tumours show resistance to the action of retinoids by mechanisms still largely unknown.<sup>6</sup>

“Retinoids have been shown to inhibit prostate cancer cell growth *in vitro* and to suppress prostate carcinogenesis through a signalling pathway that involves both nuclear hormone receptors and cytoplasmic carriers”

The cellular retinol binding proteins (CRBPs) are an important component of retinoid activity, and these proteins are thought to participate in the metabolism of retinoids within the cell by presenting the ligand to specific metabolic enzymes.<sup>7–8</sup> CRBPs are monomeric proteins of approximately 15.5 kDa, belonging to a family of small cytoplasmic proteins that specifically interact with hydrophobic ligands, the intracellular lipid binding proteins. In humans, three of these retinol carriers have been described: CRBP1, which displays wide tissue expression, and CRBP2, and CRBP3, which are expressed in a tissue specific manner.<sup>8</sup> Although, their precise function is complex and not yet fully understood, CRBPs are thought to facilitate the formation of retinyl esters for storage or conversion of retinol to retinoic acid.<sup>9</sup> Indeed, recent studies of knockout mice indicate a crucial role for CRBP1 in maintaining normal hepatic retinol

storage.<sup>10</sup> These studies demonstrated that CRBP1 is needed to facilitate the conversion of retinol to retinyl ester, thus slowing retinol turnover in the liver.<sup>10</sup>

The possible role of altered CRBP1 expression in human carcinogenesis is still largely unknown. However, CRBP1 downregulation has been associated with the malignant phenotype, most notably in breast and ovarian cancer.<sup>9–11–12</sup> Loss of CRBP1 expression was reported in 22% and 27% of invasive and *in situ* breast carcinomas, respectively,<sup>11</sup> whereas in ovarian cancer this proportion reaches 35%.<sup>12</sup> Recently, Esteller *et al* suggested that aberrant methylation at the promoter region may be one of the mechanisms underlying CRBP1 silencing in tumour cell lines and in primary tumours.<sup>13</sup>

Retinoids have been shown to inhibit prostate cancer cell growth *in vitro* and to suppress prostate carcinogenesis through a signalling pathway that involves both nuclear hormone receptors and cytoplasmic carriers.<sup>14</sup> Because the role of CRBP1 in this process has not been previously investigated, we aimed to characterise CRBP1 expression in a series of prostate carcinoma (PCa) lesions and paired high grade prostatic intraepithelial (HGPIN) lesions from patients undergoing radical prostatectomy. Furthermore, CRBP1 promoter methylation status was analysed to investigate a possible association with aberrant gene expression. To the

**Abbreviations:** BPH, benign prostatic hyperplasia; CRBP, cellular retinol binding protein; HGPIN, high grade prostatic intraepithelial neoplasia; LOH, loss of heterozygosity; MSP, methylation specific polymerase chain reaction; PBS, phosphate buffered saline; PCa, prostate carcinoma; PCR, polymerase chain reaction; PIN, prostatic intraepithelial neoplasia; PSA, prostate specific antigen

best of our knowledge no such study has been performed to date.

## MATERIAL AND METHODS

### Patients

Thirty six patients primarily treated with radical prostatectomy at the Portuguese Oncology Institute – Porto, Portugal for clinically localised prostate adenocarcinoma and simultaneously harbouring foci of HGPIN were selected for our study. Each tumour was graded and staged according to the Gleason grading system<sup>15</sup> and the TNM staging system,<sup>16</sup> respectively. For control purposes, morphologically normal tissue was procured from each of the 36 radical prostatectomy specimens and also from 13 prostates collected from cystoprostatectomy specimens from patients with bladder cancer, which did not harbour prostate cancer. Tissue samples were also obtained from 32 randomly selected patients with benign prostatic hyperplasia (BPH), who had undergone transurethral resection of the prostate. A representative slide of PCa, HGPIN, BPH, and normal tissue was selected for immunohistochemical analysis.

