ADAMTS1, CRABP1, and NR3C1 identified as epigenetically deregulated genes in colorectal tumorigenesis

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Abstract. Background: Gene silencing through CpG island hypermethylation is a major mechanism in cancer development. In the present study, we aimed to identify and validate novel target genes inactivated through promoter hypermethylation in colorectal tumor development. Methods: With the use of microarrays, the gene expression profiles of colon cancer cell lines before and after treatment with the demethylating agent 5-aza-2′-deoxycytidine were identified and compared. The expression of the responding genes was compared with microarray expression data of primary colorectal carcinomas. Four of these down-regulated genes were subjected to methylation-specific PCR, bisulphite sequencing, and quantitative gene expression analysis using tumors (n = 198), normal tissues (n = 44), and cell lines (n = 30). Results: Twenty-one genes with a CpG island in their promoter responded to treatment in cell lines, and were simultaneously down-regulated in primary colorectal carcinomas. Among 20 colon cancer cell lines, hypermethylation was subsequently identified for three of four analyzed genes, ADAMTS1 (85%), CRABP1 (90%), and NR3C1 (35%). For the latter two genes, hypermethylation was significantly associated with absence or reduced gene expression. The methylation status of ADAMTS1, CRABP1, and NR3C1 was further investigated in 116 colorectal carcinomas and adenomas. Twenty-three of 63 (37%), 7/60 (12%), and 2/63 (3%) adenomas, as well as 37/52 (71%), 25/51 (49%), and 13/51 (25%) carcinomas were hypermethylated for the respective genes. These genes were unmethylated in tumors (n = 82) from three other organs, prostate, testis, and kidney. Finally, analysis of normal colorectal mucosa demonstrated that the observed promoter hypermethylation was cancer-specific. Conclusion: By using a refined microarray screening approach we present three genes with cancer-specific hypermethylation in colorectal tumors, ADAMTS1, CRABP1, and NR3C1.

Keywords: ADAMTS1, adenomas, bisulphite sequencing, colorectal carcinomas, CRABP1, gene expression, methylation, microarrays, NR3C1, 5-aza-2′-deoxycytidine

1. Introduction

Impaired epigenetic regulation is as common as gene mutations in human cancer [22]. These mechanisms lead to quantitative and qualitative gene expression changes causing a selective growth advantage, which may result in cancerous transformation. Aber-
rantly hypermethylated CpG islands in the gene promoter associated with transcriptional inactivation are among the most frequent epigenetic changes in cancer.

In the large bowel, the adenoma-carcinoma sequence is paralleled by several genetic and epigenetic changes. One of these is hypermethylation of MLH1, which leads to inactivation of the DNA mismatch repair system and subsequent microsatellite instability (MSI) [20]. Approximately 15% of all sporadic colorectal carcinomas display MSI seen as insertions and deletions of short nucleotide repeats in their near diploid genome [1]. The microsatellite stable (MSS) tumors have a functional mismatch repair system but usually display chromosomal aberrations, including several common regions of loss or gain [42]. Subgroups of both types of colorectal carcinomas reveal aberrant methylation of tumor suppressor genes associated with lack of expression, although the methylation frequencies are generally higher in the MSI tumor group [29].

Furthermore, Toyota and co-workers suggested the CpG island methylator phenotype (CIMP) as the third pathway of colorectal tumorigenesis [56]. In 1999, they identified two epigenetically distinct tumor groups, one in which methylation was extremely rare (CIMP negative) and a second, in which the tumors were methylated at multiple loci (CIMP positive). This initial concept has been supported by some reports [45, 47, 59, 60], but not by others [3, 26, 61].

Aberrant DNA methylation changes have also been reported to occur early in the development of colorectal tumors [26, 44] and are therefore promising as early diagnostic markers of existing disease. The potential reversibility of DNA methylation patterns by epigenetic therapy further suggests that these are viable targets for treatment of cancer.

In the present study we have used a genome-wide approach to identify novel epigenetically regulated target genes in colorectal tumorigenesis, followed by technical, biological, and clinical validation analyses.

