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RESEARCH

Altered Expression of Multiple Genes Involved in Retinoic Acid Biosynthesis in Human Colorectal Cancer

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Abstract All-trans-retinoic acid (atRA), the oxidized form of vitamin A (retinol), regulates a wide variety of biological processes, such as cell proliferation and differentiation. Multiple alcohol, retinol and retinaldehyde dehydrogenases (ADHs, RDHs, RALDHs) as well as aldo-keto reductases (AKRs) catalyze atRA production. The reduced atRA biosynthesis has been observed in several human tumors, including colorectal cancer. However, subsets of atRA-synthesizing enzymes have not been determined in colorectal tumors. We investigated the expression patterns of genes involved in atRA biosynthesis in normal human colorectal tissues, primary carcinomas and cancer cell lines by RT-PCR. These genes were identified using transcriptomic data analysis (expressed sequence tags, RNA-sequencing, microarrays). Our results indicate that each step of the atRA biosynthesis pathway is dysregulated in colorectal cancer. Frequent and significant decreases in the mRNA levels of the *ADH1B*, *ADH1C*, *RDHL*, *RDH5* and *AKR1B10* genes were observed in a majority of colorectal carcinomas. The expression levels of the *RALDH1* gene were reduced, and the expression levels of the cytochrome *CYP26A1* gene increased. The human colon cancer cell lines showed a similar pattern of changes in the mRNA levels of these genes. A dramatic reduction in the expression of genes encoding the predominant retinol-oxidizing enzymes could impair atRA production. The most abundant of these genes, *ADH1B* and *ADH1C*, display decreased expression during progression from adenoma to early and more advanced stage of colorectal carcinomas. The diminished atRA biosynthesis may lead to alteration of cell growth and differentiation in the colon and rectum, thus contributing to the progression of colorectal cancer.

Keywords Colorectal cancer · Retinoic acid biosynthesis · Gene expression · Tumor progression

Introduction

Retinoids are biologically active derivatives of vitamin A (retinol), that regulate a wide variety of biological functions, such as cell differentiation, proliferation and apoptosis, in various tissues [1, 2]. All-*trans*-retinoic acid (atRA) is the most active cellular retinoid metabolite that may activate or repress the transcription of multiple target genes by binding to nuclear retinoic acid receptors (RARs) and retinoid X receptors (RXRs) [3]). Dietary retinoid precursors are converted into retinol primarily in the small intestine. Retinol bound to retinol-binding proteins can be transported from the liver, the major site of retinol storage, via the bloodstream to the target cells, including colorectal epithelial cells, for conversion to atRA [4].

The biosynthesis of atRA occurs in two steps, all-*trans*-retinol is reversibly oxidized to all-*trans*-retinaldehyde, which in turn is irreversibly oxidized to atRA [5, 6]. Multiple enzymes with tissue-specific expression from four oxidoreductase families are involved in atRA biosynthesis in humans [7]. Cytosolic alcohol dehydrogenases (ADHs) and microsomal NAD+−dependent retinol dehydrogenases (RDHs) oxidize retinol to retinaldehyde, whereas the conversion of retinaldehyde back to retinol is catalyzed by NADP+−dependent RDHs and cytosolic retinoid-active aldo-keto reductases (AKRs). In the second step, the oxidation of retinaldehyde to atRA is catalyzed by several cytosolic

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retinaldehyde dehydrogenases (RALDHs). atRA can be partially converted into *cis*-isomers. The delivery of atRA to specific nuclear receptors mediated by binding to cellular retinoic acid binding proteins [3].

ADHs can both oxidize various alcohols, including ethanol and retinol, and reduce various aldehydes. They are much more active in the oxidative direction, as expected for the NAD+−dependent enzymes. The human ADHs constitute a complex family of enzymes divided into five classes [8, 9]. ADH4 (also known as ADH7) has been shown to be the most catalytically efficient retinol dehydrogenase. ADH1 and ADH2 are also very active in retinol oxidation [10, 11], unlike ADH3; however, genetic studies have shown that ADH3 as well ADH1 and ADH4 may participate in the biosynthesis of atRA in vivo [6, 11, 12].

Among human NAD+−dependent RDHs, all-*trans*-retinol-specific RDH10 [13] and RoDH4 [10] are the most active retinol-oxidizing RDHs. Among NADP+−dependent RDHs, RDH12 and RDH11 are the most catalytically efficient retinal reductases [7]. At least two human AKRs, aldose reductase (AKR1B1) and small intestine aldose reductase (AKR1B10), catalyze the conversion of retinaldehyde back to retinol [10, 14]. In humans, the oxidation of retinaldehyde to atRA is catalyzed by RALDH1, −2 and −3 (also known as retinoid-active aldehyde dehydrogenases ALDH1A1, −2 and −3). Genetic studies in mice have indicated that the orthologous enzymes, Raldh1, −2 and −3, contribute to the generation of atRA in vivo [6]. atRA levels are controlled via the regulated expression of atRA-synthesizing enzymes and the specific atRA-degrading cytochrome P450 reductases (CYPs), CYP26A1, CYP26B1 and CYP26C1 [15].

Retinoic acid displays its antitumor activity via inhibition of cell proliferation, induction of cell differentiation and apoptosis. Retinoic acid is employed in several cancer therapies, especially in treatment of acute promyelotic leukemia, but also of solid cancers in humans, such as squamous cell carcinoma, neuroblastoma and hepatocellular carcinoma and others [16].

Colorectal cancer (CRC) is one of the most common cancers in the developed world. The important function of retinoids in both the maintenance of the normal large intestine epithelium and carcinogenesis has been demonstrated using animal model systems [17]. A subset of key atRA-synthesizing enzymes in normal colorectal tissues and colorectal carcinomas remains unknown. The aim of the present study was to identify the genes encoding these enzymes using a bioinformatics approach and to examine the expression levels of these genes in colorectal tumor specimens, in their adjacent non-neoplastic tissues and in colorectal cancer cell lines by reverse transcription-PCR. Our purpose was also to investigate the relationship between aberrant expression of genes involved in atRA biosynthesis and tumor clinicopathological characteristics.