### Immunohistochemical analysis

Sections (4 µm) were cut and placed in aminopropyltriethoxysilane (catalogue number A-3648; Sigma, Poole, Dorset, UK) coated slides. Immunostaining was performed using a streptavidin–biotin complex peroxidase method (Dako, Glostrup, Denmark), as described previously by Neuville *et al.*<sup>17</sup> Briefly, after dewaxing the sections, endogenous peroxidase activity was inhibited with freshly prepared 0.5% hydrogen peroxide in distilled water for 20 minutes. Next, the sections were processed in a 600 W microwave oven, at maximum power, three times for five minutes in citrate buffer (pH 6.0). Incubation with the polyclonal goat anti-CRBP1 antibody (clone N-17; Santa Cruz Biotechnology Inc, Santa Cruz, California, USA) was performed overnight at 4°C, at a dilution of 1/50 in 1% bovine serum albumin in phosphate buffered saline (PBS). According to the manufacturer's information, this antibody is specific for CRBP1 of human origin. All of the incubations were performed in a humidified chamber. Sections were developed with a peroxidase substrate solution (0.05% 3,3-diaminobenzidine tetrahydrochloride, 0.01% H<sub>2</sub>O<sub>2</sub> in PBS), counterstained with haematoxylin, dehydrated, and mounted. Appropriate positive controls were used for each antibody and negative controls consisted of the replacement of the primary antibody with 1% bovine serum albumin in PBS.

The assessment of CRBP1 expression was performed by light microscopy at ×400 magnification by two independent observers blinded to clinical and methylation analysis data. The immunostaining was evaluated in morphologically normal areas, HGPIN lesions, adenocarcinoma, and BPH, and was graded and scored by visual assessment of the intensity of the brown reaction product in the areas of interest (0, no immunostaining; 1+, immunostaining similar to normal glands; and 2+, immunostaining stronger than in normal glands).

### Methylation analysis

Snap frozen tissue specimens, corresponding to those analysed by immunohistochemistry, were used for methylation specific polymerase chain reaction (MSP) analysis. Sections (5 µm thick) were cut and stained to identify the areas of HGPIN, adenocarcinoma, normal tissue, and BPH. Then, the tissue block was trimmed to maximise the yield of target cells (> 70% of target cells). Subsequently, an average of 50 × 12 µm thick sections were cut and every fifth section was stained to ensure a uniform proportion of target cells and

to exclude contamination by neoplastic cells in normal and BPH tissue samples.

Genomic DNA was extracted according to the method described by Ahrendt *et al.* In short, DNA was digested overnight at 48°C with proteinase K (0.5 mg/ml) in 1% sodium dodecyl sulfate, Tris (1M, pH 8.8), EDTA (0.5M, pH 8.0), and NaCl (5M), followed by phenol/chloroform extraction (2 vol/1 vol) and ethanol precipitation.<sup>18</sup>

### Bisulfite treatment

Bisulfite treatment of DNA converts unmethylated cytosines to uracil, but the methylated ones remain as cytosines. Sodium bisulfite conversion of 2 µg of genomic DNA was performed by the modification of a previously described method.<sup>19</sup> In brief, DNA was denatured in 0.2M NaOH for 20 minutes at 50°C. A volume of 500 µl of freshly made bisulfite solution (2.5M sodium metabisulfite and 125mM hydroquinone, pH 5.0) was added to each sample and incubation was continued at 50°C for three hours in the dark. Modified DNA was purified using the Wizard DNA purification resin according to the manufacturer (Promega Corp, Madison, Wisconsin, USA) and eluted in 45 µl of water at 80°C. After treatment with NaOH (final concentration 0.3M) for 10 minutes at 37°C, isolation was continued with 75 µl 7.5M ammonium acetate, followed by an incubation step of five minutes at room temperature. Finally, the modified DNA was precipitated by adding 2.5 volumes of 100% ethanol and 2 µl glycogen (5 mg/ml). Each resulting DNA pellet was washed with 70% ethanol, dried, resuspended in 30 µl of 5mM Tris/HCl (pH 8.0), and stored at –20°C.