2. Material and methods

2.1. Cell cultures and tissue samples

Twenty colon cancer cell lines were included in the present study, nine MSI: Co115, HCT15, HCT116, LoVo, LS174T, RKO, SW48, TC7, and TC71, and 11 MSS: ALA, Colo320, EB, FRI, HT29, IS1, IS2, IS3, LS1034, SW480, and V9P [23]. All cell lines were cultured in DMEF-12 medium (GIBCO, Invitrogen Carlsbad, CA) with 10% fetal bovine serum (GIBCO), 2 mM L-glutamine (GIBCO), 100 U/ml penicillin G, and 100 µg/ml streptomycin (GIBCO) and harvested before confluence. Two MSI cell lines (HCT15, SW48) and two MSS cell lines (HT29, SW480) were cultured in parallel with and without 10 µM 5-aza-2′-deoxycytidine (Sigma-Aldrich Company Ltd., Dorset, UK) in the medium for 72 hours. DNA was extracted from the cell lines and their 5-aza-2′-deoxycytidine treated counterparts by a standard phenol-chloroform procedure, and total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA). The RNA quality was measured by a 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA) and the RNA concentration was determined using ND-1000 Nanodrop (NanoDrop Technologies, Wilmington, DE).

DNA from 53 colorectal carcinomas (25 MSS and 28 MSI) from 52 patients, including one patient with hereditary non-polyposis colorectal cancer (HNPCC), 63 adenomas (61 MSS and 2 MSI) from 52 patients, normal mucosa samples from 22 colorectal cancer patients (taken from distant sites from the primary carcinoma), and another 22 normal colorectal mucosa samples from cancer-free individuals was subjected to methylation analysis. The carcinomas (mean age 68 years) and the first group of normal mucosa samples (mean age 64 years) were taken from colorectal cancer patients admitted between 1987–1989 to one of seven hospitals located in the south-east region of Norway [34]. For nine of these 22 normal mucosa samples the corresponding primary tumor was also included in the present study. The adenomas were obtained from volunteers (mean age 67 years) participating in a polyp screening program [55]. The normal mucosa samples from cancer-free individuals were obtained from deceased persons (mean age 54 years, including 8 individuals ≥60 years). The majority of the total set of normal samples (27/44) consisted of mucosa only, whereas the remaining samples were taken from the bowel wall. The research bio-bank is registered at the Norwegian Institute of Public Health according to the Norwegian biobank legislation and the project was approved for filing and processing personal health data by the national Data Inspectorate.

Ten cell lines from various tissues were also included in the present study (prostate, n = 3; testicular germ cell tumor, n = 3; and ovary, n = 4). Additionally, clinical series of prostate (n = 20) and kidney (n = 20) carcinomas from the University Hospital of Porto, and a series of testicular germ cell tumors (n = 42) from patients admitted to the Norwegian Radium Hospital, Oslo, were screened for methylation.
2.2. Gene expression microarrays

Eighteen cell lines and four 5-aza-2'-deoxycytidine treated counterparts were analyzed by cDNA microarrays. Labeled cDNA was synthesized from 30 µg total RNA in an oligo dT-primed polymerization with SuperScript™II RNase H reverse transcriptase (Invitrogen) in the presence of either Cy3 (reference) or Cy5 (test) labeled dUTP (Amersham Pharmacia, Piscataway, NJ). Test and reference cDNA was mixed and hybridized onto cDNA microarrays (The microarray core facilities, The Norwegian Radium Hospital, Oslo, Norway), containing 15486 spots representing 12688 unique cDNA clones from 8461 genes. The human universal reference RNA (containing a mixture of RNA from ten different cell lines; Stratagene, CA, USA) was mixed 4:1 with total RNA from four combined colon cancer cell lines (two MSI, HCT116 and LoVo, and two MSS, HT29 and SW480) and used as a common reference for all samples. The fluorescence intensities of the spots were detected by a laser confocal scanner (Agilent Technologies). For each array element, a ratio between the background subtracted relative fluorescence intensities of the test and reference was calculated (GenePix Pro 6.0; Axon Instruments, Union City, CA, USA). The ratios in all samples were post-processed and normalized by the locally weighted scatterplot smoothing (LOWESS) algorithm [62] using BASE [46]. Array elements up-regulated more than two-folds after 5-aza-2'-deoxycytidine treatment in at least three of four cell lines, were considered to be potential targets for DNA methylation.