Materials and Methods

Clinical Specimens and Cell Lines

Paired colon and rectum tumor tissues samples and adjacent histologically normal tissues were examined from 80 patients with colorectal cancer. Resected tumors were staged according to the American Joint Committee on Cancer (AJCC) tumor-node-metastasis (TNM) system for patients (sixth edition). Supplementary Table S1 shows all relevant clinicopathological information of CRC patients. None of the patients received radio- or chemotherapy before surgery. In addition, six patient-matched colonic normal mucosa, hyperplastic and adenomatous polyps were also studied. Surgical specimens (Blokhin Cancer Research Center, Russian Academy of Medical Sciences and Medical Center of Central Bank of Russian Federation, Moscow) were immediately frozen and stored at −70 °C until use. Informed consent was obtained before the samples were dissected. Using histological analysis, samples with more than 80 % neoplastic cells were selected for the study. The human CRC cell lines HCT-116, RKO and HT-29 were also used in this work.

Transcriptomic Data-Mining Approaches for Identifying Potential Genes Involved in atRA Biosynthesis

We have analyzed various available transcriptomic data: expressed sequence tags (ESTs), RNA sequencing (RNA-Seq) and microarrays. The EST-based gene expression profiling in normal and tumor tissues was performed using dbEST (http://www.ncbi.nlm.nih.gov/nucest). The primary adult tissues, not cell lines, were selected and pooled for the analysis. The EST library quality was estimated based on its size (the threshold was 500 ESTs per library) and the availability of the known reference housekeeping genes, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), beta-actin (*ACTB*) and beta2-microglobulin (*β2M*). Data were recalculated to the total EST counts and normalized to the reference genes (Supplementary Table S2).

The RNA-Seq-based gene expression data were downloaded from RNA-Seq Atlas (http://medicalgenomics.org/rna_seq_atlas/). The initial results were obtained from data for 11 normal human tissues, including colon, which were pooled from multiple donors. This database covered 32384 specific transcripts corresponding to 21399 unique genes. Prepared libraries were sequenced on an Illumina GAII sequencer generating 50 million reads per tissue and further analyzed using BWA mapping to reference human hg18 genome assembly. RefSeq transcript coordinates and associated gene symbols were downloaded from the UCSC genome browser. The gene expression levels were estimated followed by normalization to RPKM values (Reads Per Kilobase per Million mapped reads) measured in reads per

kilobase of exon per million mapped sequence reads (Supplementary Table S2).

The microarray-based analysis of gene expression in colorectal normal and cancer tissues was performed using Oncomine Cancer Microarray database (http://www.oncomine.org/). In order to compare the gene expression in tumor samples relative to the corresponding patient-matched normal tissues, gene expression data from a same study, performed with the same methodology, were used. The gene expression data were log transformed, median centered per array and the standard deviation was normalized to one per array. A gene was considered as overexpressed or downregulated when its mean expression value in tumor samples was significantly higher or lower to its mean value in the normal tissue using a *t*-test ($P \leq 0.005$) and the fold change was ≥2. Datasets used in this study are summarized in Supplementary Table S3. For each gene maximal fold changes both for overexpression and downregulation were estimated.

RNA Isolation and Reverse Transcription-PCR

Total RNA was isolated by using the RNeasy Mini kit (Qiagen, USA, cat. no. 74106). First-strand cDNA was synthesized using 1 μg of total RNA, random primers (Syntol, Russia, cat. no. P014) and SuperScript™ III reverse transcriptase (Invitrogen, USA, cat. no. 18080–044). The mRNA levels were initially determined by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) using gene-specific primers (Supplementary Table S4). The densitometric measurements of the bands were performed using the GeneProfiler program for image densitometry (http://www.scananalytics.com) and normalized to those of the *β2M* and *ACTB* reference genes.

Real-Time Quantitative Polymerase Chain Reaction

Real-time quantitative polymerase chain reaction (RT-qPCR) was performed in an Applied Biosystems (ABI) 7500 Fast Real-Time PCR System using EvaGreen® dye (Biotium, Inc., USA, cat. no. 31014–1) according to the manufacturer's instructions. Each plate included at least three 'negative' (no template) controls for each gene. The primers for all genes were the same as for the conventional RT-PCR (Supplementary Table S4). The threshold cycle (Ct) value of each reaction was determined and normalized to that of the housekeeping genes *β2M* and *ACTB*. Dissociation curve analysis was performed to detect non-specific hybridization products.

Statistical Analysis

Statistical analysis of mRNA level differences in normal and tumor tissues was performed using Statistica 10.0 software. The non-parametric Wilcoxon T-criterion was used for comparison of expression level differences and Fisher's exact test - to evaluate the correlation between the mRNA level changes and clinicopathological characteristics. *P*-values< 0.05 regarded as statistically significant.

Results

Identification of Putative Genes Implicated in atRA Biosynthesis in the Colorectal Tissues Based on Microarray-, RNA-Seq- and EST Databases Analysis

The initial set of atRA-synthesizing genes was obtained from literature searches and included 32 genes: seven *ADH*s (*ADH1A*, *−B* and *–C*, *ADH2*, *ADH3*, *ADH4*, *ADH6*), 16 *RDH*s (*RDH10*, *RDHL*, *RDH5*, *RoDH*, *RoDH4*, *RDHE2*, *XOR*, *RDH8*, *RDH11-14*, *RDH17*, *DHRS4*, *DHRS4L2*, *SDRO*), two *AKR*s (*AKR1B1* and *-B10*), three *RALDH*s (*RALDH1*, *−2* and *−3*), three *CYP*s (*CYP26A1*, *−B1* and *–C1*) and gene encoding cellular retinol-binding protein CRBP1 (Supplementary Table S2). To identify candidate genes involved in atRA generation in colorectal normal and tumor tissues, we have analyzed various available transcriptomic data. The initial set of atRA-synthesizing genes was filtered according to their expression level in normal human adult colon (based on EST and RNA-Seq data). Only 13 atRA-generating genes were detected in normal colorectal tissues (Fig. 1a). Most of them exhibited differential expression levels in CRC samples according to both EST and microarray data (Fig. 1b). Comparison of gene expression profiling between these two approaches demonstrated their overall good agreement (R^2 was equal to ~0.8).

