Research Article

Gradual Histopathologic Changes During Development of Colorectal Cancer

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Abstract

Background: Colorectal cancer is one of the most common causes of mortality in the world.
Objectives: The aim of this study was to investigate the histopathologic changes including hyperchromatism, tissue lymphocyte infiltrations (TILs), aberrant crypt foci (ACF), microvessel density (MVD), p53, Bcl-2 and CD31 changes during colorectal cancer development.
Methods: Subcutaneous injections of dimethyl hydrazine DMH were administered to rats (40 mg/kg body weight) for 10 weeks. Rats were fed by food and water until 40th week and sacrificed two by two within 10, 15, 20, 25, 30 and 40 weeks after the start of treatment. Thin paraffinized sections were applied to anti-CD31, anti-Bcl-2 and anti-p53 staining procedures. MVD and ACF were reported as mean value of three HPFs.
Results: Hyperchromatism, TILs and angiogenesis were the most common initial histologic changes which started at 10th week of DMH treatment. Hyperchromatism's severity increased earlier than other changes and reached the highest value at the 25th week. The highest value of all variants occurred in the 40th week except the TILs which started to achieve the highest value in week 30 and increased until 40th week. A diminished amount of p53 was observed at week 40, however, increased intensity of CD31 and Bcl-2 were seen between 30th and 40th week.
Conclusions: In conclusion, TILs and angiogenesis might be the important earliest factors contributing to colorectal cancer progression.

Keywords: Colorectal Cancer, Tumor-Infiltrating Lymphocytes (TILs), Angiogenesis, Hyperchromatism, Microvessel Density (MVD), Aberrant Crypt Foci (ACF)

1. Background

Many countries are afflicted by colorectal cancer which is one of the most common causes of death (1). Cancer is a multistep process including the cellular, morphological and genetic changes. Cancer progression is defined as phenotypical and biological changes such as hyperchromatism, during which the tumor becomes aggressive and gains more malignant potential (2). Some noticeble genetic changes such as p53 and Bcl-2 gene mutation occur in colorectal cancer. Mutated p53 is found in dyplastic lesions even before the formation of neoplastic lesions (3). The function of this well known tumor suppressor gene is altered in many cancers (4). Similar to p53, Bcl-2, it reg-

ulates some important cell cycle events such as different types of apoptosis and necrosis. The mutated Bcl-2 is seen in many types of cancers, such as colorectal cancer (5, 6).

The starting point of carcinogenesis in colorectal cancer is the formation of pre neoplastic polyps in the colonic mucosa called aberrant crypt foci (ACF) (7). The morphological changes of crypts including crypt width and height changes and the thickness of crypts' lining leads us to detect the ACFs (2). Dysplastic ACFs demonstrate more proliferation ability in the upper parts of crypts (8) and less apoptosis in the whole length of crypts (9). Decreasing the goblet cells, nuclear atypia, increasing the pericryptal space and thickening of epithelial cells are also found in dysplastic ACFs (7, 8).

Angiogenesis increase during colorectal cancer development in order to feed growing tumors but the exact premalignant stage of colorectal cancer when angiogenesis is activated remains unclear. In this study the angiogenesis is assessed by microvessel density (MVD) using CD31 antibody, a valuable marker for detection of new vascular formation ([10]). Angiogenesis and cancer-related inflammation are the main constituents of a cancerous microenviroment. The cancer-related inflammation can be activated by various genetic changes which consequently form some types of neoplasm. In other types of cancers the inflamed microenvironment is present before the cancerous lesion develops ([11]). Tumor-lymphocytic infiltrations (TLIs) which are often associated with the better survival rates in colorectal cancer are defined as the lymphocytic migration into the cancerous tissue ([12]). This prognostic factor is easily differentiated from other inflammatory cells by histologic features of the small round cells ([13]). The semi-quantitative index, TLIs, has inverse correlation with tumor stage ([14]).

2. Objectives

The aim of this study is to investigate the histopathologic changes including hyperchromatism, TILs, ACF and MVD during colorectal cancer development.