Microarray analysis of colorectal carcinomas \( (n = 18) \) and normal mucosa samples \( (n = 3; \text{taken from sites in distance from the tumors}) \) was performed in a parallel study (unpublished). The resulting data set was used to identify the \textit{in vivo} expression level of the genes that responded to 5-aza-2'-deoxycytidine treatment in at least three of four cell lines, were considered to be potential targets for DNA methylation. Bisulphite treatment of DNA leads to sequence variations as unmethylated but not methylated cytosines are converted to uracil [8]. DNA from cell lines and colorectal carcinomas was bisulphite treated as previously described [11,16], whereas DNA from the adenomas was bisulphite treated according to the protocol of the CpGenome™DNA modification kit (Intergen Boston, MA) [51]. The promoter methylation status of \textit{ADAMTS1}, \textit{CRABP1}, \textit{NDRG1}, and \textit{NR3C1} was subsequently analyzed by methylation-specific PCR (MSP), a method allowing for distinction between unmethylated and methylated alleles [19,10]. All primers were designed with MethPrimer [27] (with the exception of \textit{NR3C1} bisulphite sequencing primers, see acknowledgements). Their sequences are listed in Table 1, along with the product fragment length, primer location, and annealing temperature for each PCR. The fragments were amplified using the HotStarTaq DNA Polymerase (QIAGEN Inc., Valencia, CA), and all results were confirmed with a second independent round of MSP.

2.3. Bisulphite treatment and methylation-specific PCR

Bisulphite treatment of DNA leads to sequence variations as unmethylated but not methylated cytosines appear as thymines, whereas 5-methyl cytosines appear as cytosines in the final sequence [8]. For genes \textit{ADAMTS1}, \textit{CRABP1}, and \textit{NR3C1}, we subjected a subset of the cell lines to bisulphite sequencing \( (n = 13, n = 9, \text{and } n = 10, \text{respectively}) \), based on the MSP results. Most analyzed cell lines were methylated as assessed by the MSP analyses, but a minimum of one unmethylated and one monoallelic methylated cell line were also included in the panel sequenced for each gene. Primer sequences and PCR conditions are listed in Table 1. All fragments were amplified with the HotStarTaq DNA Polymerase and eluted from a 2% agarose gel by the MinElute™Gel Extraction kit (QIAGEN). The samples were subsequently sequenced using the dGTP BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA) in an ABI Prism 377 Sequencer (Applied Biosystems). The approximate amount of methyl cytosine of each CpG site in the various fragments was calculated by comparing the peak height of the cytosine signal with the sum of the cytosine and thymine peak height signals, as previously described [35].
Table 1. PCR primers used for methylation-specific PCR and bisulphite sequencing

| Primer set | Sense primer | Antisense primer | Fragment location |
|------------|--------------|------------------|------------------|
| ADAMTS1 MSP-M | GTGAGTAATATCGTAGTTAAGGCGG | CTAAAACAAAAAACGCTCTAAAACG | −128 to −25 |
| ADAMTS1 MSP-U | GTGAGTAATATTGTAGTTAAGGTGG | AAAACAAAAAACACTCTAAAACACC | −128 to −27 |
| ADAMTS1 BS | GAGAGGGGAGAGTTTTGAGTAGAGT | ACTCTAAATTATTAAAATTAACAATTTCTA | −128 to −27 |
| CRABP1 MSP-M | GGAGGTTTTTTAGTTGGAGAGC | CTCGCAAAACGAAAACTAACG | −193 to +18 |
| CRABP1 MSP-U | GAGGTTTTTTAGTTGGAGAGTGG | AACTCACAAAACAAAAACTAACACT | −193 to +20 |
| CRABP1 BS | AGGGAGGTGGAGGTTTTTTAGT | CACCAACTTACCCAATACCTTAAAC | −201 to +158 |
| NDRG1 MSP-M | GAGTCGATTTATAATTCGGGTTTC | GAAATTTATTTACGTCCGAACGA | −256 to +10 |
| NDRG1 MSP-U | GAGTTGATTTATAATTTGGGTTTTG | ACAAAATTTATTTACATCCAAACAA | −256 to +12 |
| NR3C1 MSP-M | TCGGTTTCGTTCGTTCGTTTAGGTC | CGTCCCGATCCCAACTACTTCGAC | −336 to −140 |
| NR3C1 MSP-U | TTGGTTTTGTTTGTTTGTTTAGGTT | CCATCCCAATCCCAACTACTTCAAC | −336 to −139 |
| NR3C1 BS | GTTGTTAAGYGTTATTAATAGGTTG | CTCCATAAATAAAAAAAAAACC | −477 to −25 |

Abbreviations: MSP, methylation-specific PCR; BS, bisulphite sequencing; M, methylated-specific primers; U, unmethylated-specific primers; Frg . Size, fragment size; An. Temp, annealing temperature (in degrees celcius).