The retinol-oxidizing step of the atRA biosynthesis pathway was associated primarily with the expression of the *ADH1B*, *ADH1C* and *ADH3* genes in normal colorectal tissues (Fig. 1a). The *RDHL* gene (also known as *DHRS9*) was detected with a relatively lower abundance according to the RNA-Seq data; however, the EST data demonstrated a higher level of *RDHL* expression. We have also detected the less abundant RDH10 and RDH5 mRNAs in normal colorectal tissues (Fig. 1a). ADH1B, ADH1C and RDHL mRNA levels were significantly downregulated in CRC samples. RDH10 mRNA levels were slightly increased in colorectal tumors (Fig. 1b).

The mRNAs encoding the retinaldehyde-reducing enzymes, RDH11, AKR1B1 and AKR1B10, were detected in normal colorectal cells (Fig. 1a). The most abundant mRNA, AKR1B10, was characterized with the greatest decline in expression levels in CRC (Fig. 1b). RALDH1 and the less abundant RALDH3 mRNAs were both differentially expressed in colorectal carcinomas relative to the corresponding normal tissues. A slight upregulation of the CYP26A1 mRNA encoding the atRA-degrading enzyme was observed.

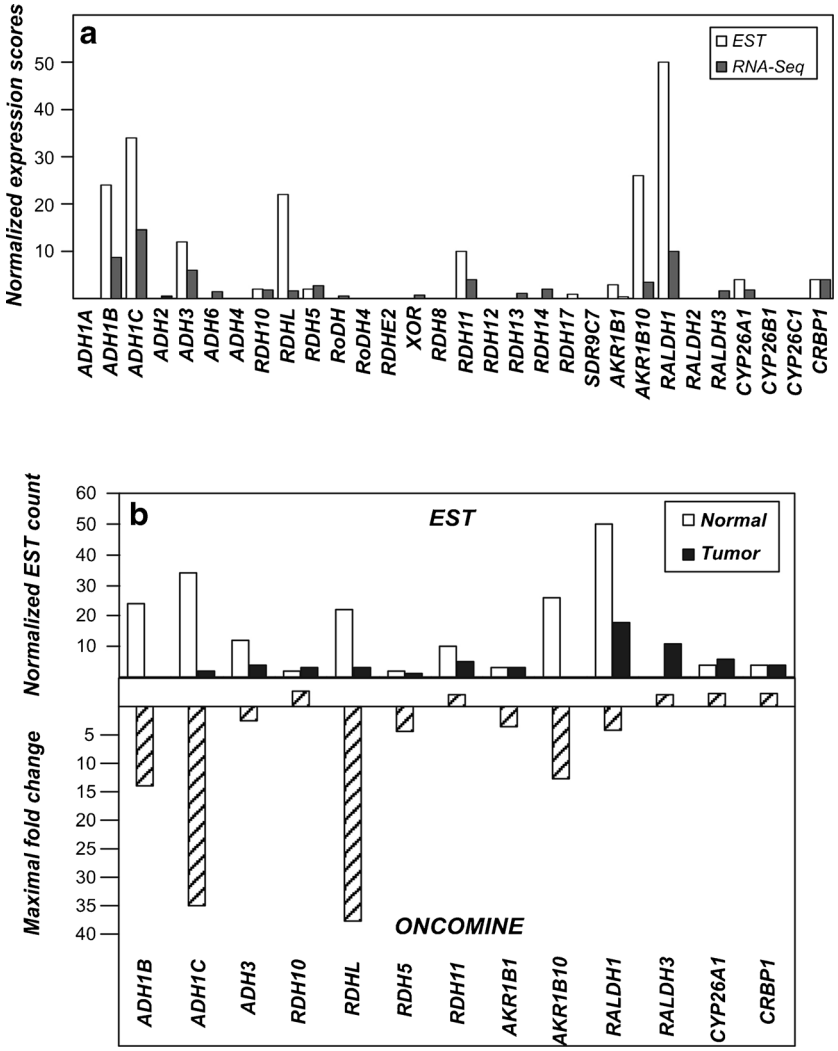

Fig. 1 Transcriptomic analysis of differential expression of genes involved in the atRA biosynthesis in normal and malignant human colorectal tissues. **a** Gene expression profiling in normal adult colon using RNA-Seq and EST data analysis. The EST data were produced using high-quality spliced ESTs from pools of cDNA libraries. EST counts were initially normalized to 100,000, then to median EST counts of the reference *ACTB* and *β2M* genes. The RPKM values are shown according to RNA-Seq Atlas. **b** Differential expression of genes encoding atRA-generating enzymes and CRBP1 in colorectal carcinomas in comparison with normal tissues. The EST-based data are shown by black bars (carcinomas) and white bars (normal mucosa). The transcriptomic profiling was performed as for data in Fig. 1a. The microarray-based ONCOMINE data were analyzed in the search of gene overexpression and/or downregulation in colorectal tumors. The highest fold-changes of mRNA levels of each gene in the selected ONCOMINE studies are shown as the striped bars. Abbreviations: *atRA* all-*trans*-retinoic acid, *RNA-Seq* RNA Sequencing, *EST* Expressed Sequence Tags, *ACTB* beta-actin, *β2M* beta2-microglobulin, *RPKM* Reads Per Kilobase per Million mapped reads, *CRBP1* cellular retinol-binding protein

The levels of CRBP1 mRNA remained unchanged in tumor tissues relative to normal tissues according EST data and slightly increased according ONCOMINE data (Fig. 1b).

The simultaneous analysis of three fundamentally different transcriptomic databases allowed us to both identify the colorectal-associated gene set potentially involved in atRA generation and predict the cancer-associated gene expression variations at each step of the biochemical process. This set was supplemented by the genes encoding highly active enzymes, which were not detected in the colorectal tissues by transcriptomic data analyses (*ADH1A*, *ADH2*, *ADH4*, *RoDH4*, *RDH12* and *RALDH2*). Semi-quantitative RT-PCR and real-time RT-qPCR were employed to confirm the expression patterns of these selected genes.

