Original Article

Association of Global DNA Hypomethylation with Clinicopathological Variables in Colonic Tumors of Iraqi Patients

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ABSTRACT

Background/Aim: Colorectal cancer (CRC) ranks sixth among the most common 10 cancers in Iraq. It is a foremost public health dilemma and there is improved interest in understanding the fundamental principles of its molecular biology. DNA methylation in cancer has become the issue of passionate investigation. As compared with normal cells, the malignant cells show major disruptions in their DNA methylation patterns. We aimed to assess the association of global DNA hypomethylation in colonic adenomas and carcinomas of Iraqi patients, measured by immunohistochemistry of 5-methylcytosin, with different clinicopathological variables. **Patients and Methods:** Thirty tissue paraffin blocks from patients with colorectal adenomas, 30 tissue paraffin blocks from patients with colorectal adenocarcinomas, and 30 samples of apparently normal colonic tissue taken from autopsy cases as a control group were included in the present study. From each block, two sections of 5 μm thickness were taken, one section was stained with Hematoxylin and Eosin for revision of histopathological diagnosis and one section was immunohistochemically stained for 5-methylcytosine (5mC) and digitally analyzed by AperioImageScope software. **Results:** The mean digital value of 5mC immunohistochemical expression was sequentially decreased during neoplastic progression from normal colonic tissue into adenoma and then to carcinoma. The mean digital value of 5mC expression was significantly lower in large size adenomas (≥1 cm), and those with severe dysplasia. Concerning carcinoma cases, 5mC expression was significantly lower in stage C2. **Conclusions:** The immunohistochemical evaluation of 5mC yields refined information on colorectal tumor biology in adenoma and carcinoma. Global DNA hypomethylation reflected by low immunohistochemical expression of 5-mC is associated with advanced colorectal adenomatous polyps suggesting that it is an early event in colorectal carcinogenesis. Also this hypomethylation can reflect bad prognosis of patients with colorectal cancer by its correlation to higher tumor stage.

Key Words: Colorectal adenoma, colorectal carcinoma, DNA hypomethylation, 5-methylcytocin

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Colorectal cancer (CRC) is a major public health problem and ranks sixth among the most common 10 cancers in Iraq; therefore, there is renewed interest in understanding the basic principles of its molecular biology.[1,2] There is growing evidence in early detection of this disease with novel screening methods to reduce compliance and increase specificity of available methods.[3] The disease begins as a small benign adenomatous polyp, which develops into a large advanced adenoma with high-grade dysplasia and then progresses to an invasive carcinoma. The clinical behavior of colorectal carcinomas results from interactions at many levels.[4]

Despite vast evidence for the mutational basis of cancer, epigenetic changes, which are events that alter the way of gene expression without affecting genetic coding, also may participate in carcinogenesis. Methylation of DNA, which

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takes place at the cytosine residues of cytosine guanine (CpG) dinucleotides with the aid of an enzymatic reaction that produces 5-methylcytosine (5mC), is a well-characterized mechanism for epigenetic gene regulation.[5] 5mC is an imperative suppressor of transcription in the genome. When present in promoters, 5mC is associated with stable, long-term transcriptional silencing. This may happen by either blocking positive transcription factors, or through promoting the binding of negative transcription factors. 5mC is bound by several classes of proteins that facilitate transcriptional repression.[6]

In carcinogenesis, epigenetic silencing of important tumor suppressor genes transcription by CpG island methylation is biologically equivalent to acquiring an inactivating mutation.[7] Hypomethylation is the second type of methylation that is associated with carcinogenesis. Hypomethylation causes activation of oncogenes such as C-MYC and H-RAS.[8] Hypomethylation also causes disruption of expression of the APC gene in colon.[9]

The term DNA hypomethylation refers to progressive and global reduction in the number of methylated cytosine bases (5mC) in the genome of tumor cells, which was first demonstrated in the 1970s. Among hypomethylation-associated mechanisms, a pattern of hypomethylation at the repetitive sequences in satellite or pericentromeric regions of tumor cells may affect neoplastic progression by making chromosomes more susceptible to breakage resulting in genomic instability. In addition, hypomethylation may cause disruption of normal gene structure and function with resulting chromosomal instability by reactivating previously silenced retrotransposons.[10]

The aim of the present work is to study the association of global DNA hypomethylation in colonic adenomas and carcinomas of Iraqi patients; measured by immunohistochemistry of 5mC; with different clinicopathological variables, including age and gender of patients, site, size, gross morphology, histopathological types, degree of dysplasia of colorectal adenomas, and grade and stage of colorectal adenocarcinomas.