2.5. Quantitative gene expression analyses

We used TaqMan real-time fluorescence detection (Applied Biosystems, Foster city, CA) to quantify mRNA levels in the colon cancer cell lines, as previously described [14,18]. cDNA was generated from two µg total RNA using the SuperScript™ II reverse transcriptase enzyme (Invitrogen) and oligo dT primers (Medprobe) according to the manufacturers’ protocol. cDNA from the genes of interest (ADAMTS1, Hs01095534_g1; CRABP1 Hs00171635_m1; and NR3C1, Hs00230818_m1) and the endogenous controls (ACTB, Hs99999903_m1 and PGK1, Hs00943173_gH) were amplified separately by the ABI Prism 7000 Sequence Detection System following the protocol recommended by Applied Biosystems. All samples were analyzed in duplicates. The quantitative expression levels were measured against a standard curve generated from dilutions of cDNA from the human universal reference RNA (containing a mixture of RNA from ten different cell lines; Stratagene). In order to adjust for the possibly variable amounts of cDNA input in each PCR, we normalized the expression quantity of the target genes with the quantity of the housekeeping gene PGK1, which was found to be the most suitable (see Results).

2.6. Statistics

All 2 × 2 contingency tables were analyzed using Fisher’s exact test. For the statistical analyses of the quantitative gene expression, a 2 × 3 table and Chi-square test were used. The gene expression was divided in two categories: low expression included samples with gene expression < median value for the gene in question across all cell lines, high expression includes samples with gene expression > median. Methylation status was divided in three categories: unmethylated, partial methylation, and methylated. All P values were derived from two tailed statistical tests using the SPSS 11.5 software (SPSS, Chicago, IL, USA).

3. Results

3.1. Identification of novel candidate genes potentially epigenetically deregulated in colorectal carcinomas

Ninety-three array elements were up-regulated two fold or more after 5-aza-2’-deoxycytidine treatment
Fig. 1. Gene expression of potential candidates for inactivation by DNA hypermethylation in colon cancer cell lines (A), 5-aza-2′-deoxycytidine treated cell lines (B), and primary colorectal carcinomas (C). The panels illustrate the gene expression in alphabetically order as assessed by microarray analyses. Each ratio is presented by a color for visualization. The gene expression of 21 genes containing a CpG island in their promoter was induced by 5-aza-2′-deoxycytidine treatment in colon cancer cell lines (B) and was simultaneously down-regulated in primary colorectal carcinomas relative to normal colon mucosa (C). Genes submitted to methylation analyses are highlighted in red.
in at least three out of the four cell lines analyzed (HCT15, HT29, SW48, and SW480). Among the 93 elements, 88 were present on Agilent oligo arrays used to analyze a panel of 18 primary colorectal carcinomas and three normal colon tissues (unpublished). The median of 60 of these genes was down-regulated across the panel of tumor samples relative to normal colon tissue and 21 of them contained a CpG island in their 5’ region (based on the definition and search algorithm presented by Takai and Jones [53]), thus representing potential epigenetically deregulated target genes. Included in this list was APC, which is one of the known gene targets for inactivation by hypermethylation in colorectal cancer. Among the 21, we selected ADAMTS1, CRABP1, NDRG1, and NR3C1 (Fig. 1), encoding proteins with potential roles in tumor development [12,15,17,21,24,32,33,38,40,41,49,58], as the first candidates to be analyzed. The genes were subjected to promoter methylation analysis in colon cancer cell lines. Prior to the analyses, the microarray cDNA clones responding to 5-aza-2′-deoxycytidine treatment were sequenced, and their identity confirmed.

