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RESEARCH

The Influence of Methylated Septin 9 Gene on RNA and Protein Level in Colorectal Cancer

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Abstract Colorectal cancer is one of the leading death causes in the world. Specificity and sensitivity of the present screening methods are unsuitable and their compliance is too low. Nowadays the most effective method is the colonoscopy, because it gives not only macroscopic diagnosis but therapeutic possibility as well, however the compliance of the patients is very low. Hence development of new diagnostic methods is needed. Altered expression of septin 9 was found in several tumor types including colorectal cancer. The aim of this study was to detect the methylation related mRNA and protein expression changes of septin 9 in colorectal adenoma-dysplasia-carcinoma sequence and to analyze its reversibility by demethylation treatment. Septin 9 protein expression showed significant difference between normal and colorectal cancer (CRC) samples ($p<0,001$). According to biopsy microarray results, septin 9 mRNA expression decreased in the progression of colon neoplastic disease ($p<0,001$). In laser microdissected epithelial cells, septin 9 significantly underexpressed in CRC compared to healthy controls ($p<0,001$). The expression of septin9_v1 region was higher in the healthy samples, while septin9_v2, v4, v4*, v5 over-expression were detected in cancer epithelial cells compared to normal. The septin 9 mRNA and protein levels of HT29 cells increased after demethylation treatment. The increasing methylation of septin 9 gene during colorectal adenoma-dysplasia-carcinoma sequence progression is reflected in the decreasing mRNA and protein expression, especially in the epithelium. These changes can be reversed by demethylation agents converting this screening marker gene into therapeutic target.

Keywords Colorectal cancer · mRNA expression · Peripheral blood · Protein expression · Septin 9

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Introduction

Colorectal cancer is one of the leading death causes in men and women worldwide. In 2004, there were 2 886 800 cases of diagnostised cancer (376 400 CRC) and 1 711 000 of cancer caused death (203 700 CRC) in the European countries. However, the most common cancer is lung cancer in Europe followed by colorectal cancer with 13,2% incidence [1]. In the United States the new cancer cases were 1 372 910 (145 290 CRC) and the estimated deaths were 570 280 (56 290 CRC) in 2005 [2]. Specificity and sensitivity of the present screening methods are unsuitable and their compliance is too low. The current non-invasive screening is the fecal occult blood test (FOBT), which can detect the presence of blood in the stool, but its efficiency is not sufficiently high. Nowadays the most effective method is the colonoscopy, because it gives not only macroscopic diagnosis, but therapeutic possibility as well, however the compliance of the patients is very low. Hence development of new diagnostic methods is needed.

Genetic and epigenetic alterations in colorectal neoplasia have been extensively studied as markers for specific detection of the disease. Recently, several molecular assays get into the focus, which can be suitable for demonstration

biomarkers from peripheral blood. Detection of disease-specific DNA methylation alterations can be a new possibility, because DNA methylation plays role in early tumorigenesis. Numerous genes and/or their regulatory regions such as CpG islands in promoters have been found hypermethylated in neoplasia including colorectal cancer. Hypermethylation of Aristaless-like homeobox-4 (ALX4) gene was detected by Ebert et al. in surgically removed colon adenoma and cancer tissue specimens, furthermore in serum and plasma samples using methylation specific polymerase chain reaction [3]. They also demonstrated high frequency of ALX4 gene methylation in a large set of gastrointestinal cancers suggesting that the methylation of this gene may serve as a marker for gastrointestinal adenocarcinomas [4]. High methylation level of septin 9 (SEPT9) gene was also identified in colorectal cancer samples using methylation specific real-time PCR. In these studies, CRC could be detected with 68%–72% sensitivity and 89%–90% specificity. Positive rates for plasmas from the other cancers and non-cancerous samples were low, therefore the septin 9 can be a positive marker for colorectal cancer non-invasive screening [5, 6].

Septin 9 mRNA and protein expression have not been analyzed in parallel yet, hence the effects of septin 9 methylation status to its mRNA and protein level is not clarified. In this study, we investigated septin 9 mRNA and protein expression in colorectal diseases. Our aims were the quantification and characterization of septin 9 mRNA and protein expression in every stage of the colorectal adenoma-dysplasia-carcinoma sequence to establish the different expression pattern of septin 9 from normal to cancerous tissues. Furthermore we evaluated the septin 9 mRNA and protein expression changes in HT29 colon adenocarcinoma cells after demethylation agent treatment.