PATIENTS AND METHODS

Sixty patients having colorectal tumors were enrolled in the present study including 30 adenomas and 30 carcinomas. Paraffin blocks were collected from those patients for the period from December 2013 to February 2015. The control group included 30 samples of apparently normal colonic tissue taken from autopsy cases [Figure 1a]. These specimens were processed and paraffin embedded in the same center.

The clinicopathological parameters were obtained from patients' admission case sheets and pathology reports. This study agreed to the terms of ethical considerations according to the form prepared for this purpose by the Iraqi Ministry of Health. It was also approved by the Committee of Ethical Standards in the Faculty of Medicine, Al-Nahrain University, one of the colleges affiliated to the Ministry of Higher Education and Scientific Research, Iraq.

From each block, two sections of 5 μm thickness were taken, one section was stained with Hematoxylin and Eosin (H and E) and slides were revised for the histopathological type, grade, and stage (according to Astler-Coller staging system) of colorectal adenocarcinomas; and for the histopathological type and grade of dysplasia of colorectal carcinomas.

The other section was immunohistochemically stained using three steps: Indirect streptavidin method for anti-5-mC antibody, clone 33D3, manufactured by Abcam, which recognizes the modified base 5-methylcytidine found in DNA of plants and vertebrates. The procedure was carried out in accordance with the manufacturer's instructions:

1. Five micrometer sections were obtained from formalin fixed–paraffin-embedded tissue blocks and mounted on Fisher brand positively charged slides
2. Slide baking: The slides were placed in a hot air oven at 65°C overnight
3. Deparaffinization and rehydration: The slides were immersed sequentially in the following solutions at room temperature for the indicated times:
   A. Xylene for 5 min
   B. Fresh xylene for 5 min
   C. Absolute ethanol for 5 min
   D. 95% Ethanol for 5 min
   E. 90% Ethanol for 5 min
   F. 70% Ethanol for 5 min
   G. 50% Ethanol for 5 min
   H. Distilled water for 5 min
4. Antigen retrieval: The tissue sections were placed in the jar containing 200 mL and heated in: (1) Microwave (700 W) for 9 min, (2) microwave (350 W) for 15 min. Slides were left in the retrieval solution containing jar, and allowed to cool for 20 min at room temperature. Slides were removed from the antigen retrieval solution and placed in Tris–HCl washing buffer solution (TBS) for 5 min
5. PAP pen was used to draw a circle around the tissue section
6. To quench the endogenous peroxidase of the tissue, 3% hydrogen peroxide block reagent (ready to use) was used, 2–3 drops of peroxidase block were applied onto the tissue to cover the tissue sections, then the slides were placed in the humid chamber and incubated at

room temperature for 10 min after that the slides were rinsed gently in TBS for 5 min. The excess buffer was tapped immediately and the slides were drained and blotted gently

7. Primary antibody: 50–100 μL (according to size of tissue on the slide) of primary antibody 5mC (diluted with common antibody diluent in ratio of 1:100) were applied onto the tissue sections and incubated in humid chamber at 37°C overnight, and then slides were rinsed in TBS for 5 min. Then excess buffer was drained and blotted as before
8. Secondary (biotinylated link) antibody was used to conjugate to the primary antibody, 1–2 drops of the secondary antibody were applied onto the tissue sections then the slides were incubated in the humid chamber at 37°C for 30 min, then slides were rinsed in TBS for 5 min. Then excess buffer was drained and blotted as before
9. Streptavidin–HRP reagent (streptavidin conjugated to horseradish peroxidase) was used to form streptavidin–biotin complex, 1–2 drops of the Streptavidin–HRP reagent were applied covering the tissue sections and placed in the humid chamber, incubated at 37°C for 30 min. After that the slides were rinsed in TBS for 5 min then excess buffer was drained and blotted as before
10. Substrate-chromogen solution: Streptavidin–biotin complex was incubated with substrate-chromogen solution until desired staining intensity had developed. DAB (3,3′-diaminobenzidine) was used to precipitate a color, giving brown color. Substrate-chromogen solution was prepared by adding one drop of the DAB chromogen to 1 mL of substrate buffer, using the provided graduated test tube, then the prepared solution was mixed well and by using the provided transfer pipette, enough drops were applied on each section covering the whole specimen, the slides were incubated in humid chamber at room temperature for 10 min. After that the slides were rinsed gently in TBS for 2 min then rinsed in distilled water for 2 min
11. Counter stain (Mayer's hematoxylin): Slides were immersed in a bath of Mayer's hematoxylin for 15 s. After that the slides were rinsed gently in tap water for 2 min
12. Mounting medium: 1–2 drops of faramount aqueous medium (DAKO, Denmark) were applied onto the wet sections, and the sections were quickly covered with coverslips and left to dry overnight.