### 3.2. Methylation status of novel candidate genes in vitro and in vivo

The promoters of ADAMTS1, CRABP1, and NR3C1 were hypermethylated in 17/20 (85%), 18/20 (90%), and 7/20 (35%) colon cancer cell lines, respectively, whereas NDRG1 was unmethylated in all samples as assessed by the MSP analysis (Table 2 and Fig. 2). Thirty-seven of 52 (71%) colorectal carcinomas were hypermethylated for ADAMTS1, with equal frequencies in MSI and MSS tumors (Table 2). Moreover, 23/63 (37%) of the colorectal adenomas, 2/22 (9%) of the normal mucosa samples taken from distant sites from tumors, and none (0/21) of the normal mucosa samples from cancer-free individuals, were hypermethylated. The primary carcinomas represented in the nine pairs of matched normal and cancer tissue were methylated in 6/8 (75%), 4/7 (57%), and 4/7 (57%), for ADAMTS1, CRABP1, and NR3C1, respectively, whereas the corresponding normal mucosa samples were unmethylated. CRABP1 was significantly more frequently methylated among the MSI carcinomas (22/28; 79%) than the MSS group (3/23; 13%; \(P < 0.001\)), and 7/60 (12%) of the adenomas, 1/22 (5%) of the normal mucosa samples taken from distant sites from tumors, and none (0/21) of the normal mucosa samples from cancer-free individuals, were hypermethylated. The methylation frequency of NR3C1 was also significantly higher in MSI carcinomas (12/28; 43%) than in MSS carcinomas (1/23; 4%; \(P = 0.003\)). Two of 63 (3%) adenomas and none of the normal mucosa samples were hypermethylated for NR3C1.

Both ADAMTS1 and CRABP1 were hypermethylated in a subset of cancer cell lines from various other tissues (3/13 and 9/13, respectively), but were unmethylated in 82 tumors from prostate, testis, and kidney. NR3C1 was unmethylated in the same cell lines and was therefore not subjected to methylation analysis in these primary tumors.

MSP analyses of the four 5-aza-2′-deoxycytidine treated colon cancer cell lines verified that ADAMTS1, CRABP1, and NR3C1 were demethylated in 80% (8/10) of the originally methylated cases. ADAMTS1 was still methylated in 5-aza-2′-deoxycytidine treated SW480 cells and no change in gene expression was detected by the microarray analyses. However, in drug treated SW48 cells, NR3C1 expression was upregulated compared with untreated cells even in the absence of visible demethylation.

### 3.3. Bisulphite sequencing

Bisulphite genomic sequencing of ADAMTS1, CRABP1, and NR3C1 in selected colon cancer cell lines showed that all cytosines at non-CpG sites were converted to thymine. This is seen in Fig. 3 along with the detailed sequencing results and the initial promoter methylation status as assessed by MSP. In general, there seemed to be a good association between the MSP scoring and the bisulphite sequences. Five out of nine cell lines methylated in ADAMTS1, as assessed by MSP, displayed only fully methylated CpG sites, whereas the remaining four cell lines displayed partial methylation (the presence of both methylated and unmethylated cytosine) in one to six CpG sites. Among these, the TC71 cell line had five partially methylated CpG sites including sites 17 to 19, which were fully methylated in all other methylated cell lines. In the CRABP1 gene promoter, the majority of CpG sites in the methylated cell lines were also fully methylated in the bisulphite sequencing electropherograms. Some partial methylation was seen around CpG sites 6 to 11 as five out of the six methylated cell lines, as assessed by MSP, displayed both methylated and unmethylated cytosines of two to five of these CpG sites. RKO was the only methylated cell line, as assessed by MSP analysis, harboring partial methylation for NR3C1 and only at two of the 58 successfully
Table 2
Promoter hypermethylation of candidate genes in colon cancer cell lines, colorectal carcinomas, adenomas, and normal mucosa

| Sample     | MSI status | ADAMTS1 | CRABP1 | NDRG1 | NR3C1 |
|------------|------------|---------|--------|-------|-------|
| Co115      | MSI        | M       | M      | U     | M     |
| HCT115     | MSI        | M       | M      | U     | U/M   |
| HCT116     | MSI        | M       | M      | U     | U     |
| LoVo       | MSI        | U/M     | U/M    | U     | U     |
| LS174T     | MSI        | U       | U      | U     | U     |
| RKO        | MSI        | M       | M      | U     | M     |
| SW48       | MSI        | M       | M      | U     | M     |
| TC7        | MSI        | U/M     | U/M    | U     | U     |
| TC71       | MSI        | M       | U/M    | U     | U     |
| ALA        | MSS        | M       | U/M    | U     | U     |
| Colo320    | MSS        | U       | M      | U     | U     |
| EB         | MSS        | M       | M      | U     | M     |
| FRI        | MSS        | U       | U/M    | U     | U     |
| HT29       | MSS        | M       | M      | U     | M     |
| LS1       | MSS        | M       | M      | U     | U     |
| LS2       | MSS        | M       | M      | U     | U     |
| LS3       | MSS        | M       | M      | U     | U     |
| LS1034     | MSS        | M       | M      | U     | U/M   |
| SW480      | MSS        | M       | U       | U     | U     |
| V9P        | MSS        | U/M     | M      | U     | U     |
| Carcinomas | MSI and MSS* | 37/52 (71%) | 25/51 (49%) | Nd | 13/51 (25%) |
|           | MSI        | 20/28 (71%) | 22/28 (79%) | Nd | 12/28 (43%) |
|           | MSS        | 17/24 (71%) | 3/23 (13%) | Nd | 1/23 (4%) |
| Adenomas   | MSI and MSS** | 23/63 (37%) | 7/60 (12%) | Nd | 2/63 (3%) |
| Normal(a)  | 2/22 (9%) | 1/22 (5%) | Nd | 0/22 (0%) |
| Normal(b)  | 0/21 (0%) | 0/21 (0%) | Nd | 0/20 (0%) |