3. Methods

3.1. Chemichals

1, 2- Di methyl hydrazin (DMH) was purchased from Sigma-Aldrich (Sigma-Aldrich, Broendby, Denmark). anti-CD31, anti-Bcl-2 and anti- p53 were purchased from Glostrup company (Dako, Glostrup, Denmark).

3.2. Animals Experiments

Fourteen, 4 weeks old Wistar male rats weighing 170 - 200 g, were obtained from animal house of Science College, Urmia University. The study was approved by Ethics Committee of Urmia Medical University, Iran (URM.REC.1395.1120). The humidity and tempreture (20 - 25°C) of the room in which Rats' cages were kept were controlled. The cycle of 12 hours light/ 12 hours dark, food and water available all the time were provided ([15]). DMH in a solution including EDTA 1 mmol/L and PBS was administered as subcutaneous injections (40 mg/kg body weight) for 10 weeks. Rats were fed by food and water untill 40th week and twelve rats were sacrificed two by two within 10, 15, 20, 25, 30 and 40 weeks after the start of treatment and exposure to the DMH ([16]).

3.3. Inclusion Criteria for Animal Models

1- All rats were male in gender to eliminate pregnancy possibility
2- The age of rats was 4 weeks
3- All rats weighed 170 - 200 g.

3.4. Exclusion Criteria for Animal Models

1- Female rats
2- Rats older and fatter than the suggested criteria

3.5. Sample Size

According to $\alpha = 0.05$ and power = 80%, sample size in each group was determined as 7 ([15]).

3.6. Immunohistochemichal Analysis and TILs

Thin paraffinized sections were applied to anti-CD31, anti-Bcl-2 and anti-p53 staining procedure. One section of more tumoral morphology was stained with anti-CD31 antibody and the angiogenesis was assessed as the mean value of hotspots and reported as MVD ([10]). The mean number of stained microvessels in three high-power fields (HPFs) was calculated ([17]). TILs were easily assessed by histopathologic features of lymphocytes which have a small spherical nucleus including abundant dark staining chromatin with not much cytoplasm ([13]).

3.7. Aberrant Crypt Foci (ACF) Detection

After dissection, the colons were removed and washed gently in PBS buffer. A longitudinal direction cut from cecum to anus was done and the tissue samples were kept in a filter paper supplemented with 10% formalin. After 24 hours, the samples were stained in 2% methylene blue and were prepared for ACF count ([8], [18]).

3.8. Statistical Analysis

All statistical analysis was performed by one-way analysis of variance (ANOVA) using SPSS software version 19.0 (SPSS Inc., Chicago, IL, USA). P value less than 0.05 were considered statistically significant.

4. Results

4.1. ACF Identification

ACFs were recognized under the microscope ($\times$ 40) on the base of morphologic features such as crypt enlargement and elongation ([19]). The results demonstrate that the treatment with DMH causes formation of cancerous lesions and significant increased number of ACF in comparison with healthy control group (P < 0.05).

4.2. Histological Analysis

The results of histological analysis including hyperchromatism, TILs, MVD, destruction of epithelium, epithelial cells shedding and ACF are shown in Table 1. Hyperchromatism, TILs and MVD are the first histologic changes which start at 10th week of DMH treatment. Hyperchromatism's severity increases more and obtain the highest value at the 25th week. The highest value of all variants occured in the 40th week except the TILs, which started to receive the highest value in week 30 and increase till 40th week. Angiogenesis received the highest score in 40th week and epithelial cells shedding, destruction of epithelium, ACF, TILs and hyperchromatism followed in descending order. The number of cancer free cells (A), goblet cells (B), normal crypts (C), abnormal chromatin (D), abnormal mitosis (E), and polymorphism of epithelial cells (F) are shown in Figure 1. For histological evaluation, goblet cells and normal crypts, which were largely seen in the healthy colorectal samples, decreased during the development of cancer. As expected, abnormal mitosis as well as altered chromatin pattern, free cancer cells, abnormal mitosis and epithelial polymorphism increased in DMH-treated groups during the treatment. Altogether, the DMH-treated cancer group, demonstrated the following features: enhancement of MVD, ACF and epithelial cells around and within it, reduction of goblet cells while normal crypts contained a significant number of these cells, increased polymorphism and TILs (Figure 2).