Digital analysis of 5mC immunohistochemical staining using AperioImageScope sofware

This difference in staining intensity between the two types of cells was confirmed by image analysis, in which the average integrated optical density of the nuclei in the neoplastic tissue and that of nuclei in the normal tissue are reported for each patient and control, demonstrating a constant and significantly lower intensity for the former type of cell.

Using a light microscope (Human, Germany), each immunohistochemically stained slide was scanned with 10× objectives for the positive brown nuclear immunostaining and with 40× objective, three fields that reflect the best of the overall immunostaining of the entire slide were chosen and captured using a Sony digital camera (Sony DSC-W330 14.1MP Digital Camera). Captured images of 1392 × 1040 pixels were saved on PC in an uncompressed JPG format.

Each image was analyzed by AperioImageScope (Version 10). The Aperio positive pixel count algorithm can be used to quantify the amount of a specific stain present in a slide image. This algorithm has a set of default input parameters when first selected. These inputs have been preconfigured for brown color quantification in the three intensity ranges (weak positive, positive, and strong positive). For each case, three images were measured and the average of measurement was taken. The measurements for each case were saved in an Excel file.

Statistical analysis

Data were analyzed using Statistical Package for Social Sciences (SPSS) program version 17 and Microsoft Office Excel 2010. Numeric data were expressed as mean ± SEM (SD); frequency was used to express discrete data. Analysis of variance was used to analyze numeric data, whereas Chi-square test was used to analyze discrete data, and Least Significant Difference test (LSD) was used for multiple comparisons. *P*-value of less than 0.05 was considered significant.

For Aperio software, the result of the positive pixel count algorithm was showed by selecting the annotations window from the view menu in themenu bar. This window showed the number of the positive pixelsinto three intensity ranges (weak, positive, and strong), number ofnegative, total number (positive + negative), and the percentage of positivity (number of positive/number of total, multiplied by 100); these measurements were saved as Excel 2010 spreadsheet file.

RESULTS

The clinicopathological parameters of colorectal adenoma and carcinoma cases included in the present study are summarized in Tables 1 and 2, respectively.

Histologically, in H and E sections, the cytologic hallmark of epithelial dysplasia of colorectal adenoma is nuclear

Table 1: Clinicopathological parameters of colorectal adenomas

Parameters	Values
Age	
Mean	57.5±6.97 years
Range	45-71 years
Gender	
Male	19 (63.3%)
Female	11 (36.7%)
Male: Female	1.7:1
Site (%)	
Right colon	7 (23.3)
Left colon	10 (33.3)
Rectosigmoid colon	13 (43.3)
Size	
Mean	0.75±0.311 cm
Range	0.4-1.5 cm
Histopathological types (%)	
Tubular	20 (66.7)
Tubulovillous	8 (26.6)
Villous	2 (6.7)
Dysplasia (%)	
Mild	17 (56.7)
Moderate	9 (30.0)
Severe	4 (13.3)