The Expression of Genes Encoding Retinol-Oxidizing Enzymes in Colorectal Carcinomas

The mRNA levels of the 19 genes potentially involved in atRA production in the colorectal tissues were assessed by semi-quantitative RT-PCR in 30 pairs of samples from patients with CRC (Table 1). The ADH1B and ADH1C mRNAs

Table 1 Gene expression profiling of all-trans-retinoic acid-generating genes in colorectal cancer specimens by semi-quantitative and real-time quantitative RT-PCR

Gene	mRNA level changes* (% of specimens, fold change, *P* value)		
	Down	Up	Unchanged
ADH1B	**85 % (68/80)** **28 (2.05–2224) *P*<0.00001**	5 % (4/80) 3.0 (2.0–4.6)	10 % (8/80)
ADH1C	**81 % (65/80)** **41 (2.03–2455) *P*<0.00001**	2.5 % (2/80) 3.4 (2.6–4.3)	16.5 % (13/80)
ADH3	17 % (5/30) 2.7 (2.0–5.0)	10 % (3/30) 2.5 (2.0–3.0)	**73 % (22/30)**
RDHL	**90 % (27/30)** **7 (2.0–10.0) *P*<0.00001**	0 %	10 % (3/30)
RDH5	**50 % (15/30)** **5.2 (2.5–10.0) *P*<0.005**	3 % (1/30) 3.5	47 % (14/30)
RDH10	23 % (7/30) 3.8 (2.1–6.5)	33 % (10/30) 4.8 (2.05–12.9)	44 % (13/30)
RDH11	3 % (1/30) 3.5	10 % (3/30) 3.1 (3.0–6.0)	**87 % (26/30)**
AKR1B1	7 % (2/30) 2.25 (2.0–2.5)	0 %	**93 % (28/30)**
AKR1B10	**87 % (69/80)** **20 (2.36–2043) *P*<0.00001**	0 %	13 % (11/80)
RALDH1	**60 % (18/30)** **2.7 (2.0–2.9) *P*<0.005**	0 %	40 % (12/30)
RALDH2	**50 % (15/30)** **6.2 (2.0–10.0) *P*<0.005**	33 % (10/30) 6.1 (3.0–10.0)	17 % (5/30)
RALDH3	3 % (1/30) 3.5	37 % (11/30) 3.5 (3.0–10.0)	**60 % (18/30)**
CYP26A1	13 % (4/30) 2.3 (2.0–5.0)	**50 % (15/30)** **5.75 (2.0–10.0) *P*<0.005**	37 % (10/30)
CRBP1	3 % (1/30) 8.3	3 % (1/30) 4.0	**94 % (28/30)**

*Shown are the geometric mean and ranges (in parentheses) of fold changes in mRNA levels

The predominant mRNA level changes are shown in bold and *P*-values are estimated for down or overexpression. *Abbreviations*: *ADH1B* and *ADH1C* alcohol dehydrogenases; *RDHL*, *RDH5*, *RDH10* and *RDH11* retinol dehydrogenases; *AKR1B1* and *AKR1B10* aldo-keto reductases; *RALDH1*, *RALDH2* and *RALDH3* retinaldehyde dehydrogenases; *CYP26A1* cytochrome P450 26A1, *CRBP1* cellular retinol-binding protein, *ACTB* beta-actin, *β2M* beta2-microglobulin

were expressed at very high levels in normal colon and rectum tissues, whereas the ADH1A, ADH2 and ADH4 mRNAs were not detected. The levels of ADH3 mRNA were approximately eight times lower than the levels of ADH1B and ADH1C. The expression levels of the ADH1B, ADH1C and RDHL mRNAs were significantly decreased in a majority of the 30 tumor samples. A fold change greater than two was considered significant. The expression levels of ADH3 mRNA remained unchanged in most of the tumor samples. Among the genes encoding NAD+−dependent RDHs, only *RDH10*, *RDHL* and *RDH5* were expressed in the colon and rectum. The mRNAs of these genes were detected at much lower levels in normal colorectal tissues compared with the mRNA levels of *ADH1B* and *ADH1C*. The ADH1B and ADH1C mRNA expression levels displayed the largest changes in CRC samples. Therefore, we performed a detailed expression analysis of these two mRNAs by RT-qPCR in the extended panel of 80 paired samples (Fig. 2, Table 1). RDH10 mRNA was expressed in normal colorectal tissues at a significantly lower level than the two ADH1 mRNAs (approximately 8-fold lower); however, as RDH10 enzyme exhibited very high catalytic efficiency for retinol oxidation [13], we also examined the RDH10 mRNA levels by RT-qPCR (Table 1). The ADH1B and ADH1C mRNA levels were reduced by at least 2-fold in 85 % and 81 % of tumor samples, respectively, even at early stages (Fig. 2, Table 1, $P<0.00001$). A statistically significant decrease in the expression level of at least one of these genes was observed in 96 % of tumor samples. The changes in RDH10 mRNA levels were not significant when compared with the two ADH1 isoforms (Table 1).

Thus, the expression levels of the genes *ADH1B*, *ADH1C*, *RDHL* and *RDH5* significantly decreased in CRC samples,