Materials and Methods

Patients and Samples

Biopsy samples were taken from 7 healthy, from 13 patients with adenoma (six with low-grade dysplasia, seven with high-grade dysplasia) and from 15 with colorectal cancer during endoscopic intervention and stored in RNALater Reagent (Qiagen Inc., Germantown, US) at −80°C. Additionally, surgical specimens were collected from 6 adenoma and 6 colorectal cancer (moderately differentiated, Dukes B stage, left side adenocarcinoma) patients and 6 histologically normal tissues near to the resection end for laser microdissection assay. Histological diagnoses were determined by a pathologist. Tissues were immediately frozen in liquid nitrogen and stored at −80°C. All patients provided written informed consent.

Cell Culture

1,5 million HT29 colon adenocarcinoma cells/tissue culture flasks were raised in RPMI-1640 medium containing 10% FCS (Sigma-Aldrich, St. Louis, US) at 37°C in 5% CO2. Cultured HT29 cells were treated by 10uM and 20 uM 5-aza-2′-deoxycytidine (5-aza) (Sigma-Aldrich) demethylation agent for 72 h in FCS-free medium. Molecular biology grade water and acetic acid were used as control in 1:1 ratio. Total RNA was applied for whole genomic microarray analysis. In parallel, 40 000 20 uM 5-aza-2′-deoxycytidine treated and control cells/slide were cytocentrifuged and fixed for immunocytochemical analysis.

Laser Microdissection

Frozen tissue specimens were cut onto PALM membrane mounted glass slides at −20°C. The slides were stored −80°C until the microdissection. The frozen sections were fixed in ethanol series, and were stained with cresyl violet (Sigma-Aldrich). Five thousand epithelial cells and 10 000 stromal cells were collected from six different histological regions of the colon (from healthy, adenomatous and tumorous epithelium and stroma) using PALM laser microdissector (PALM, Bernried, Germany).

Real-time PCR was applied in order to examine the expression of septin 9 splice variants in LCM (laser capture microdissection) epithelium samples. Beta-actin was used as reference gene. One μg of the isolated total RNA was reverse transcribed using the Transcriptor First Strand cDNA Synthesis Kit (Roche). Real-time PCR was performed using LightCycler DNA Master SYBR Green I (Roche) and primers for septin 9 splice variants. The sequences of the applied primers were the same as previously described by Scott et al. [7]. After enzyme activation at 95°C for 5 min, 45 PCR cycles were performed (denaturation at 95°C for 10 s, annealing at 61°C for 10 s and extension at 72°C for 20 s) which was followed by a melting curve analysis.

Microarray Analysis

Total RNA was isolated from the biopsy samples and from HT29 cells using RNeasy Mini Kit (Qiagen), from catapulted cells using RNeasy Micro kit (Qiagen) according to the manufacturer's instructions. The quantity of the extracted RNA was controlled by spectrophotometer (NanoDrop, Thermo Fisher Scientific Inc., Waltham, US) and quality checking was performed by capillary gelelectrophoresis (2100Bioanalyzer and RNA 6000 Pico Kit, Agilent Inc, Santa Clara, US).

In case of the biopsy and cell culture samples, first and second strand cDNAs were synthesized from 2,6216±0,662 ug

total RNA, then were amplified and labeled by in vitro transcription. In case of LCM samples, Two-Cycle Target Labeling and Control Kit (Affymetrix Inc., Santa Clara, US) was applied according to the manufacturer's descriptions. After fragmentation, 8 ug of cRNA from each samples was hybridized into HGU133 Plus2.0 arrays (Affymetrix) at 45°C for 16 h. Microarrays were washed and stained using streptavidin-phycoerythrin (Invitrogen, Carlsbad, US). The fluorescent signals were detected using GeneChip Scanner 3000. Data files of biopsy microarrays were used in a previously published study in different comparisons [8] and are available in the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo) (GSE4183, normal: GSM95473-GSM95480, adenoma: GSM95481-GSM95495, CRC: GSM95496-GSM95510). Data files of microarrays from laser microdissected samples are available in the GEO database as well (http://www.ncbi.nlm.nih.gov/geo) (GSE15960, normal: GSM400186-GSM400191, adenoma: GSM400174-GSM400179, CRC: GSM400180-GSM400185).