### CRBP1 promoter methylation status

DNA methylation patterns in the CpG island of the CRBP1 gene promoter were determined by MSP.<sup>20</sup> For polymerase chain reaction (PCR) amplification, 2 µl of bisulfite modified DNA was added in a final volume of 25 µl PCR mix containing 1× PCR buffer (16.6mM ammonium sulfate, 67mM Tris, pH 8.8, 6.7mM MgCl<sub>2</sub>, and 10mM 2-mercaptoethanol), dNTPs (each at 1.25mM), 1 U Hot Start Taq DNA polymerase (Qiagen, Hilden, Germany), and primers (300 ng each reaction). The CRBP1 primer sequences used in our study were those described previously by Esteller *et al.*<sup>13</sup> MSP was carried out using the following conditions: one cycle at 95°C for one minute, followed by 35 cycles of one minute at 95°C, one minute at 62°C, and one minute at 72°C, with a final extension for five minutes at 70°C. Leucocyte DNA collected from healthy individuals was used as a negative control. The same leucocyte DNA was methylated *in vitro* with SssI bacterial methyltransferase (New England Biolabs Inc, Hitchin, Hertfordshire, UK) and used as a positive control for methylated alleles. The PCR products were directly loaded on to a non-denaturing 6% polyacrylamide gel, stained with ethidium bromide, and visualised under ultraviolet illumination.

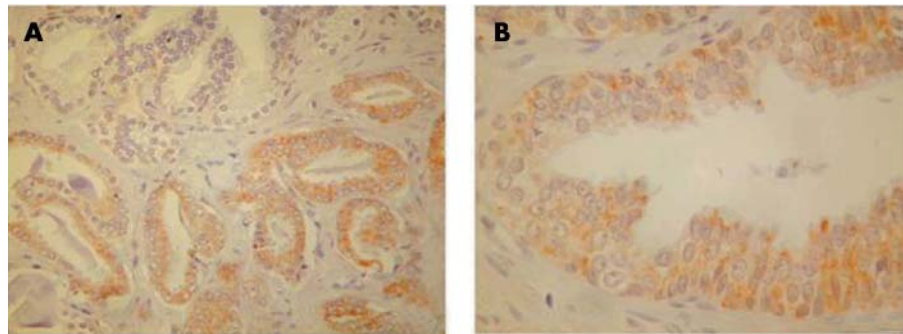
### Statistical analysis

The Mann-Whitney U test was used to compare the serum prostate specific antigen (PSA) concentrations between patients with prostate cancer and BPH. All remaining comparisons for significance were performed by means of the  $\chi^2$  test or Fisher's exact test, as appropriate. All p values represent two tailed tests and were considered significant at 0.05.

## RESULTS

### Clinicopathological data

We studied 36 patients with clinically localised prostate adenocarcinoma with a median age of 64 years (range, 40–74). As control groups, 32 patients with BPH (median age,



**Figure 1** Immunohistochemical expression of CRBP1 in prostate: (A) overexpression in adenocarcinoma, contrasting with low expression in a normal gland (upper left); (B) overexpression in a high grade prostatic intraepithelial neoplasia lesion (streptavidin–biotin complex peroxidase method with haematoxylin counterstain).

68 years; range, 54–80) and 13 patients submitted to cystoprostatectomy for bladder cancer (median age, 61 years; range, 49–80) were included. The median values of pre-operative serum PSA were 8.0 ng/ml (range, 3.35–16.9) and 5.23 ng/ml (range, 0.4–32.5) for patients with cancer and BPH, respectively, and this difference reached significance ( $p = 0.01$ ). The median Gleason score of the prostate adenocarcinomas was 6 (range, 4–9). Of the radical prostatectomy specimens, three cases were staged as pT2a, 17 as pT2b, 13 as pT3a, and three as pT3b.

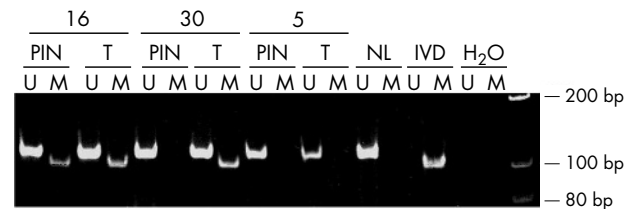
### Immunohistochemical analysis

In all BPHs, the cytoplasmic expression of CRBP1 was similar to that seen in the normal glands (fig 1A).