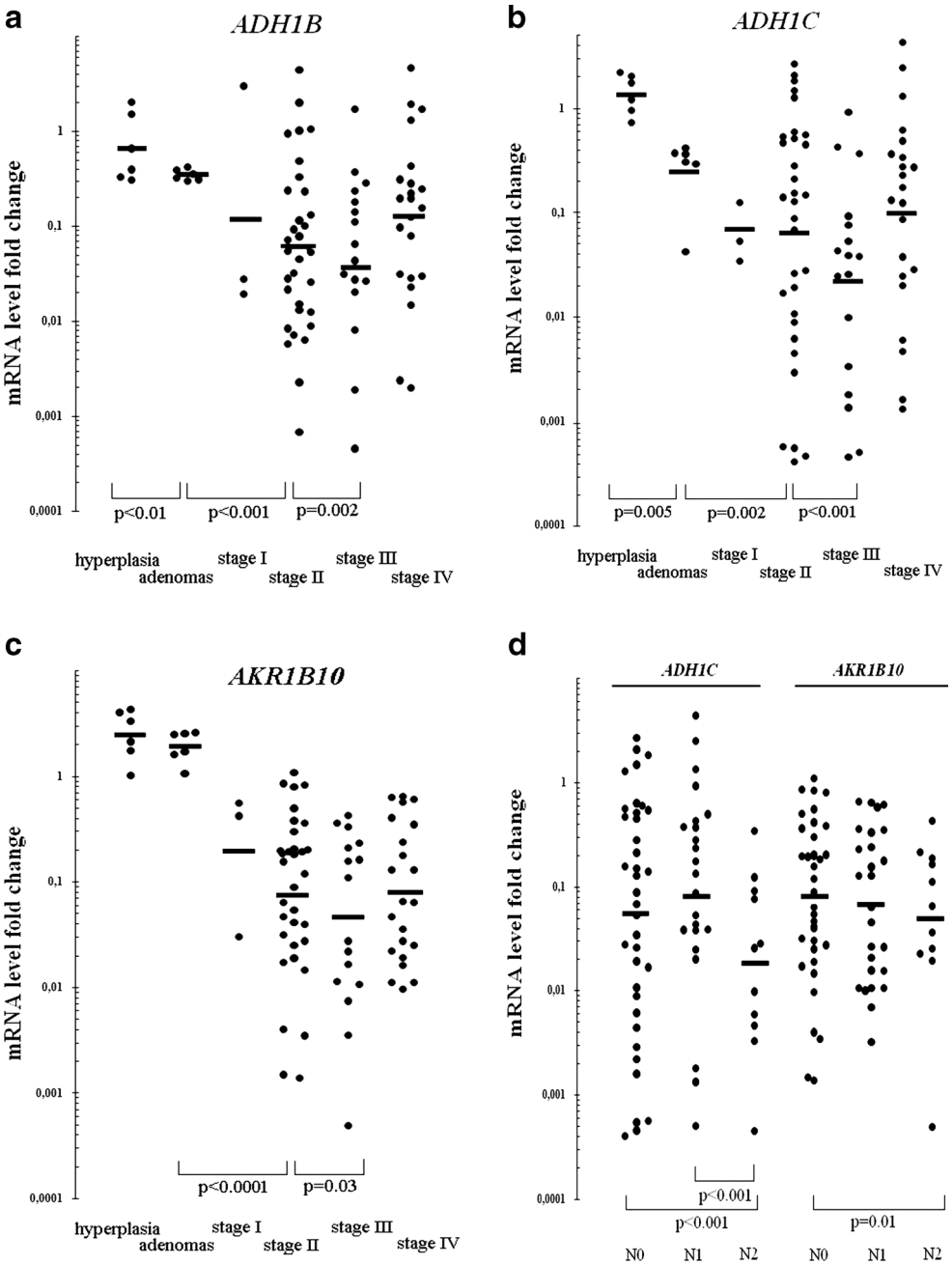

Fig. 2 The changes in mRNA expression level during colorectal cancer progression (a,b,c) and lymph node metastasis formation (d) determined by RT-qPCR. **a**,**b**, **c** Correlation between *ADH1B*, *ADH1C* and *AKR1B10* genes expression and cancer progression. These graphs show the relative mRNA expression (log transformed) in colonic hyperplasia tissues, adenomas and carcinomas, divided by groups according to their pathological stage of progression (I-IV). Each point corresponds to one sample. Averages are marked by a horizontal black line in each group. The corresponding P-values are given below in the figure, except for the stage I group that is represented in only three cases. **d** Correlation between *ADH1C* and *AKR1B10* genes expression and lymph node metastasis. N0 – no regional lymph node metastasis; N1 – metastasis to one to three regional lymph nodes; N2 – metastasis to four or more regional lymph nodes. Abbreviations: *ADH1B* alcohol dehydrogenase 1B gene, *ADH1C* alcohol dehydrogenase 1C gene, *AKR1B10* aldo-keto reductase 1B10 gene

and only *RDH10* showed the decreased expression in about one-fourth of the tumor samples and the enhanced expression levels in one-third of the tumors.

The Expression of Genes Encoding Retinaldehyde-Reducing and Retinaldehyde-Oxidizing Enzymes in Normal Colorectal Tissues and Colorectal Carcinomas

Of the genes encoding all the known retinaldehyde-reducing NADP+—dependent RDHs, we have only detected the expression of the *RDH11* gene in the large intestine. The levels of RDH11 mRNA were not altered in most of the tumor samples (Table 1). The expression levels of AKR1B1 mRNA also remained unchanged in most of the tumor samples. The significantly more abundant mRNA, AKR1B10, displayed the largest expression level changes in the CRC samples. We performed the more detailed expression analysis for this mRNA by real-time RT-qPCR in 80 paired samples (Fig. 2c). In contrast to the RDH11 and AKR1B1 mRNAs, the levels of the predominantly expressed AKR1B10 were markedly decreased in a majority of the tumor samples, even at early stages (87 %, $P<0.00001$).

In humans, the oxidation of retinaldehyde to atRA is catalyzed by three retinoid-active retinaldehyde dehydrogenases,

RALDH1, −2 and −3. The levels of RALDH1 mRNA were approximately 4-fold higher than either RALDH2 or RALDH3 mRNA levels. The expression levels of RALDH1 mRNA were reduced in 60 % of the tumor samples (Table 1); however, the reduction was not significant. Thus, the expression levels of RALDH1 mRNA, which was predominantly expressed in the large intestine, decreased in most of the tumor samples.

Altered Expression of Genes Encoding the atRA-Degrading Enzyme CYP26A1 and Retinol-Binding Protein CRBP1 in Colorectal Carcinomas

From the mRNAs of the three known atRA-degrading enzymes (CYP26A1, −B1 and –C1), only CYP26A1 mRNA was detected in normal colorectal tissues, and its expression was enhanced in half of the tumor samples. The expression levels of mRNA encoding the retinol-binding protein, CRBP1, remained unchanged in most of the tumor samples (94 %, Table 1).

