Human Colorectal Cancer Stage-dependent Global DNA Hypomethylation of Cancer-associated Fibroblasts

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Abstract. Background/Aim: Cancer-associated fibroblasts (CAFs) play an important role in tumor development and progression. The prevailing consensus favors the view that a specific epigenetic signature underpins the stable CAF phenotype. The aim of the present study was to assess global DNA methylation in CAFs during the adenoma–carcinoma sequence in non-familial sporadic human colorectal cancer (CRC). Patients and Methods: Immunohistochemical staining of nuclear 5-methylcytosine (5'-meCyt) was performed in matched samples of colonic tumor tissue and normal colonic mucosa excised from six patients with adenomas and four with adenocarcinomas. The staining intensity was expressed semi-quantitatively as the immunohistochemical staining score (ISS). Results: ISS values of human colonic CAFs and adenomatous samples were 14.00±2.2 and 14.08±1.8, respectively, showing no statistically significant difference. In contrast, a marked trend was found towards global DNA hypomethylation in CAFs from adenocarcinomatous specimens compared to matched normal mucosa: ISS: 9.25±2.44 (range=6-11) vs. 16.17±0.75, respectively, p<0.03. Conclusion: Final stages of cancer development in CRC are associated with global DNA hypomethylation in stromal CAFs.

Traditionally, studies of neoplastic growth have focused on molecular events in tumor cells, whereas the stromal fibroblasts surrounding the tumor mass were considered mere bystanders and, consequently, their role in cancer development and progression have remained largely neglected. Accruing information is now available showing that these mesenchymal cells are conscripted and educated by the adjacent cancer cells and as cancer-associated fibroblasts (CAFs) they participate in a malevolent alliance with their rogue neighbors, promoting their unrelenting growth and invasive program (1-4). Of note, however, evidence [reviewed in (5)] shows that this is not invariably the case: stromal components may act to impede early-stage tumorigenesis. In this scenario, the desmoplastic response represents an initial host defense designed to restrain the growth of the incipient tumor. A characteristic hallmark of CAFs is that they can be propagated in vitro and in vivo for extended periods of time without loss of their cancer-promoting phenotype (6, 7). Much evidence favors the prevailing opinion that this phenotypic stability is due to cancer-imposed epigenetic changes rather than to clonal somatic mutations (8-12) [reviewed in (3, 4, 13-14)].

Epigenetics refers to somatically inheritable changes in gene expression that do not require changes in DNA nucleotide sequence. A salient example of epigenetics is the control of gene activity by DNA methylation, i.e. the addition of a methyl group to the 5' carbon of the pyrimidine cytosine within a DNA CpG dinucleotide sequence, an enzymatic reaction catalyzed by DNA methyltransferases (DNMTs) (Figure 1). Methylation extends through the promoter and start site of a gene and typically prohibits the critical interaction of transcription factors with DNA-response elements. Thereby, DNA site-specific hypermethylation is associated with heterochromatin and gene silencing. Conversely, DNA hypomethylation results in transcriptionally active euchromatin leading to brisk gene expression (15).

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Global DNA hypomethylation has been described in CAFs derived from a large number of solid tumors including human gastric carcinomas (10, 11). An early work reported global DNA hypomethylation in the stromal proteoglycan versican gene in human colonic carcinoma cells (16), an interesting observation since full-length human recombinant versican expression in cultured murine fibroblasts was shown to induce a myofibroblast-like phenotype (17), a well-recognized hallmark of CAFs.

The aim of the present study was to assess global DNA methylation in CAFs at the various stages of the human non-familial sporadic CRC adenoma–adenocarcinoma sequence using a validated immunohistochemical assay that reports the staining intensity of 5'-methyl cytosine (5'-meCyt) in the cell nucleus.

Materials and Methods

Patients. Colonic tissues were obtained from biopsies taken from eight patients at various stages of the CRC adenoma–carcinoma sequence who underwent colonoscopy at the Institute of Gastroenterology and Hepatology, Soroka University Medical Center, Beer Sheba, Israel. This group consisted of two patients with adenocarcinomas and six with adenomas. Specimens of colonic adenocarcinomas were also obtained from two patients who underwent surgery at the Surgery Division, Soroka University Medical Center, Beer Sheba, Israel, and, collectively, the colonic adenocarcinoma group consisted of four patients. The adenoma group consisted of three patients with tubular adenomas (benign polyps) and three with tubulovillous adenomas (prone to malignancy) diagnosed by a senior pathologist.