Fifteen PCA lesions showed loss of CRBP1 expression, whereas of the remaining 21 cases with CRBP1 expression, six showed a similar degree of immunoreactivity to that of normal glands, and 15 showed increased CRBP1 immunoreactivity (fig 1A). No significant association was found between CRBP1 expression and the Gleason score or the pTNM stage (table 1).

With regard to HGPIN, 18 cases showed loss of CRBP1 immunoreactivity, nine cases displayed a similar degree of immunoreactivity to that of normal glands, and nine cases showed increased CRBP1 immunoreactivity (fig 1B).

No differences were noted between HGPIN and PCA regarding CRBP1 expression ( $p = 0.34$ ). Discrepant patterns of CRBP1 expression between paired PCA and HGPIN lesions were seen in eight cases. Specifically, in four cases the PCA displayed CRBP1 overexpression, whereas HGPIN showed loss of expression, in three cases the PCA showed increased expression and HGPIN showed normal expression, and in



**Figure 2** Illustrative example of methylation specific polymerase chain reaction for the CRBP1 promoter region in high grade prostatic intraepithelial neoplasia (PIN) and adenocarcinoma (T). Lanes U and M correspond to unmethylated (109 bp) and methylated (99 bp) reactions, respectively. In patient 16, both lesions are methylated, whereas in patient 5 both are unmethylated. In patient 30, methylated alleles were only detected in the adenocarcinoma. In each case, normal leucocyte DNA was used as a negative control for methylation (NL), DNA from normal leucocytes methylated with an excess of SssI bacterial methyltransferase was used as a positive control for methylation (IVD), and water was used as a negative polymerase chain reaction control ( $H_2O$ ). The HiLo marker is depicted on the right side.

one case the HGPIN lesion overexpressed CRBP1 but in the respective PCA this expression was lacking.

### Methylation analysis

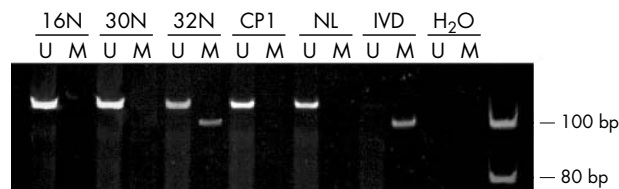
Overall, CRBP1 promoter hypermethylation was found in 17 cases of PCA and three of 35 HGPIN (fig 2). In one HGPIN lesion, the methylation status could not be assessed owing to insufficient DNA. No significant association was found between loss of CRBP1 expression and CRBP1 promoter hypermethylation, either in PCA or HGPIN (table 1). The frequency of CRBP1 methylation in prostate adenocarcinoma was higher than in HGPIN, and this difference reached

**Table 1** Correlation between CRBP1 expression and histopathological parameters and methylation status in prostate adenocarcinoma and HGPIN lesions

	CRBP1 immunoreexpression			p Value
	Increased	Normal	Absent	
Prostate adenocarcinoma (n = 36)				
Gleason score				0.68
4–6	10 (27.8%)	4 (11.1%)	12 (33.3%)	
7–10	5 (13.9%)	2 (5.6%)	3 (8.3%)	
Pathological stage				0.89
pT2a and pT2b	8 (22.2%)	3 (8.3%)	9 (25.0%)	
pT3a and pT3b	7 (19.4%)	3 (8.3%)	6 (16.7%)	
Methylation status				0.54
CRBP1 methylated	6 (16.7%)	4 (11.1%)	7 (19.4%)	
CRBP1 unmethylated	9 (25.0%)	2 (5.6%)	8 (22.2%)	
HGPIN lesions (n = 35)*				
CRBP1 methylated	0 (0%)	2 (5.7%)	1 (2.9%)	0.60†
CRBP1 unmethylated	9 (25.7%)	6 (17.2%)	17 (48.5%)	

\*In one HGPIN lesion the methylation status could not be assessed; †the p value was calculated by grouping together the cases with normal and increased CRBP1 expression.  
HGPIN, high grade prostatic intraepithelial neoplasia.