Table 2: Clinicopathological parameters of colorectal carcinomas

Parameters	Values
Age	
Mean	66.67±7.56 years
Range	49-80 years
Gender	
Male	20 (66.7%)
Female	10 (33.3%)
Male: Female	2:1
Site (%)	
Right colon	6 (20.0)
Left colon	10 (33.3)
Rectosigmoid colon	14 (46.7)
Gross morphology (%)	
Fungating	12 (40)
Ulcerative	14 (46.7)
Annular	4 (13.3)
Histopathological types (%)	
Mucinous	5 (25)
Nonmucinous	25 (75)
Grade (%)	
Well differentiated	4 (13.3)
Moderately differentiated	23 (76.7)
Poorly differentiated	3 (10)
Stage (Astler-Coller staging system) (%)	
B2	7 (23.3)
C1	15 (50)
C2	8 (26.7)

hyperchromasia, elongation, and stratification. These changes are most easily appreciated at the surface of the adenoma and are often accompanied by the presence of large nucleoli, eosinophilic cytoplasm, and a reduction in the number of goblet cells. Notably, the epithelium fails to mature as cells migrate from crypt to surface [Figure1b–d]. The H and E sections of colorectal carcinomas revealed that most of the cases are well-to-moderately differentiated adenocarcinoma [Table 2 and Figure 1e and f].

Immunostaining of 5mC is localized in the nuclei of the cells as brown colored staining. The staining pattern of pleomorphic nuclei in the neoplastic area was distinctly different from that observed in the normal counterpart. The morphologically altered nuclei displayed densely labeled spots within faintly labeled areas, whereas normal nuclei were darker and uniformly stained [Figure 2].

The mean digital value of the percentage of positivity of 5mC immunohistochemical expression (5mC%) in colorectal carcinoma was (0.26 ± 0.11), with a range of 0.12–0.53; in colorectal adenoma was (0.54 ± 0.12), ranging from 0.31 to 0.75 and the control group showed a value of (0.59 ± 0.11), ranged from 0.33 to 0.75. There was a significant difference in the mean digital value of 5mC between carcinoma, adenoma, and control groups ($P < 0.0005$) as shown in Table 3.

The mean digital value of the percentage of positivity of 5mC immunohistochemical expression (5mC%) was lower in colorectal carcinoma in comparison to colorectal adenomas and control group with mean differences of (−0.27567 ± 0.03) and (−0.32567 ± 0.03), respectively; and both differences are highly significant ($P < 0.0005$), whereas 5mC% was nonsignificantly lower in colorectal adenomas in comparison to control group ($P = 0.099$) with a mean difference of (−0.05 ± 0.03) as shown in Table 4.

Regarding the association of 5mC immunohistochemical expression with clinicopathological parameters of the studied cases, 5mC% was significantly different according to degree of dysplasia of adenomas ($P < 0.0005$) with those of severe dysplasia showing the lowest mean digital values of 5mC% (0.3900 ± 0.02677). It was also significantly lower in adenomas ≥1 cm in size with a mean digital value of 5mC% (0.4386 ± 0.02790) ($P = 0.009$). There was no significant difference in 5mC% according to age, gender, site, and histopathological types of adenomas ($P > 0.05$) as shown in Table 5.

In colorectal carcinomas, 5mC% was significantly different according to stage ($P = 0.015$) with the least value shown in stage C2 (0.2200 ± 0.04563); the highest stage in the present study. The other clinicopathological parameters, including age, gender, site, gross morphology, and histopathological