4.3. Immunohistochemistry Assay

The DMH-treated cancer group demonstrated increased expression of CD31 (Figure 3), reduced p53 expression (Figure 4) and enhanced Bcl-2 expression during the colorectal cancer development (Figure 5).

5. Discussion

DMH is used in many researches to induce colorectal cancer. It takes 5 to 7 months to form dysplastic ACFs which is the key clinicopathologic change in colorectal cancer (8, 20, 21). The present study demonstrates the histopathologic changes during the development of colorectal cancer in the 10th to 40th weeks of DMH treatment. In this study, abnormal crypts which demonstrate structural and morphological changes appear at week 20; although a decreased number of goblet cells is observed at week 10 (Figure 2). Hyperchromatism, TILs and angiogenesis changes start in 10th week but epithelium changes and dysplasia in crypts appear in 15th week. Hyperchromatism rise to the high index in 25th week however TILs get to the high index of changes in 30th week. The highest amount of

all changes were in 40th week. The relationship between angiogenesis and clinical prognosis encourages many researchers investigate the MVD alterations. MVD is the most acceptable marker for cancer angiogenesis (22). Increased MVD which is assessed by the antibody against CD31 is related with angiogenesis and lymphatic vessel proliferation and is an important prognostic factor of colorectal cancer (17, 23, 24). Even the studies which demonstrate that quantification of MVD ha no prognostic value state that angiogenesis is an important step during tumor progression (10). In the present study, increased intensity of CD31 is seen between 30th and 40th week. Several researchers have demonstrated the relationship among mutations of p53 and Bcl-2 and poor clinical outcome (10, 25, 26) even if no correlation exists between p53 gene status and angiogenesis (10). Inhibition of apoptosis which is regulated by genes such as Bcl-2 can lead to the growth and progression of colorectal cancer (26). In this study, diminished amounts of p53 is observed at week 40, however, increased intensity of CD31 and Bcl-2 is seen between the 30th and 40th week. A low number of TILs is associated with poor prognosis of colorectal cancer (27). As in the present study, TILs increase during cancer development to restrict the tumor growth and invasion.

Considering the impact of the study on the important factors involved in the development of cancer and the treatment based on these factors, more animal and human samples can be used to generalize the findings of this study. More specific IHC markers for colorectal cancer can be used for therapeutic purposes.

5.1. Conclusions

The results of this study demonstrate the main histopathologic changes during the progression of colorectal cancer. Increase in TILs and angiogenesis, which were the first changes, could be the basis of dysplastic crypts and ACF formation. In conclusion, TILs and angiogenesis might be the important earliest factors that contribute to colorectal cancer progression.

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Footnotes

Authors' Contribution: Vahid Nejati and Jamileh Abedi: study concept and design and acquisition of data; Maedeh Vakili Saatloo and Maryam Koohsoltani: analysis and interpretation of data, drafting of the manuscript and critical

Table 1. Analysis of Semi-Quantitative Abnormalities of Colorectal Tissue During Cancer Development[a]

Parameters	Groups, Mean ± SE						
	Healthy Control	Cancer Group (10th Week)	Cancer Group (15th Week)	Cancer Group (20th Week)	Cancer Group (25th Week)	Cancer Group (30th Week)	Cancer Group (40th Week)
Hyperchromatism	0	0.36 ± 0.08	0.79 ± 0.16	1.2 ± 0.11	1.75 ± 0.14	1.75 ± 0.1	1.83 ± 0.16
TILs	0	0.15 ± 0.07	0.51 ± 0.13	0.86 ± 0.18	1.53 ± 0081	1.76 ± 0.08	1.91 ± 0.08
MVD	0	0.1 ± 0.08	0.15 ± 0.2	0.26 ± 0.03	0.4 ± 0.05	1.25 ± 0.14	2
Destruction of epithelium	0	0	0.14 ± 0.02	0.074 ± 0.092	1.2 ± 0.15	1.43 ± 0.12	1.93 ± 0.09
Epithelial cells shedding	0	0	0.2 ± 0.057	0.63 ± 0.044	0.93 ± 0.08	1.45 ± 0.12	1.97 ± 0.01
ACF	0	0	0.36 ± 0.05	0.86 ± 0.08	1.43 ± 0.07	1.78 ± 0.05	1.91 ± 0.08