**Figure 3** Example of methylation specific polymerase chain reaction for the CRBP1 promoter region in morphologically normal prostate tissue from radical prostatectomy specimens (N) and cystoprostatectomy specimens (CP). Lanes U and M correspond to unmethylated (109 bp) and methylated (99 bp) reactions, respectively. Sample 32N shows both unmethylated and methylated alleles, whereas samples 16N, 30N, and CP1 are completely unmethylated. Normal leucocyte DNA was used as a negative control for methylation (NL), DNA from normal leucocytes methylated with an excess of SssI bacterial methyltransferase was used as a positive control for methylation (IVD), and water was used as a negative polymerase chain reaction control (H<sub>2</sub>O). The HiLo marker is depicted on the right side.

significance ( $p = 0.0004$ ). In two HGPIN lesions with CRBP1 methylation, the respective PCa was also methylated, whereas in the remaining case of methylated HGPIN the paired adenocarcinoma was unmethylated.

Only one of 36 morphologically normal prostate tissue samples derived from patients simultaneously harbouring prostate cancer displayed CRBP1 promoter methylation, and none of the 13 normal prostate tissue samples collected from the cystoprostatectomy specimens showed CRBP1 promoter methylation (fig 3).

All BPH lesions analysed were completely unmethylated at the CRBP1 promoter.

## DISCUSSION

Altered retinoid metabolism is thought to play an important, yet not fully understood, role in the development of several human tumours.<sup>2-5</sup> Moreover, the effects of retinoids mediated by nuclear receptors seem to depend upon the function of CRBPs.<sup>7-8</sup> Owing to the lack of data concerning the relevance of CRBP1 in prostate tumorigenesis, we assessed CRBP1 expression and the methylation pattern of this gene in PCa and precursor (HGPIN) lesions. We show here that both altered CRBP1 expression and promoter hypermethylation are common events in prostate cancer.

Our immunohistochemical results show that CRBP1 expression is frequently lost in PCa and HGPIN (41.7% and 50%, respectively), being a more frequent event than reported for breast invasive and in situ carcinomas or ovarian cancer.<sup>11-12</sup> Cvetković and co-workers showed that the results of quantitative PCR to measure CRBP1 expression were in complete agreement with the immunohistochemical analysis performed in ovarian carcinoma.<sup>12</sup> Hence, the approach used in our study to assess CRBP1 expression is reliable and it also allows for immunolocalisation analysis, precluding the need for laser capture microdissection of prostate lesions. The high proportion of loss of CRBP1 expression both in PCa and HGPIN suggests that this alteration may be an important event in prostate carcinogenesis. In breast cancer cell lines, it has been hypothesised that CRBP1 would be involved in the promotion of apoptosis through an increase in all-trans retinoic acid synthesis.<sup>11</sup> Because suppression of apoptosis seems to play a key role in prostate carcinogenesis,<sup>14</sup> the observed loss of CRBP1 in prostate neoplastic cells could disrupt a retinoic acid mediated apoptosis pathway.

Interestingly, 15 of 36 PCa cases and nine of 36 cases of HGPIN showed CRBP1 overexpression, compared with normal glands. Such a finding has not been described in other epithelial neoplasms, but was reported in leiomyosarcomas, particularly in the epithelioid variant.<sup>21</sup> A significant

association was then established between CRBP1 overexpression and tumour differentiation,<sup>21</sup> but such an association was not found among our patients with prostate cancer, taking the Gleason score as a measure of neoplastic differentiation (data not shown). At first glance, CRBP1 overexpression may seem a disadvantageous acquisition by prostate cancer cells, but this change could represent a side effect of the altered expression of other genes in a subset of prostate tumours, rather than a primary event in prostate carcinogenesis. A probable candidate would be the gene encoding transforming growth factor  $\beta$ 1, because this gene is frequently upregulated in prostate cancer,<sup>22</sup> and it has been reported to induce CRBP1 overexpression.<sup>23</sup> Alternatively, an increase in CRBP1 cytoplasmic values could result from an abnormal (that is, less effective) pathway of protein degradation, as previously reported for other proteins.<sup>24</sup>

“Because suppression of apoptosis seems to play a key role in prostate carcinogenesis, the observed loss of CRBP1 in prostate neoplastic cells could disrupt a retinoic acid mediated apoptosis pathway”