Biopsy specimens of intact colonic mucosa were obtained from macroscopically normal colonic mucosa located at least 5 cm from the tumor margin. All tissue specimens were fixed in formalin and embedded in paraffin, and 4 µm tissue sections were used for further studies. All patients signed an informed consent form. The study was approved by the Ethics Committee of Soroka University Medical Center in compliance with the ethical standards of the Helsinki Declaration: Clinical numbers: 0140-12-SOR, 033-14-SOR.

Immunohistochemistry. Figure 1 shows the epigenetic reaction leading to methylation of the DNA nucleotide cytosine. All chemicals of highest purity grade available were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA) unless stated otherwise. Nuclear 5'-meCyt was detected using a mouse monoclonal antibody (162 33 D3; Calbiochem, Merck Millipore, Darmstadt, Germany) raised against 5-meCyt conjugated to ovalbumin, tested previously in human gastric CAFs (10) and in a human colonic myofibroblast cell line (Ling E, unpublished observation). The protocol was as described by Jiang et al. (10) with minor modifications. Briefly, tissue sections were deparaffinized in xylene and rehydrated in graded alcohol series. Antigen retrieval was performed in boiling 100 mM citrate buffer (pH 6.0) for 20 min with subsequent cooling at room temperature for an additional 20 min. Following wash in phosphate-buffered saline (PBS), the tissue sections were exposed to 3.5 N HCl for 15 min at room temperature to induce DNA denaturation. Following washing in PBS and 20-min incubation with 0.3% H$_2$O$_2$ in absolute methanol for quenching endogenous peroxidase activity, and an additional wash in PBS, tissue sections were incubated with 1.5% horse serum for 60 min at room temperature and then with anti-5-meCyt at 1:500 dilution in 1% albumin solution in PBS for 24 hours at 4°C. Antibody detection was performed using Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) with diaminobenzidine substrate according to the manufacturer’s instructions. Slides were counterstained with hematoxylin, dehydrated and mounted. The majority of slides contained both pathological and normal tissue sections, where such slides were not available, both control and pathological tissue sections were treated identically.

Pathological evaluation. The intensity of nuclear staining of fibroblasts adjacent to adenomas or carcinomas was compared to that of resident fibroblasts in the lamina propria of normal colonic mucosa. Additionally, the intensity of staining of fibroblasts was compared to staining of normal and malignant epithelial cells and of lymphocytes found in the stromal tissue. These types of cells (epithelial cells and accompanying lymphocytes) were chosen to serve as internal control for two reasons: firstly, the nuclear staining of these cells for 5'-meCyt is well documented (18) and, secondly, the variability of staining of epithelial cells and lymphocytes was low. Nuclear staining intensity was scored as low (score 1), moderate (score 2) and intense (score 3), respectively. The high intensity score of 3 was defined as the staining intensity of cells in the adjacent epithelial cells and of lymphocytes. Cell scoring for nuclear 5'-meCyt staining was performed by a senior pathologist using a double-headed light microscope. The scoring system was based on global assessment of staining in all fibroblast nuclei in the entire high-power field compared with that in epithelial cells in the same field. The intensity of staining was evaluated in six randomized high-power fields of 2 mm$^2$ per slide and scoring was expressed as the average density per 1 mm$^2$ field.

Statistical analysis. Wilcoxon signed-rank test was used to evaluate the association between dependent variables. Each slide was assigned a final score which was based on the sum of individual intensity scores of each power field. Four groups were defined for statistical purposes: adenomatous specimens versus normal tissue specimens, and adenocarcinomatous samples versus normal tissue samples. The nuclear staining intensity scores of tumor surgical specimens were pooled with those of biopsy specimens. The statistical analysis was performed using IBM SPSS software (SPSS
Results

Global DNA methylation in stromal fibroblasts of colonic tubular and tubulovillous adenomas. Tissue specimens of six patients with colonic adenomas were examined. In all samples, the nuclei of epithelial cells (normal, adenomatous and carcinomatous), stromal fibroblasts and lymphocytes within the lamina propria stained positively for 5-meCyt (data not shown). The mean nuclear intensity score of 5'-meC staining of fibroblasts in normal colonic mucosa was 14.08±1.83 (range=12-16), whereas the respective value for CAFs in the stroma of colonic adenomas was found to be 14.00±2.2 (range=11-17): This difference was not statistically significant.

Global DNA hypomethylation in stromal fibroblasts of colonic adenocarcinomas. Colonic mucosa specimen of four patients with colonic adenocarcinomas were examined. In all samples, nuclei of epithelial cells, stromal fibroblasts and intraepithelial lymphocytes stained positively for 5'-meC; however, a markedly reduced nuclear staining intensity of stromal CAF adenocarcinoma was consistently found compared to their counterparts in normal mucosa. The score of nuclear staining intensity revealed a significant ($p<0.03$) loss of nuclear staining of colonic adenocarcinoma CAFs compared to stromal fibroblasts surrounding the normal colonic mucosa (Figure 2): The nuclear staining intensity score in CAFs was 9.25±2.44 (range=6-13) vs. 16.17±0.75 (range=15-17) in normal stromal fibroblasts.