Immunohistochemistry

Paraffin-embedded ten healthy, ten villous adenoma and ten colorectal cancer (Dukes A, B, C) tissues were used for immunohistochemical analysis. 4 um thick tissue sections were cut and stained with septin 9 polyclonal antibody in 1:50 dilution (Abnova, Walnut, US) and goat anti-mouse IgG (H+L) (Invitrogen) secondary antibody in 1:100 dilution. Nuclear co-staining using Hoechst No. 33258 (Sigma-Aldrich) was performed. Immunoreactivities for septin 9 were detected using digital microscope (Mirax Viewer V.1,11,43,0, Zeiss, Gottingen, Germany) and the slides were scanned using Mirax Desk with AxioCam Mrm Rev.3 camera (Zeiss). Septin 9 staining was evaluated in 250 to 400 epithelial cells of each case. Septin 9 immunopositivity of normal colon tissue was compared with the staining in adenoma and colon tumor tissue. The percentage of positive cells were determined (labeling index LI) both in the surface and in the glandular epithelium. Only the epithelial cells showing perinuclear septin 9 staining were considered as positive cells.

Cytocentrifuged control and 5-aza-2′-deoxycytidine treated HT29 cells were incubated in methanol-hydrogen peroxide mixture in order to block the endogenous peroxidase activity. Bovine serum albumin (Sigma-Aldrich) was used for inhibition of aspecific binding. The samples were stained by septin 9 polyclonal antibody in 1:50 dilution (Abnova). EnVision™+/HPR anti-mouse secondary antibody (Dako, Glostrup, Denmark) were applied. DAB (diaminobenzidine)+substrate chromogen system was applied for visualization of the staining. Nuclear co-staining using hematoxylin solution (Sigma-Aldrich) was performed. Slides were scanned using Mirax Desk (Zeiss) and evaluated using digital microscope (Zeiss). Percentage of septin 9 positive cells was determined (labeling index-LI) in at least 1000 cells.

Statistical Analysis

In case of microarray analysis, GCRMA with quantile normalization and median polish summarization methods were applied. After preprocessing, SAM (Significance Analysis of Microarray) was applied on the three different sample types (biopsy, laser microdissected and HT29 cell culture). The expression differences were characterized by logFC (log intensity differences) and p values (probability level of the different stages). Further statistical examinations were carried out on adequate transcripts by ANOVA and by TukeyHSD. Expression differences were featured by p values and presented on boxplots.

Results

Septin 9 mRNA Expression in Biopsy Samples

Septin 9 mRNA expression levels in biopsy samples were detected using four different Affymetrix IDs (207425_s_at, 41220_at, 208657_s_at and 1559025_at) of the gene (www.affymetrix.com/analysis/index.affx). 207425_s_at transcript significantly differentially expressed in normal colon versus low-grade, high-grade dysplastic adenoma and Dukes A, B stage colorectal cancer. According to this mRNA expression difference, healthy samples can be separated from the other diagnostic groups. Furthermore, significant septin 9 expression differences were detected between low-grade dysplastic adenoma versus Dukes CD stage CRC, high-grade dysplastic adenoma versus Dukes CD stage CRC and Dukes AB stage CRC versus Dukes CD stage CRC (adjusted p values >0,01) (Fig. 1a).

The expression of the 1559025_at transcript was similar in healthy and low-grade dysplastic adenoma biopsies, but decreased in high-grade dysplasia and in malignant samples (Fig. 1b). This expression difference was statistically significant, but it was minimal. However, clear separation can be seen between healthy, low-grade dysplastic adenoma versus high-grade adenoma and CRC samples.

The expression of other AFFY IDs did not alter (41220_at, 208657_s_at) in the different sample groups.