Abbreviations: U, unmethylated; M, methylated; MSI, microsatellite stable; MSS, microsatellite unstable; Nd, not done.

*MSI and MSS carcinomas grouped together.

**Only two adenomas were MSI. The remaining 61 were MSS. Here they are presented together.

(a)Normal mucosa samples from individual colorectal cancer patients (taken from distant sites from the primary carcinoma).

(b)Normal colorectal mucosa samples from healthy individuals.

Fig. 2. Representative methylation-specific PCR results from the analysis of ADAMTS1, CRABP1, NDRG1, and NR3C1 in sixteen colon cancer cell lines. A visible PCR product in lanes U indicates the presence of unmethylated alleles whereas a PCR product in lanes M indicates the presence of methylated alleles. NB, normal blood (positive control for unmethylated samples); IVD, in vitro methylated DNA (positive control for methylated samples); neg, negative control (containing water as template); U, lane for unmethylated MSP product; M, lane for methylated MSP product. Each gel panel is a merge of two to three gel panels mainly run on the same agarose gel.
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A) ADAMTS1

B) CRABP1

C) NR3C1
amplified CpG sites. The three remaining cell lines, Co115, HT29, and SW48, displayed full methylation at all sites.

3.4. Quantitative gene expression of ADAMTS1, CRABP1, and NR3C1 in colon cancer cell lines

Figure 4 shows the relative expression levels of CRABP1 and NR3C1 in the colon cancer cell lines \( (n = 20) \). The expression levels are displayed as ratios between the individual genes and the endogenous control PGK1 and multiplied by a factor of 1000. The housekeeping genes PGK1 and ACTB had comparable expression levels overall, but since the median standard deviation in PGK1 was less than in ACTB (0.12 and 1.69, respectively), the gene expression of PGK1 was chosen for normalization of the target genes. For ADAMTS1, there was a trend toward lower gene expression in methylated cell lines compared with the unmethylated cell line Colo320, but the difference was not statistically significant. Colo320 demonstrated a high level of gene expression, whereas in the remaining two unmethylated cell lines, ADAMTS1 was not expressed at all. The majority of the methylated cell lines demonstrated little or no expression of ADAMTS1 with the exception of TC71 and LS1034, which had elevated gene expression levels, although still 3–4 fold lower than seen in Colo320. Among the three cell lines displaying both methylated and unmethylated DNA molecules for ADAMTS1, only V9P expressed the gene and again at a low level (data not shown). For CRABP1, there was a strong association between gene expression and methylation status \( (P = 0.005) \). Cell lines containing both methylated and unmethylated DNA molecules revealed the highest CRABP1 expression. Promoter hypermethylation of NR3C1 was also associated with reduced gene expression \( (P = 0.031) \). NR3C1 was not expressed at all in four of the five fully methylated cell lines, and also the gene expression in the methylated Co115 was close to zero.
3.5. Promoter methylation and association to clinical variables

For the colorectal carcinomas there was a strong association between methylation and right-sided tumor location as 15/19 (79%) tumors methylated in CRABP1 and 10/13 (77%) tumors methylated in NR3C1 were located in the right side of the colon \( (P = 0.001 \) and \( P = 0.002 \), respectively). Further, more methylation was found among the lowly differentiated tumors than among medium to highly differentiated tumors. For CRABP1 this was statistically significant \( (P = 0.029) \). We saw more methylation among tumors from females (11/27; 41%) than in those from males (2/24; 8%) for NR3C1 \( (P = 0.01) \). There was no statistically significant association between methylation and age nor methylation and Duke’s stage.