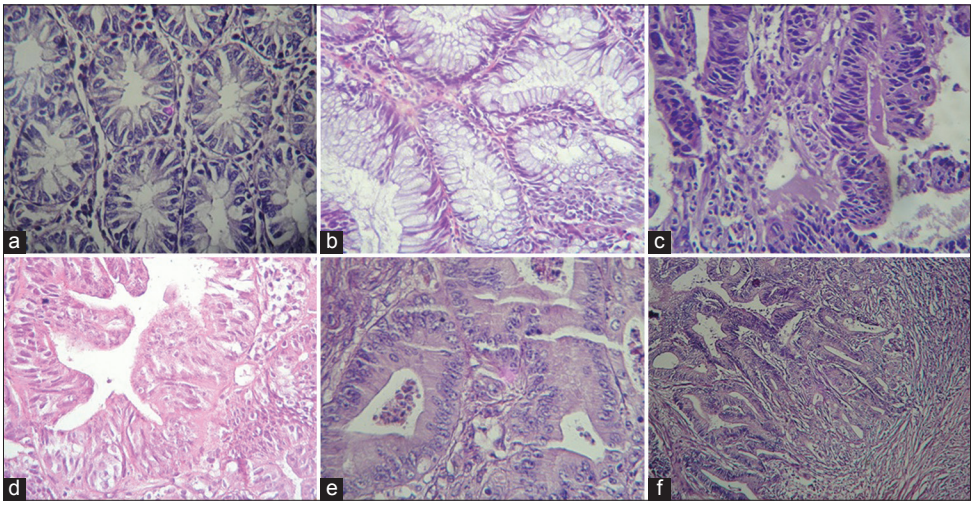

Figure 1: H and E-stained sections. (a) Normal colonic tissue (control group) (40×). (b) Tubular adenoma with mild dysplasia (40×). (c) Tubulovillous adenoma with moderate dysplasia (40×). (d) Tubulovillous colorectal adenoma with severe dysplasia (40×). (e) Well-differentiated adenocarcinoma (H and E) (40×). (f) Moderately differentiated adenocarcinoma (H and E) (10×)

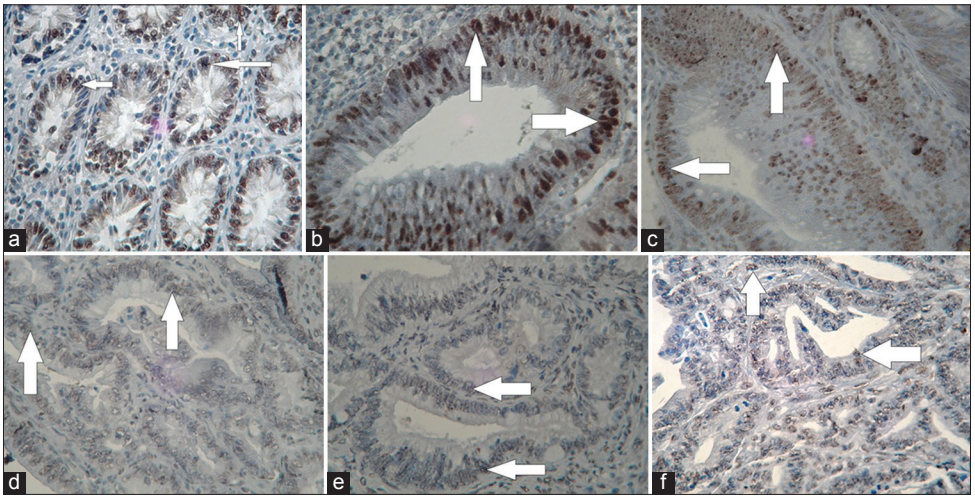

Figure 2: Immunohistochemical staining of 5-methylcytosine (5mC). (a) Normal colonic tissue with brown imunohistochemical nuclear staining of 5mC with strong positive intensity (white arrows) (40×). (b) Tubular adenoma with mild dysplasia showing brown imunohistochemical nuclear staining of 5mC with strong positive intensity (white arrows) (40×). (c) Tubulovillous adenoma with moderate dysplasia showing brown imunohistochemical nuclear staining of 5mC with positive intensity (white arrows) (40×). (d) Tubulovillous adenoma with severe dysplasia showing brown imunohistochemical nuclear staining of 5mC with weak positive intensity (white arrows) (40×). (e) Well-differentiated adenocarcinoma showing brown immunohistochemical nuclear staining of 5mC with weak positive intensity (white arrows) (40×). (f) Moderately differentiated adenocarcinoma showing brown immunohistochemical nuclear staining of 5mC with weak positive intensity (white arrow) (40×)

types showed nonsignificant association with 5mC% as shown in Table 6.

Classification of the cases of carcinoma, adenoma, and control groups into different scores of 5mC intensity showed highly significant difference in the distribution of cases among study groups (carcinoma, adenoma, and control) according to intensity scores (weak positive, positive, and strong positive) ($P < 0.0001$). Strong positive intensity was mainly seen in control cases (20, 66.7%) in comparison with adenoma (4, 13.3%) and carcinoma (2, 6.7%). The highest number of cases with positive intensity was mainly seen in adenoma (11, 36.8%) in comparison with carcinoma (4, 13.3%) and control (6, 20%). Carcinomas showed the largest number of cases with weak positive intensity (24, 80%) in comparison with adenoma (15, 50%) and control (4, 13.3%) [Figures 2 and 3].