Septin 9 mRNA Expression and Splice Variant RT-PCR Analysis in Laser Microdissected Samples

Septin 9 mRNA expression was detected in stromal and epithelial cells from surgical samples using four different AFFY IDs (207425_s_at, 41220_at, 208657_s_at and

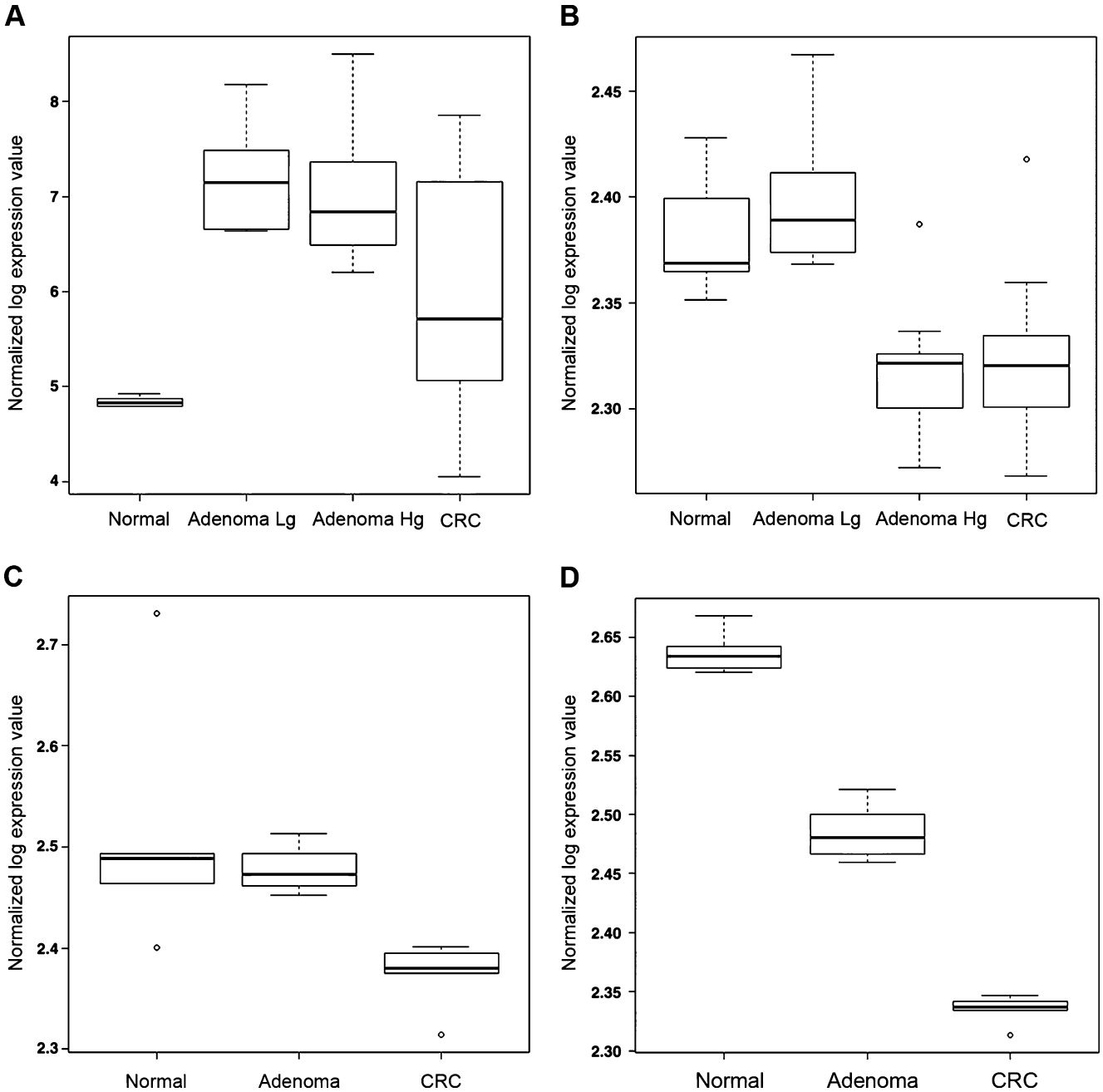

Fig. 1 Normalized log expression values of 207425_s_at transcript (A) and 1559025_at transcript (B) in biopsy samples. Normalized log expression values of 1559025_at in laser microdissected stroma (C) and epithelium samples (D)

1559025_at) of septin 9 gene (www.affymetrix.com/analy sis/index.affx). Only 1559025_at showed differences between the sample groups. In stromal cells, septin 9 mRNA levels did not show obvious differences between healthy, adenoma and cancer samples. In healthy and adenoma samples, similar septin 9 expression was found, while cancer stromal samples showed lower expression levels (Fig. 1c). In epithelial cells, small mRNA expression reduction was detected according to the disease progression. The septin 9 mRNA level was significantly lower in adenoma epithelial cells compared to normal (adjusted p value <0,05), and it was more down-regulated in CRC (adjusted p value <0,01) (Fig. 1d).