In most (28 of 36) cases, the pattern of CRBP1 expression in paired PCa and HGPIN lesions was concordant, supporting the purported adenocarcinoma precursor role of HGPIN.<sup>25</sup> However, in seven cases, CRBP1 was overexpressed in the adenocarcinoma whereas decreased or normal values were seen in the paired HGPIN. This observation, in addition to the remaining discordant case in which the HGPIN lesion displayed CRBP1 overexpression and the adenocarcinoma showed loss of expression, could be explained by the well known genetic heterogeneity that characterises prostate tumours.<sup>26</sup>

Aberrant methylation was a frequent finding in our series of prostate adenocarcinomas (42.2%). This frequency is similar to that reported for gastric cancer by Esteller *et al*, making prostate adenocarcinoma one of the cancers where this gene is most frequently methylated.<sup>13</sup> However, we did not find a correlation between methylation and the lack of CRBP1 expression, whereas in that study a close relation between CRBP1 methylation and lack of expression was shown in several tumour cell lines and preneoplastic lesions.<sup>13</sup> Thus, additional mechanisms may be responsible for CRBP1 silencing in prostate carcinogenesis, although no mutations or loss of heterozygosity (LOH) have yet been described for this gene. Indeed, frequent LOH has been reported at 3p24–26 and 3p12–22 in prostate adenocarcinoma,<sup>27</sup> but not at 3q23, the chromosomal band that CRBP1 has been mapped to.<sup>28</sup> An alternative explanation would be the existence of a critical level of methylation for the silencing of CRBP1, as we have suggested previously for GSTP1.<sup>29-30</sup> Strikingly, in ovarian cancer, the prevalence of the

## Take home messages

- Altered expression and promoter hypermethylation of the CRBP1 gene, which encodes cellular retinol binding protein 1, occur frequently in prostate carcinoma, although CRBP1 hypermethylation is not an early event in tumorigenesis
- Both adenocarcinoma and high grade prostatic intraepithelial neoplasia show frequent CRBP1 overexpression
- The molecular mechanisms underlying altered CRBP1 expression in prostate cancer deserve further study

lack of CRBP1 expression (35%) reported by Cvetković and colleagues<sup>12</sup> contrasts with the notable absence of CRBP1 methylation seen by Esteller *et al.*<sup>13</sup> Moreover, several cases of prostate adenocarcinoma with both CRBP1 promoter methylation and protein expression were found in our series. A possible explanation for this finding would be monoallelic expression of CRBP1, in which case only one allele would be methylated. Interestingly, monoallelic expression of RARβ2 in breast cancer has also been suggested for cases in which LOH was found at this gene locus.<sup>31</sup> The relevance of other genetic events for the high frequency of loss of CRBP1 expression seen by immunohistochemical analysis clearly warrants further study.

CRBP1 methylated alleles were found in only one sample of normal prostate tissue derived from patients with prostate cancer, and the respective tumour showed the same methylation status. Aberrant promoter methylation has been reported in normal tissues from several sites and was eventually related to aging.<sup>32</sup> However, the occurrence of a single case of CRBP1 methylation in morphologically normal tissue from a patient simultaneously harbouring a prostate cancer with the same alteration should raise the possibility of contamination, although precautions were taken to prevent this event. Furthermore, the possibility that this epigenetic change might occur in morphologically normal cells as a potential premalignant characteristic cannot be ruled out because CRBP1 methylation was not seen in normal prostate tissue derived from patients without prostate cancer, or in BPH tissue samples.

Our study indicates that CRBP1 methylation in HGPIN is a considerably less frequent event than in adenocarcinoma (8.6% *v* 47.2%). Hence, this epigenetic alteration does not seem to occur frequently in the early steps of prostate carcinogenesis. It is worthwhile noting that both colorectal and gastric adenomas frequently display methylation of the CRBP1 promoter region, and this frequency parallels that seen in their invasive counterparts.<sup>13</sup> These findings suggest that the acquisition of CRBP1 aberrant methylation may occur at different steps of the tumorigenic process, depending upon the biological characteristics of each neoplasm.

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