Thus, the expression patterns of almost all of the atRA-generating genes that were identified using a bioinformatic approach were confirmed by reverse transcription-PCR. The exception was the *RALDH2* gene, which was only detected using RT-PCR, but at relatively low levels.

Altered Expression of Genes Involved in atRA Biosynthesis in Colorectal Cancer Cell Lines HCT-116, RKO and HT-29

The expression patterns of the genes implicated in atRA biosynthesis in the human colorectal cancer cell lines, HCT-116, RKO, HT-29, and primary colorectal carcinomas were generally similar. In all colorectal cell lines, the expression levels of the mRNAs of each gene examined were normalized to the ACTB and β2M mRNA levels. We compared the results from the colorectal cancer cell lines to those of the normal human colon and rectum tissues. The mRNA levels of the genes *ADH1B*, *ADH1C*, *RDHL*, *AKR1B10* and *RALDH1* were reduced in all cell lines, and the mRNA levels of the genes *RDH10*, *CYP26A1* and *CRBP1* were not significantly altered. All three cell lines showed very low expression levels of the *ADH1C* gene (Fig. 3).

The Expression of ADH1B, ADH1C and AKR1B10 in Patient-Matched Colonic Hyperplastic and Adenomatous Polyps

In addition to colorectal carcinomas, six patient-matched samples of hyperplastic and adenomatous polyps were also examined. The expression levels of the most abundant in normal colorectal tissues mRNAs, *ADH1B*, *ADH1C* and *AKR1B10*, were determined in hyperplastic tissues and adenomas compared with normal mucosa. The results of RT-qPCR have shown that the relative expression levels of *ADH1B* and

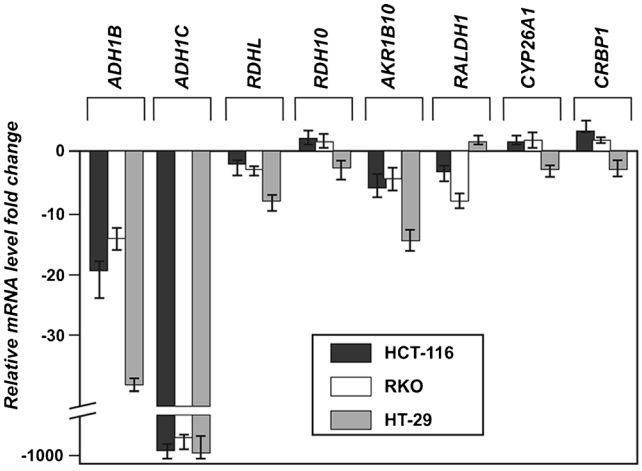

Fig. 3 Real-time quantitative PCR-analysis of the ADH1B, ADH1C, RDHL, RDH10, AKR1B10, CYP26A1 and CRBP1 mRNA levels in human colon cancer cell lines HCT-116, HT-29 and RKO. Data were normalized to *ACTB* and *β2M* and calculated relative to the normal colon tissues. The average mRNA levels in the control normal colon samples were arbitrarily set to 1 and the fold changes are shown. Abbreviations: *ADH1B and ADH1C* alcohol dehydrogenases 1B and 1C, *RDHL* and *RDH10* retinol dehydrogenases L and 10, *AKR1B10* aldo-keto reductase *1B10*, *CYP26A1* Cytochrome P450 26A1, *CRBP1* cellular retinol-binding protein, *ACTB* beta-actin, *β2M* beta2-microglobulin

ADH1C mRNAs were lower in all adenomas than in adjacent normal mucosa at least 2-fold. The mean values of the expression reduction (fold change) were for *ADH1B* – 2.9 (2.7–3.1) and for *ADH1C* – 4.1 (2.4–23.3) (Fig. 2a and b). There were no significant differences in expression of these genes between hyperplastic tissues and normal mucosa. The level of *AKR1B10* mRNA remained unchanged in adenomatous polyps compared with normal mucosa (Fig. 2c).

Correlation Between Aberrant Expression of ADH1B, ADH1C and AKR1B10 in CRC and Clinicopathological Characteristics

The most highly expressed in normal colonic mucosa mRNAs, *ADH1B*, *ADH1C* and *AKR1B10*, displayed the largest expression level changes in CRC. We determined the correlation between their expression data and clinicopathological characteristics. Tumors with unknown stage/N, T, M status (4 samples) or undefined differentiation status (17 samples) and synchronous tumors (2 samples) were excluded from the corresponding statistical calculation. No significant relationships were observed between *ADH1B*, *ADH1C* and *AKR1B10* expression and patients' gender, age, T factor (tumor depth), differentiation status or tumor localization ($P>0.05$). To clarify the possible relationship between aberrant expression of *ADH1B*, *ADH1C* and *AKR1B10* in CRC and tumor progression we determined the correlation between the changes in their expression and pathologic tumor stages as well as the metastatic status. In summary, 74 tumor samples

from early stage I to advanced stage IV (stage I (n=3), stage II (n=32), stage III (n=17), and stage IV (n=22) were analyzed. Six patient-matched samples of normal mucosa, hyperplastic and adenomatous polyps were also analyzed.

The mean values of the expression reduction (fold change) were as follows: for *ADH1B* - adenomas, 2.9, stage I, 8.6, stage II, 17.1, stage III, 27.3, stage IV, 8.13; for *ADH1C* - adenomas, 4.1, stage I, 14.6, stage II, 15.8, stage III, 46.7, stage IV, 10.1, and for *AKR1B10* - adenomas, 1.9, stage I, 5.2, stage II, 13.3, stage III, 21.7, stage IV, 12.6. The highest values for expression decrease of these genes were detected in the group of stage III tumors. These results showed that the level of *ADH1B*, *ADH1C* and *AKR1B10* mRNAs gradually decreased according pathologic stage except for stage IV (Fig. 2). *ADH1B* and *ADH1C* genes display decreased expression during progression from adenoma to early and more advanced stage of colorectal carcinomas (I-II-III).