Real-time PCR assay was performed from laser micro-dissected healthy and colorectal cancer epithelial samples using SEPT9_v1, v2, v4, v4* and SEPT9 _5 splice variant primers. SEPT9_v2, v4, v4* and v5 splice variants over-expressed in the tumorous epithelium compared to normal (v2: 17,4-fold, v4: 2-fold, v4*: 12,5-fold and v5: 2-fold). Lower SEPT9_v1 expression was observed in cancer than in normal epithelial cells (v1: 0,5-fold) (Fig. 2).

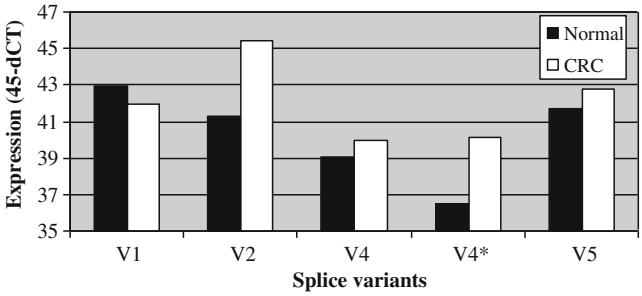

Fig. 2 Expression of septin 9 splice variants in laser microdissected epithelial cells

Immunohistochemistry in Colon Tissues

In normal samples, septin 9 protein strongly expressed both in the surface and in the glandular epithelium. The immunopositivity was stronger in the surface epithelium than in glandular. Septin 9 immunostaining was weaker in the adenomatous epithelium (25,78%±7,6%) than in the normal (43,6%±8,1%) ($p<0,05$). In adenoma samples, septin 9 protein expressed not only in the perinuclear area, but diffuse septin 9 positivity was also detected in the total cytoplasm. Septin 9 showed very weak intensity in the tumor tissue (8,89%±2,14%). Perinuclear positivity was not found in tumor tissues, but immunopositive molecules formed small particles in the cytoplasm (Fig. 3).

Septin 9 mRNA Expression in 5-aza Treated HT29 Cells

Small septin 9 expression differences were found between control and 5-aza treated HT29 cells. Little septin 9 mRNA elevation was detected in demethylation agent treated cells (mean: 4,548±0,645) compared with the controls (mean: 4,475±0,055), but this expression alteration was not significant (adjusted p value >0,05). This can be the result of demethylation caused by 5-aza-2′-deoxycytidine. Ex-