DISCUSSION

To the best of our knowledge, this is the first study in Iraq that deals with 5mC immunohistochemical expression in

Table 3: Mean digital values of 5-methylcytosine (5mC) immunohistochemical expression in control group, colorectal adenomas, and carcinomas

	Groups	Mean	SD	*P* value
5mC (%)	Carcinoma	0.2643	0.11288	<0.0005
	Adenoma	0.5400	0.12205	
	Control	0.5900	0.11375	

N: Number of cases, SD: Standard deviation

Table 4: Multiple comparisons of mean digital values of 5-methylcytosine (5mC) immunohistochemical expression in controls, adenomas, and carcinomas

Dependent variable	(I) Groups	(J) Groups	Mean difference (I–J)	SD	*P* value
5mCC%	Carcinoma	Adenoma	−0.27567	0.03003	<0.0005
		Control	−0.32567	0.03003	<0.0005
	Adenoma	Carcinoma	0.27567	0.03003	<0.0005
		Control	−0.05000	0.03003	0.099
	Control	Carcinoma	0.32567	0.03003	<0.0005
		Adenoma	0.05000	0.03003	0.099

SD: Standard deviation

Table 5: Association of 5-methylcytosine (5mC) immunohistochemical expression to clinicopathological parameters of colorectal adenomas

Clinicopathological parameter	Mean 5mC%	SD	*P* value
Age group			
<50 year	0.5300	0.10794	0.761
50-59 year	0.5592	0.13444	
>59 year	0.5233	0.12055	
Gender			
Male	0.5574	0.13523	0.314
Female	0.5100	0.09338	
Site			
Right	0.6114	0.14645	0.201
Left	0.5080	0.09964	
Rectosigmoid	0.5262	0.11744	
Size			
<1 cm	0.5709	0.11782	0.009
≥1 cm	0.4386	0.07381	
Histopathological type			
Tubular	0.5495	0.11109	0.799
Tubulovillous	0.5275	0.15854	
Villous	0.4950	0.12021	
Degree of dysplasia			
Mild	0.6229	0.08823	<0.0005
Moderate	0.4500	0.04873	
Severe	0.3900	0.05354	

SD: Standard deviation

colorectal adenoma and carcinoma and its association to different clinicopathological parameters.

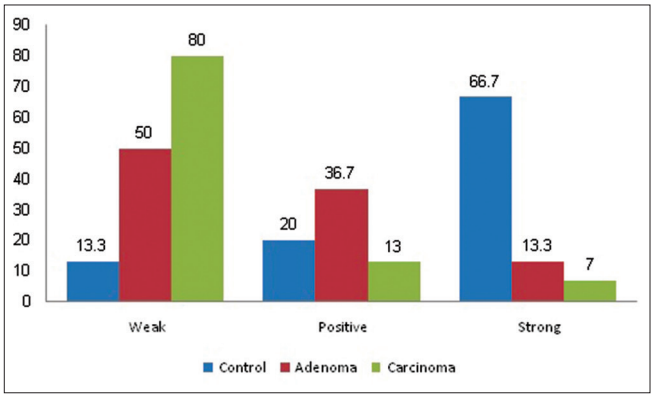

Figure 3: Classification of 5 mC intensity into (weak positive, positive and strong positive) according to the tabulated values of Aperio Image Scope and its association with the study groups (control, adenoma, and carcinoma)

Identification of factors associated with the genesis and progression of colorectal cancer is of immense importance in the development of methods for prevention and treatment. Polypectomy currently constitutes the best strategy for prevention of colorectal cancer. The knowledge of parameters reflecting the biological behavior of tumors, associated with the severity and degree of progression, is an important determinant of prognosis and improvement in cancer therapy.[11]