We determined the correlation between the changes in the expression level of *ADH1B*, *ADH1C* and *AKR1B10* mRNA and the presence of metastases in the lymph nodes (N factor) and distant organs (M factor). No significant relationships were observed between gene expression and M factor (P>0.05). Lymph node status was not available in 37 patients (50 %), 26 patients (35 %) had N1 level (metastasis to one to three regional lymph nodes) and 11 patients (15 %) had N2 level (metastasis to four or more regional lymph nodes) level. The mean values of the expression reduction (fold change) were as follows: for *ADH1B* – N0, 19.3, N1, 20.7, N2, 9.5; for *ADH1C* – N0, 21.4, N1, 15.3, N2, 53.6 and for *AKR1B10* – N0, 12.5, N1, 14.3, N2, 20.5. Significant correlation was found between *ADH1C* down-regulation and higher N levels (P<0.001) (Fig. 2d). Expression level of *AKR1B10* in patients without lymph node methastasis was greater than that in those with lymph node methastasis (P=0.03) and significantly higher than that in patients with N2 level (P=0.01) (Fig. 2d).

Discussion

Retinoic acid, a pleiotropic regulator of gene expression, is essential in embryogenesis, epithelial differentiation, vision and the functioning of the nervous and immune system. Multiple enzymes catalyze atRA biosynthesis in humans. These enzymes exhibit different catalytic activity, substrate and cofactor preference, as well as tissue-specific patterns of expression [5–7].

A number of studies have implicated retinoids in normal colonocyte function and in colorectal carcinogenesis (i.e., inhibition of cell growth, promotion of cell adhesion and suppression of colon cancer cell invasiveness) [18, 19]. In humans, a significant level of retinoic acid is found in normal colon mucosa [20]. The dietary retinyl esters and β-carotene might be converted to retinol in the small intestine and transported to the liver for storage [4]. In contrast to the small intestine and liver, the high catalytic efficiency of cytosolic retinol oxidation has been observed in the colon [21]. Thus, retinol is the primary source for atRA biosynthesis in the large intestine (Fig. 4).

The impaired conversion of retinol to atRA has been observed in several cancer types, including colorectal [22], prostate [23], breast [24], ovarian [25], gastric [26] and other cancers. Strongly diminished atRA biosynthesis activity has been shown to occur in parallel with the downregulation of RDHL and RDH5 in many colorectal cancer cell lines, including HT-116, RKO and HT-29 [22]. However, these enzymes exhibit dual retinol/sterol substrate specificity and have

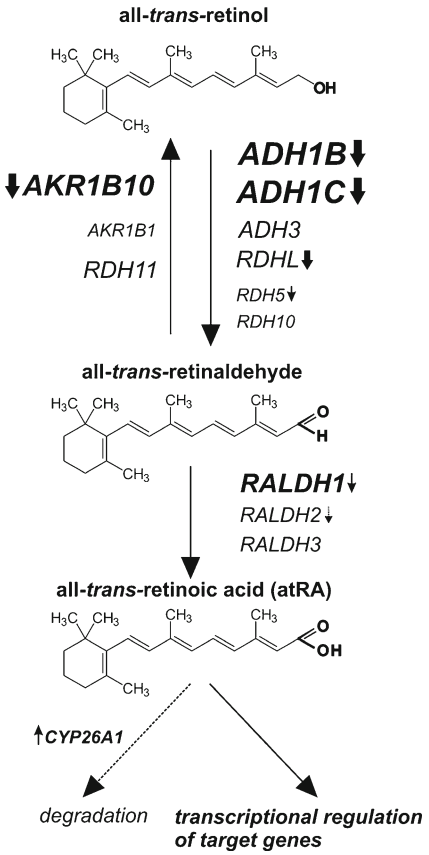

Fig. 4 Altered expression of genes involved in all-trans-retinoic acid (atRA) biosynthesis in human colorectal cancer. In the normal colorectal mucosa, all-*trans*-retinol is reversibly oxidized to all-*trans*-retinaldehyde by alcohol dehydrogenases ADH1B, ADH1C, ADH3 and NAD+−dependent retinol dehydrogenases RDH10, RDHL, RDH5. The conversion of retinaldehyde back to retinol is catalyzed by NADP+−dependent RDH11 as well as aldo-keto reductases AKR1B10 and AKR1B1. All-*trans*-retinaldehyde is irreversibly oxidized to retinoic acid by retinaldehyde dehydrogenases RALDH1, −2 and −3. atRA can be degraded by cytochrome CYP26A1. Black arrows indicate the changes in mRNA level in colorectal cancer specimens relative to normal tissues. Thickness and size of the font symbols for genes involved in atRA production reflect the levels of their expression in the normal colorectal mucosa and the thickness and size of the arrows - the levels of the mRNA expression alterations in tumors in comparison to adjacent normal tissues

low activity for retinol oxidation. A decrease in their expression levels may not be the main cause of the dramatically reduced atRA biosynthesis observed. The key enzymes contributing to atRA generation in normal colorectal tissues and colorectal carcinomas remained unknown.

It should be noted that the majority of recent studies describing the retinoid acid pathway have focused on separate genes or proteins involved in this process [22, 27, 28]. A few studies have reported the altered expression of many participants in the retinoid metabolism in several non-cancerous diseases, such as uterine fibroids [29], non-alcoholic fatty liver disease [30], and testicular feminization syndrome in mice [31]. Using bioinformatic approaches, we selected genes associated with atRA biosynthesis that are expressed in colorectal tissues. Most of the genes exhibit altered gene expression levels in colorectal tumors compared with normal tissues. RT-PCR analysis confirmed that almost all of these genes display various changes in expression levels. Two ADH1 mRNAs exhibited the largest alteration in expression levels among all of the mRNAs studied in this work. *ADH1B* and *ADH1C* are found in colorectal tissues at much higher levels than all the genes encoding the retinol-oxidizing enzymes, including RDH10. The latter has been shown to exhibit the lowest K(m) value for all-*trans*-retinol (~0.035 μM) among all NAD+—dependent retinoid oxidoreductases [13]. RDH10 is the dominant RDH in atRA metabolism during mouse embryogenesis [32]. ADH enzyme family plays an important role in atRA biosynthesis in postnatal and adult tissues [6]. Genetic studies have supported the proposal that ADH1 may be important for retinol oxidation into retinaldehyde in vivo. $Adh1^{-/-}$ mutant mice exhibit a 10-fold decrease in atRA production compared with wild-type mice [6, 11]. The treatment with the specific cytosolic ADH inhibitor, 4-methylpyrazole, resulted in the reduction of atRA formation in mice [33]. Importantly, among the different classes of ADHs, ADH1 is the most sensitive enzyme to this inhibitor [34]. ADHs are cytosolic enzymes and Parlesak with co-workers showed the high catalytic efficiency of retinol oxidation in cytosol of colonic mucosa [21]. We demonstrated that the expression levels of *ADH1B* and *ADH1C* were sharply decreased in a majority of the tumor samples, while *RDH10* showed the decreased mRNA levels only in about one-fourth of the tumor samples and the elevated mRNA levels in one-third of the CRC specimens. It is known that rectal cancer tissues showed a striking reduction in ADH1 proteins immunostaining intensity compared with normal tissues [35].