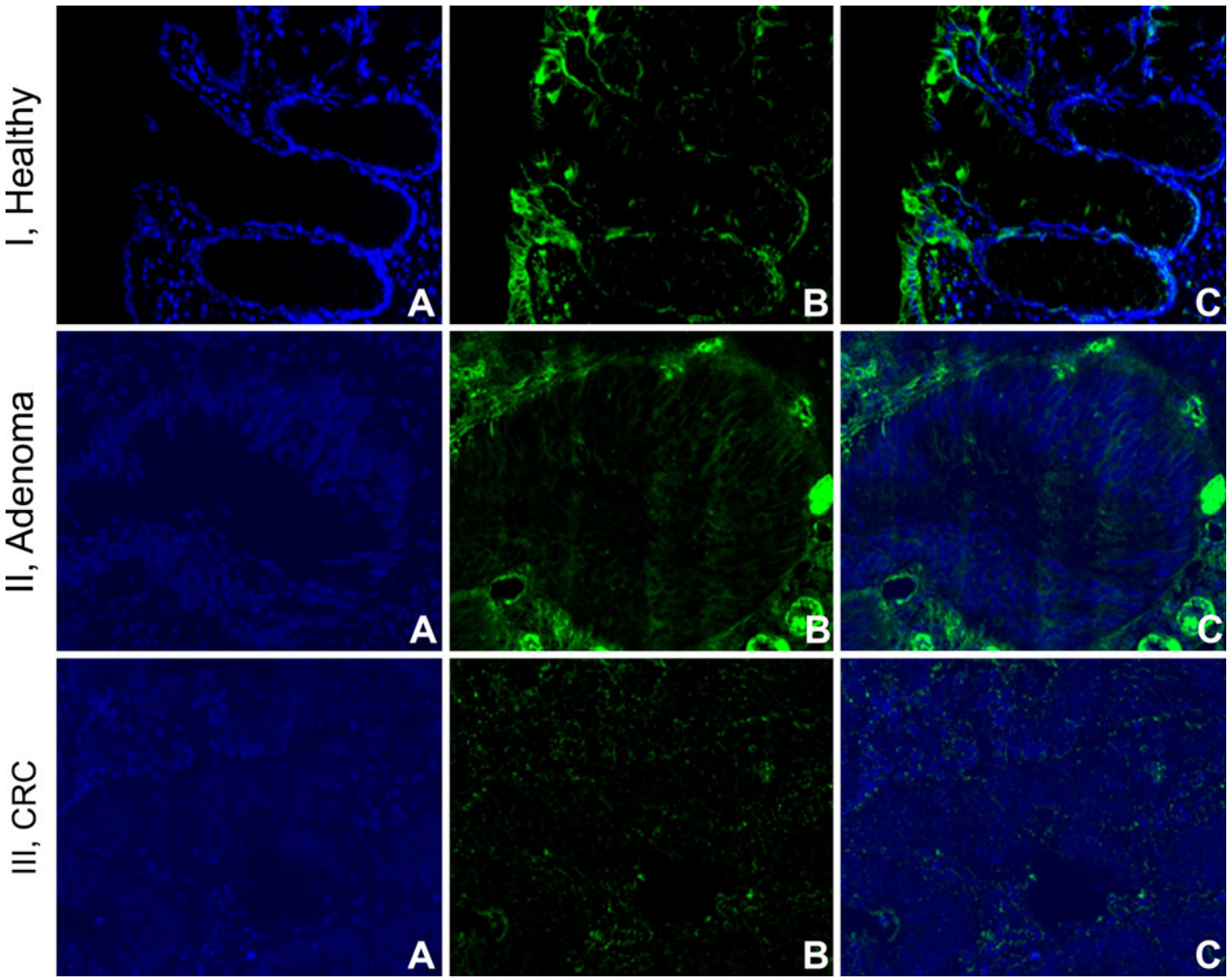

Fig. 3 Fluorescence immunohistochemical expression of septin 9 through the adenoma-dysplasia-carcinoma sequence. I: Healthy, II: Adenoma, III: CRC; A: nuclear staining, B: Septin 9 staining, C: nuclear and septin 9 staining

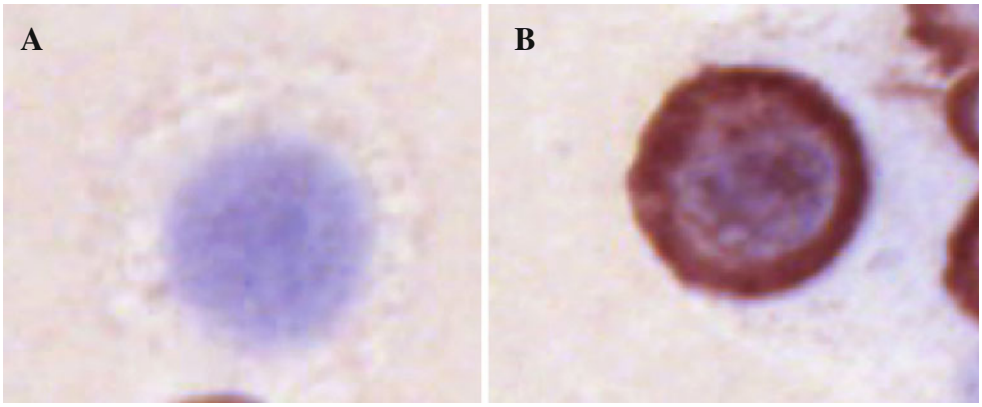

Fig. 4 Septin 9 immunohistochemistry in cytocentifuged control and demethylation agent treated HT29 cells. A: septin 9 negative, B: septin 9 positive cell with dark brown cytoplasm

panded sample number is needed in order to reach the significance $p<0,05$.