Discussion

Our results indicate that, similarly to other gastrointestinal regions (10), significant changes in the epigenetic signature are a hallmark of colonic CAFs in human CRC. Notably, global DNA hypomethylation, assessed by immunostaining of nuclear 5'-meCyt, was fully established in stromal colonic CAFs at the adenocarcinoma CRC stage. Interestingly, no difference in nuclear 5'-meCyt intensity staining was noted between colonic benign polyps or polyps prone to malignancy and normal stromal fibroblasts.

In a series of preliminary experiments (19) designed to provide direct evidence that cancer cells instruct colonic fibroblasts to acquire a CAF-like phenotype, we previously assessed global DNA methylation in a human normal colonic myofibroblast line (CCD-18Co) co-cultured in a Transwell system with HT-29 cells, a human colonic adenocarcinoma cell line. Global DNA methylation was quantified by a microplate-based assay. The exposure of colonic fibroblasts to cancer cells resulted in a marked time-dependent loss of DNA methylation. These findings, albeit preliminary, indicate that sustained paracrine cross-talk of CCD-18Co cells with cancer cells changes the epigenetics of colonic fibroblasts.

Our findings concord with those reported by Mrazek et al. (20). These investigators used genomic-wide gene-microarray expression and Illumina-based methylation analysis to study cultured colonic CAFs collected from...
stroma surrounding colonic adenocarcinomas. Their results indicated differential expression of genes and DNA methylation of promoter regions specific to CAFs. As noted in other solid tumor types (11), global DNA hypomethylation was associated with focal DNA hypermethylation.

What are the putative molecular mechanisms underpinning changes in the epigenetic landscape of colonic CAFs in terms of global DNA methylation? Neoplastic cells induce a chronic inflammatory response that builds up an inflammatory pro-tumorigenic microenvironment (TME) (21, 22). In addition, CAFs maintain the inflammatory TME by expressing a pro-inflammatory gene signature and by acting as brisk recruiters of pro-inflammatory cells. Therefore, in a vicious self-sustaining train of events, both tumor cells and CAFs contribute to the TME inflammasome (23-25).

A vast body of evidence is available showing that pro-inflammatory cytokines are able to exert their action on key components of the epigenetic machinery (12, 26, 27). Among them, transforming growth factor-β (TGFβ) plays a determinant role and findings indicate that a frequent site of TGFβ intervention is the regulation of DNMTs involved in DNA methylation [Figure 1 (28), reviewed in (29)]. Interestingly, Hawinkels and co-workers presented evidence that levels of active TGFβ1 are increased in human colonic tissue at the adenocarcinoma stage (30), to wit, at the CRC stage in which frank changes in CAF epigenetic landscape were observed in this study. Of note, a high level of TGFβ in CRC tumors correlates with disease relapse (31).

As discussed previously, DNA hypomethylation is associated with proficient chromatin and brisk activity of transcription factors. We surmise that among the newly-expressed genes are well-recognized markers of the CAF trait such as cyclooxygenase (COX2), a potential oncogenic enzyme, and fibroblastic-activation protein (FAP), an enzyme involved in the remodeling of the extracellular matrix (4, 24). Concordant with this view, FAP was shown to be expressed in CAFs surrounding colorectal carcinomas (32), and high FAP expression in CRC was shown to be associated with poor patient prognosis (33).

Epigenetic changes, unlike genetic ones, are potentially reversible and can be interfered with by drugs (14). Our observation that colonic CAFs express frank changes in their epigenetic signature predominantly at the adenocarcinoma stage of CRC may indicate a critical timing when administration of drugs able to reverse epigenetic changes in CRC could be beneficial.

A limitation of the present study is that only global DNA methylation in CRC CAFs was examined, and a deliberate characterization of the DNA methylome must include the assessment of gene promoter site-specific methylation signature. Moreover, our approach precludes any information on possible phenotypic and functional heterogeneity of colonic stromal CAFs (34). An attractive line of research, poorly explored to date, should also address specific histone post-translational modifications in stromal fibroblasts. We surmise that in addition to changes in the DNA methylome, histone changes greatly contribute to the stable CAF phenotype and, thereby, they sustain the role of CAFs in promoting the opportunistic growth agenda of cancer cells.

Conflicts of Interests

None.

References