Gene expression is modified by epigenetic factors that occur in various forms, such as DNA methylation.[12] It is known that the cell stress results in genetic and epigenetic changes and causes an altered cellular "memory" that drives disease pathology.[13] The main risk for cancer is a prolonged exposure and increased DNA damage. There is a wealth of data that supports the fact that cancerous cells have aberrant patterns of epigenetic modifications. The greatest studied epigenetic modification is DNA methylation, which consists of the methyl group at carbon 5 of the cytosine. Methylation of cytosine residues in DNA provides a mechanism for a gene control expression.[14] DNA hypomethylation was the initial epigenetic abnormality recognized in human neoplasms. DNA methylation (5mC status) is a sensitive marker of the tumerogenesis induced by the oxidative damage reactions, and very characteristic of cancer cells.[15]

The major epigenetic mechanisms that are believed to play a vital role in cancer development include DNA methylation of cytosine bases in CpG dinucleotides and post-translational modifications of histone proteins that mediate the packaging of DNA into chromatin and thus control gene expression through regulating chromatin conformation. Although these multiple epigenetic mechanisms are all involved in CRC pathogenesis and there is an interaction between

Table 6: Association of 5-methylcytosine (5mC) immunohistochemical expression to clinicopathological parameters of colorectal carcinomas

Clinicopathological parameter	Mean 5mC%	SD	*P* value
Age group			
<60 year	0.3317	0.14483	0.245
60-69 year	0.2569	0.12419	
>69 year	0.2364	0.06562	
Gender			
Male	0.2600	0.10458	0.772
Female	0.2730	0.13359	
Site			
Right	0.2033	0.06861	0.173
Left	0.2480	0.11679	
Rectosigmoid	0.3021	0.11709	
Gross morphology			
Ulcerative	0.2807	0.14003	0.370
Annular	0.3100	0.12111	
Fungating	0.2300	0.06353	
Histopathological type			
Mucinous	0.2180	0.02490	0.323
Nonmucinous	0.2736	0.12148	
Grade			
Well differentiated	0.2075	0.07411	0.161
Moderately differentiated	0.2604	0.11035	
Poorly differentiated	0.3700	0.13856	
Stage			
B2	0.3671	0.11757	0.015
C1	0.2400	0.07141	
C2	0.2200	0.12906	

SD: Standard deviation

various epigenetic mechanisms, DNA methylation and histone modifications in the gene promoter region are the most widely studied epigenetic mechanisms and they are also found to be the primary mediators of CRC epigenetic inheritance in cancer cells.[16]

Pathology has recently entered the field of personalized medicine. This brings new expectations for the accuracy and precision of tissue-based diagnosis, in particular, when quantification of histologic features and biomarker expression is required. While for many years traditional pathologic diagnosis has been regarded as ground fact, this idea is no longer sufficient in contemporary tissue-based biomarker research and clinical application. Another major advance in pathology is brought by the development of virtual microscopy technology enabling digitization of microscopy slides and presenting new opportunities for digital image analysis. Computerized vision presents an instantaneous benefit of increased capacity (automation) and precision (reproducibility).[17] In the present study, computer-assisted quantification of the staining intensity was performed on malignant, adenomous, and normal tissues of human colon to test the correlation between the immunolabeling signal and the respective histological patterns observed.

The current work has shown that the mean digital value of 5mC immunohistochemical expression was lower in colorectal carcinoma in comparison to colorectal adenoma with a mean difference (-0.27567 ± 0.03), which is highly significantly different ($P < 0.0005$).

Weak positive intensity of 5mC immunohistochemical expression (ie, low intensity) was mainly seen in carcinoma cases (80%) in comparison with adenoma (50%) and control (13.3%). The difference in the distribution of weak positive intensity of 5mC immunohistochemical expression among the study groups (carcinoma, adenoma, and control) was highly significant ($P = 0.00002$). Positive intensity (ie, moderate intensity) was mainly seen in adenoma cases (36.8%) in comparison with carcinoma (13.3%) and control (20%) with no significant difference ($P = 0.1$). Strong positive intensity (ie, high intensity) was mainly seen in control cases (66.7%) in comparison with adenoma cases (13.3%) and carcinoma cases (6.7%) with highly significant difference ($P = 0.00004$).