Immunohistochemistry in 5-aza Treated HT29 Cells

In control cells, septin 9 positive cell ratio was 56,95% (1105 positive cells from 1940), while the treated sample showed higher percentage of positive cells (63,9%, 1975 positive cells from 3089) (Fig. 4).

Discussion

Septins have been originally detected in cell division cycle mutant yeast, but nowadays it is became known that septin proteins play role in several cellular processes. There are 12 human septin genes which have been implicated in neoplasia, furthermore in neurological and infectious diseases [9]. Epigenetic alterations of septin 9 gene were found in several diseases, including ovarian and breast cancer [10–13], leukemia [14–16], urologic cancer [17] and brain tumors [18]. Septin 9 showed hypermethylation in colon cancer, as well [3–5, 19, 20].

In this study, we analyzed septin 9 protein and mRNA expression in biopsy samples, laser microdissected and HT29 adenocarcinoma cells using immunohistochemistry and whole genomic microarrays.

In biopsy assays, septin 9 significantly differentially expressed in healthy, adenoma and colorectal cancer samples, but the direction of the expression changes were different in case of the distinct Affymetrix IDs. The expression of 207425_s_at transcript was lower in the healthy biopsies than in the others (Fig. 1a). Decreasing expression tendency was detected from low-grade dysplastic adenoma in association with the disease progression. The epithelial expression of septin 9_v2, v4, v4* and v5 splice variants correlated with these findings (Fig. 2). Similarly to the previous studies [21, 22], these septin 9 variants showed overexpression in the tumors compared to normal controls. The lower intensity in healthy samples can be caused by DNA methylation in the promoter region which can block the transcription, hence the mRNA expression decreases. The expression level of 207425_s_at transcript was the same in advanced CRC like in healthy samples. Using whole biopsy samples containing both epithelial and stromal layers may explain this observation.

The other transcript represented by 1559025_at Affymetrix ID showed higher expression in healthy and low-grade dysplastic adenoma biopsy samples, while it was lower in high-grade dysplastic adenoma and CRC (Fig. 1b). Septin9_v1 expression in laser microdissected epithelial cells correlated with these results (Fig. 2), however this splice variant was found to be overexpressed in breast and ovarian tumors [21, 23]. This decreasing expression was also shown between laser microdissected healthy, adenoma and cancer samples using microarrays (Fig. 1c, d). The expression tendencies detected in stroma and epithelium suggest that the septin 9 signs mainly came from the epithelial layer.

In accordance with the mRNA expression changes, we demonstrated gradually decreasing septin 9 protein expression during the colorectal normal-adenoma-carcinoma sequence (Fig. 3). Similarly to the previous findings [18], the expression of SEPT9 protein was found to be perinuclear and cytoplasmic also in our study. In macroscopic morphology, the number of goblet cells decreased during CRC development, and it may be in correlation with septin 9 hypermethylation and dowregulation of its mRNA and protein in CRC.

Reexpression of hypermethylated and hence downregulated genes can be induced by 5-aza-2-deoxycytidine demethylation agent. After 5-aza-2-deoxycytidine treatment, increased androgen receptor expression was detected in human leukemia cells [24], and estrogen receptor beta gene demethylation was found in ovarian cancer cell lines [25]. Significant correlation was proved between oncostatin M (OSM) hypermethylation and loss of mRNA expression in colorectal cancer cell lines by demethylation of OSM by 5-

aza causing its reexpression [26]. Our demethylation agent treatment assay showed similar mRNA expression elevation as previously found. In our study, increased septin 9 mRNA and protein expression were observed in HT29 colorectal adenocarcinoma cell line which suggests the presence of DNA hypermethylation of septin 9 gene (Fig. 4).

Discovery of DNA methylation became the part of molecular biology in cancer research. DNA methylation based separation of healthy from colorectal cancer or adenoma from cancer cases can establish new therapeutic possibilities. With the help of these methods not only the early cancer detection, but individual and targeted therapy can be done in the future.

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