4. Discussion

We have used microarray gene expression profiling in combination with 5-aza-2′-deoxycytidine treatment of colon cancer cell lines to identify novel gene targets epigenetically inactivated at early stages of colorectal tumorigenesis. When cell lines are cultured with the DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine, epigenetic gene silencing by aberrant DNA methylation can be reversed. This could be seen in the present study by the reappearance of unmethylated MSP bands of originally methylated control genes. Genes up-regulated in cell lines after 5-aza-2′-deoxycytidine treatment might be potential candidates for inactivation by hypermethylation in colorectal cancer.

In order to limit the probability of selecting false positives, strict criteria were used. Only array elements up-regulated more than two-fold in at least three of the four cell lines analyzed, were further investigated. The expression level of these genes was subsequently examined in primary colorectal carcinomas, and the down-regulated ones were identified. This step-wise approach most likely explains the high success rate in validation of novel and tumor specific epigenetically regulated target genes in colorectal cancer. Genome wide DNA microarray approaches have previously been used to identify new target genes for epigenetic inactivation in colorectal cancer \[36,37,52\] as well as in other cancer types \[28,30\]. Suzuki and co-workers \[52\] cultured the colon cancer cell line RKO with trichostatin A and low-dose-5-aza-2′-deoxycytidine, inducing the expression of several genes. Complete methylation was shown for twelve genes (including three known control genes) in the RKO cell line. The processed microarray expression data from the four drug-treated colon cancer cell lines in the present study include eight of the twelve methylated genes identified by Suzuki et al. \[52\]. Five of these genes also responded to 5-aza-2′-deoxycytidine treatment in our study of colon cancer cell lines, whereas FOLH1, PCDH8, and TIMP2 did not. FOLH1 and PCDH8 had a comparable methylation pattern in primary colorectal carcinomas and their normal counterparts \[52\], and thereby these markers are not tumor specific. TIMP2 turned out to be methylated only in the RKO cell line and not in a panel of eight additional colon cancer cell lines or in primary colorectal cancers \[52\]. In summary, by using the screening and selection approach outlined in the present study, the probability of identifying epigenetic targets with biologic significance in colorectal tumorigenesis is increased.

Three of our four candidates were hypermethylated in several colon cancer cell lines as identified by MSP analysis. As this method tends to overestimate DNA methylation in certain cases \[43\], we additionally bisulfite sequenced approximately half of the cell lines. For all genes, there seemed to be a good association between the results obtained with the two methods. Rand et al. \[43\] have pointed out that incompletely converted sequences during bisulphite treatment can be co-amplified with the methylated alleles during MSP. In the present study, all cytosines at non-CpG sites in the bisulphite sequences were converted to thymine. Additionally, none of our MSP primer sets amplified untreated DNA (data not shown), which means that if unconverted sequences had been present, this would not have influenced the results. To establish whether the promoter hypermethylation of the present target genes led to transcriptional inactivation, we analyzed the quantitative gene expression in the cell lines. There was a good association between hypermethylated status and reduced gene expression, which was significant for CRABP1 and NR3C1. The relatively small sample set might partly explain the lack of statistical significance for ADAMTS1. Other mechanisms may also cause lack of expression of this gene since two of three cell lines unmethylated for ADAMTS1 showed no expression (data not shown).

DNA from cell lines is often more frequently hypermethylated than is DNA from primary tumors \[50\]. CpG island hypermethylation has also previously been demonstrated to be an early and frequent event during colorectal carcinogenesis, but only a limited number of epigenetic changes have been identified in be-
ADAMTS1 is a metalloproteinase of the ADAM (A disintegrin-like and metalloprotease) family containing a thrombospondin type I motif and was first described in 1997 as an inflammation and cancer cachexia-related gene [25]. Today, a total of 19 similar genes have been characterized in the human genome [4], and several of them have been implicated in various diseases, including cancer [40]. The carboxy-terminal half region of ADAMTS1 has been shown to suppress both tumorigenicity and experimental tumor metastatic potential [24] and by binding and sequestering VEGF165, ADAMTS1 inhibits angiogenesis and endothelial proliferation [32]. Down-regulated gene expression of ADAMTS1 is found in breast carcinomas and pancreatic cancer [41,33] but the mechanistic cause(s) is not known. This study did not find a significant association between hypermethylation and reduced gene expression, and thus the importance of methylation for regulation of gene expression of ADAMTS1 remains unclear. Investigation of ADAMTS1 methylation in breast – and/or pancreatic cancer might clarify this.