Hypomethylation of DNA sequences is observed during the early stages of tumorigenesis or in abnormal non-neoplastic tissue, such as hyperplasia.[18]Global DNA hypomethylation, which is characterized by a generalized reduction in the number of methylated cytosines within CpG sites, is found as an early and consistent event in colorectal carcinogenesis and is correlated with mechanisms that drive the early stages of the carcinogenic process including chromosomal instability, increased chromosomal mutation rates, and loss of imprinting.[19] Yamada *et al*. reported a significantly increased number of microadenomas (small colonic intramucosal lesions) in hypomethylated mouse models as compared with controls suggesting that hypomethylation may promote early-stage tumor development in the colon in mice.[20] In humans, the role of global DNA methylation in colorectal tumorigenesis has primarily been studied by comparing methylation patterns in colorectal tumor tissue, with matched adjacent normal appearing tissue obtained from the same patient[21-26] or with normal colon tissue from healthy control subjects.[21,22] These studies indicate that virtually all colorectal tumors (benign adenomas and cancers) display a higher degree of decrease in methylated cytosines within CpG sites (global hypomethylation) as compared with matched and unmatched normal appearing colon tissue.[27] It has been observed that up to 5% of cytosines are methylated in normal tissues and that this DNA methylation is essential for controlling gene expression of tissue-specific housekeeping or imprinted genes and for keeping genomic stability through silencing transposable elements of the genome.[28]

Advanced colorectal adenomas are associated with size ≥1 cm, high-grade dysplasia, and/or villous histology.[29] The present study revealed that 5mC immunohistochemical expression is significantly lower in adenomas measuring 1 cm or more, and those with severe dysplastic change. The mean value of 5mC was inversely correlated with increasing morphologic dysplasia in adenomas (mild, moderate, and severe), which is comparable with a recent study conducted by Shen *et al*.[30] There was no significant difference in 5mC immunohistochemical expression according to histopathological types of adenomas (tubular, villous, and tubulovillous) and other clinicopathological parameters, including age, gender, and site, which agrees with a previous study by Bariol *et al*.[22]

Collectively, the above results indicate that low 5mC immunohistochemical expression is associated with advanced colorectal adenomatous polyps suggesting that DNA hypomethylation is an early event in colorectal carcinogenesis.

Regarding colorectal carcinoma, the current work showed that 24 out of 30 cases (80%) displayed low 5mC expression weak intensity. These results elucidate that cancer cells are associated with a generalized disruption of the DNA methylation pattern involving an overall decrease in the level of 5-mC of particular CpG islands.[28]

A number of studies, including the current investigation, have clearly demonstrated that the genomic 5-mC content of colorectal cancers is reduced when compared with the paired normal mucosa.[22,31] Although the absolute changes in methyl content vary between studies, it is likely that this primarily reflects variations in methodology.[22]

The present research reported nonsignificant correlation between 5mC immunohistochemical expression with age, gender, site grade, and histopathological types of colorectal carcinoma; this is equivalent with other studies.[22,30] There was significant correlation between 5mC immunohistochemical expression with stage of carcinoma, which agrees with recent articles.[30,32]

From all the above observations, the immunohistochemical method described previously allows the pathologist to collect useful information on the DNA methylation status of various regions in the colorectal tissue biopsies.

A superior understanding of the epigenetic events in carcinogenesis has created a potential for "epigenetic therapies." Although epigenetic therapies are few in number, several are nowadays being studied in clinical trials or have been approved for specific cancer types.[10]

CONCLUSION

The immunohistochemical evaluation of 5mC yields refined information on colorectal tumor biology in adenoma and carcinoma. Global DNA hypomethylation reflected by low immunohistochemical expression of 5-mC is associated with advanced colorectal adenomatous polyps suggesting that it is an early event in colorectal carcinogenesis. Also this hypomethylation can reflect bad prognosis of patients with colorectal cancer by its correlation to higher tumor stage. This information could be integrated with the clinical and biologic tumoral framework for good assessment, management, and follow-up of the studied cases. Computerized digital analysis of immunohistochemical staining can resolve disagreement among different observers about the quality of staining intensity because the digital method does not classify the results into groups, but rather provides a numerical value for each individual case; thus, it increases the diagnostic and, more importantly, the prognostic sensitivity of the immunohistochemical analysis.

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Conflicts of interest
There are no conflicts of interest.

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