Some published values for the activity levels of ADH isozymes varied widely, although the average kinetic constants of retinol-active ADH1 do not differ substantially from that of RDH10 (Km (μM) 0.035–0.182) [36]. The comparison of kinetic constants must be done with great caution because their definition is often performed using different approaches. The expression levels of RDHL and RDH5 was also decreased. The expression levels of ADH3 mRNA remained unchanged in most of the tumor samples, but the contribution of the corresponding enzymes in the shift of the retinol-oxidizing reaction was significantly less compared with ADH1 when considering the expression levels and known retinoid activity. Therefore, the dramatic diminution in the expression levels of genes encoding the predominant retinol-oxidizing enzymes could result in a significant decrease in retinaldehyde production and the accumulation of retinol.

Among the genes encoding retinaldehyde-reducing enzymes, *AKR1B10* is significantly more abundant in normal colorectal tissues than the *RDH11* and *AKR1B1* genes. The retinal-reducing activity of AKR1B10 is comparable with that of RDH11 and 100-fold times higher than that of AKR1B1 [10]. AKR1B10 is upregulated in both human non-small cell lung and hepatocellular carcinomas [27, 37, 38]. In contrast, the expression levels of the *AKR1B10* gene are significantly reduced in gastric cancer [39] and CRC ([40] and in this study). Thus, we observe the dysregulation of the conversion of retinaldehyde to retinol in CRC samples compared with normal tissues.

Cytochrome CYP26A1 is capable of metabolizing atRA to inactive polar metabolites [15]. The increased expression levels of *CYP26A1* indicate that the degradation of atRA is enhanced in most of CRC samples. The overexpression of *CYP26A1*, the major player in the degradation of atRA in CRC, and the downregulation of the gene encoding the predominant retinal-oxidizing enzyme, RALDH1, could also result in a decrease in atRA formation.

The human colorectal cancer cell lines, HCT-116, HT-29 and RKO, and colorectal carcinomas showed a similar pattern of changes in the expression levels of atRA-generating genes (Fig. 4). It should be noted, that highly diminished atRA biosynthesis has been demonstrated in these cell lines [22].

The loss of RA biosynthetic genes may contribute to the lack of differentiation observed in colon adenomas and carcinomas. Jette with co-workers present evidence supporting a lack of retinoic acid biosynthesis as a mechanism contributing to the development of colon adenomas and carcinomas. The authors found that reintroduction of the tumor suppressor adenomatous polyposis coli (APC) into APC-deficient colon cancer cells increases conversion of retinol to atRA and propose that APC controls retinoic acid biosynthesis and promotes retinoid-induced program of colonocyte differentiation [22].

The functional consequences of atRA biosynthesis impairment can also involve the toxicity due to the increased retinol levels. The retinol toxicity has been demonstrated in $Adh1^{-/-}$ mice with a strongly diminished retinol metabolism to atRA [11]. Retinol can undergo hydroxylation by cytochrome P450s, thereby forming reactive oxygen species or other toxic byproducts [41]. High levels of retinol may likely disrupt many cellular processes through non-specific binding of

retinol. Studies on ADH knockout mice have indicated that ADH1 is a major protective factor against retinol toxicity [11]. CRBP1 regulates the cellular uptake of retinol and plays a role in retinol storage. As the expression levels of CRBP1 mRNA remain primarily unchanged in most of the tumor samples examined, the storage capacity for retinol may not be increased despite its accumulation in CRC.

Reductions in the expression of the most abundant in normal colonic tissues *ADH1B* and *ADH1C* genes have been observed in adenomatous colonic polyps, which are considered to be preneoplastic lesions. Downregulation of these genes highly correlate with progression along the normal mucosa-adenoma-advanced tumor stages sequence. Reductions in *AKR1B10* expression also correlated with different pathological stages, showing consistent down-regulation along with cancer progression. We found a correlation between *ADH1C* and *AKR1B10* gene expression and the degree of node metastasis (Fig. 2). Lymph node involvement is one of the most important prognostic factors for colorectal cancer. Thus, the changes in the expression of these genes may play an important role in the CRC development and progression.

Most of the enzymes involved in atRA biosynthesis are multifunctional. ADHs, AKRs and RALDHs may be also involved in protection against alcohols and aldehydes [42, 43]. Their decreased expression levels in the large intestine could lead to various effects associated with both atRA deficiency and the toxicity of alcohols and aldehydes.

In conclusion, our analysis of the expression levels of genes involved in atRA biosynthesis in normal human colorectal tissues and colorectal carcinomas showed that this process is disrupted at each step of the pathway. Significant alterations in the expression levels of key genes involved in retinol oxidation can result in retinol accumulation and a decrease in atRA production. The most abundant of these genes, *ADH1B* and *ADH1C*, display decreased expression during progression from adenoma to early and more advanced stage colorectal carcinomas. The diminished atRA biosynthesis may lead to alteration of cell growth and differentiation in the colon and rectum, thus contributing to the progression of colorectal cancer.

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Conflict of Interest The Authors declare that there is no conflict of interest.

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