The cellular retinoic acid binding proteins (CRABP) have a high affinity for retinoic acid and belong to a family of small cytosolic lipid binding proteins. Two highly homologous forms of CRABP have been characterized, CRABP1 and CRABP2, with about 75% amino acid identity [5]. Although their exact functions are not completely understood, regulation of the availability of retinoic acid to its nuclear receptors and subsequent enhancement of the retinoic acid effect on regulating target gene expression are both plausible [38]. Retinoic acid, a metabolite of vitamin A, plays a role in cell cycle arrest, cell differentiation, and to a certain extent apoptosis by altering gene transcription, and there is increasing evidence that retinoic acid metabolism may be altered during carcinogenesis [38]. Moreover, several reports have documented alterations in the expression of retinol and retinoic acid binding proteins in various tumor types [38]. CRABP1 was initially shown to be inactivated by promoter hypermethylation in papillary thyroid carcinomas and in two colon cancer cell lines [21] and lately in colorectal carcinomas [39]. In the present study, we demonstrate that methylation of CRABP1 is present both in benign and malignant tumors of the large bowel, in contrast to normal mucosa, showing that this change is an early tumorigenic event. Furthermore, all cell lines with CRABP1 hypermethylation had reduced or no gene expression, which indicates that the promoter hypermethylation leads to transcriptional inactivation. The mouse Crabp1 gene,
which reveals 99.3% amino acid identity to human CRABP1 [5], demonstrates developmentally regulated gene expression through methylation changes in its 5'-flanking region [58]. Taken together, these lines of evidence support that CRABP1 expression is epigenetically deregulated early in the establishment of colorectal tumors.

The nuclear receptor subfamily 3, group C, member 1 (NR3C1) gene encodes the glucocorticoid receptor, which resides in the cytoplasm in a multiprotein complex. Upon binding to glucocorticoid, the protein translocates into the nucleus where it functions as a transcription factor and participates in the regulation of several molecular processes such as inflammation, cell growth, differentiation, and glucocorticoid-induced apoptosis [12]. The ligand-activated glucocorticoid receptor is especially efficient in killing certain cells of the lymphoid lineage, and glucocorticoids are therefore included in essentially all chemotherapy protocols for lymphoid malignancies [13]. Glucocorticoids can also repress cell cycle progression in a number of other cell types, including neoplastic thymic epithelial cells [12] and non-small cell lung cancer cells [15]. In general, people have a variable sensitivity to glucocorticoids, which in part can be explained by genetic changes reported in the NR3C1 gene, including several polymorphisms and some mutations [7]. The level of NR3C1 expression is a critical determinant for glucocorticoid sensitivity [48], and thus transcriptional inactivating promoter hypermethylation in tumors will render them glucocorticoid resistant. This study is, to our knowledge, the first reporting promoter hypermethylation of NR3C1 and subsequent loss of expression. In primary colon cancers, the immunohistochemical expression of NR3C1 has previously been correlated with the expression of the cell cycle-related molecules pRb and p16 [54]. This was corroborated in the present study as NR3C1 hypermethylation was associated with tumors also harboring hypermethylation of CDKN2A (encoding the p16 protein; data not shown). Simultaneously, NR3C1 hypermethylation seemed to be mutually exclusive with the presence of mutations in the central tumor suppressor TP53 (data not shown), in accordance with a previous study reporting crosstalk between these two proteins [49]. On the other hand, these associations might simply reflect the MSI phenotype of the primary tumors. Hypermethylation of NR3C1 was associated with MSI positive tumors, whereas mutations in TP53 were associated with MSS tumors [6]. Similar observations were done for the CRABP1 gene. Methylated CRABP1 was found in tumors with methylated CDKN2A and wild type TP53. Although the functional and clinical importance of NR3C1 inactivation in colorectal tumorigenesis is not known, the inactivation of this receptor might be of clinical importance in the treatment of cancer patients receiving glucocorticoids as part of the standard treatment protocol, such as lymphoma and acute lymphatic leukemia patients. Interestingly, frequent down-regulation of NR3C1 is found in such tumors in public available microarray datasets [2] (see SOURCE for the expression of NR3C1 in lymphomas: http://source.stanford.edu/cgi-bin/sourceSearch; see GDS363 report deposited in NCBI's Gene Expression Omnibus; GEO, http://www.ncbi.nlm.nih.gov/geo/). Methylation of NR3C1 should therefore be studied in these malignancies.

In summary, by using a refined microarray screening approach we present three genes with cancer-specific hypermethylation in colorectal tumors, ADAMTS1, CRABP1, and NR3C1.

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