Abbreviations: ACF, aberrant crypt foci; MVD, microvessel density; TILs; tissue lymphocyte infiltrations.
[a] Values are defined as: 2 = highest, 1.5 = high, 1 = moderate, 0.5 = weak, 0 = no abnormality is seen.
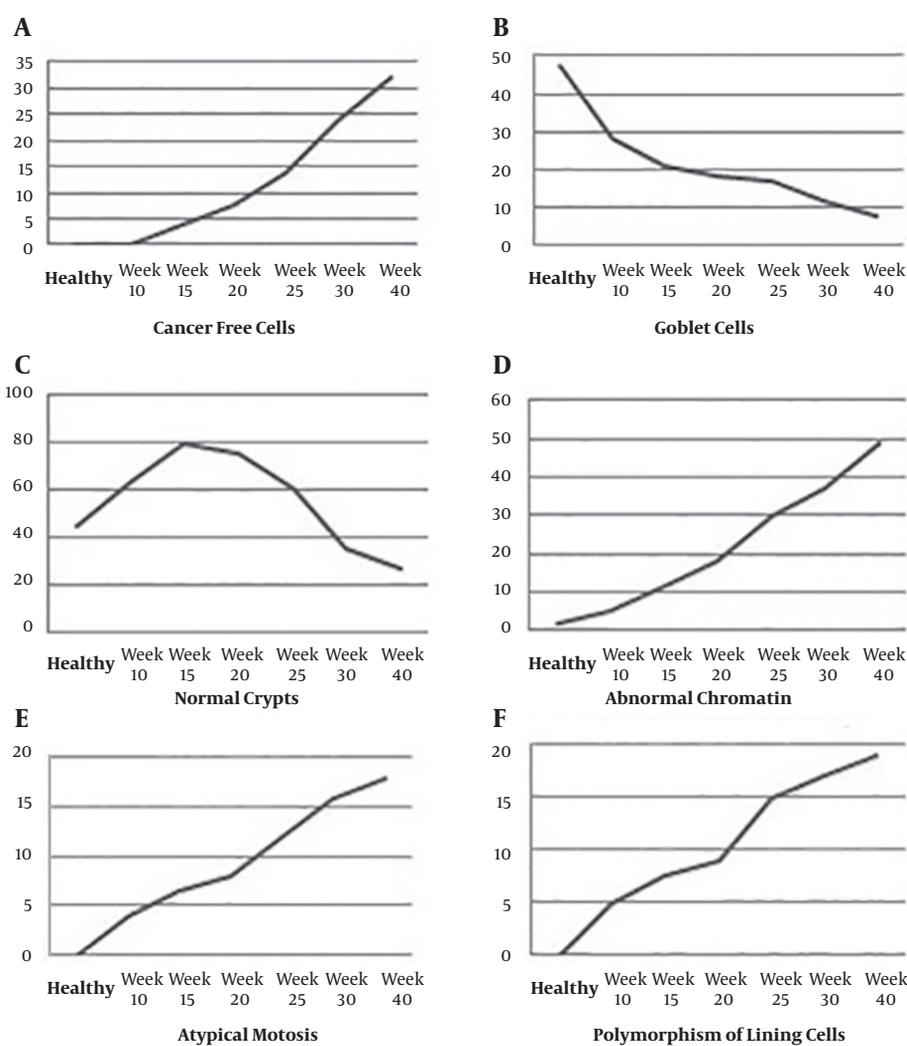
Figure 1. Number of cancer free cells (A), goblet cells (B), normal crypts (C), abnormal chromatin (D), abnormal mitoses (E), and epithelial cell polymorphisms (F) from 10th to 40th weeks of DMH- treatment
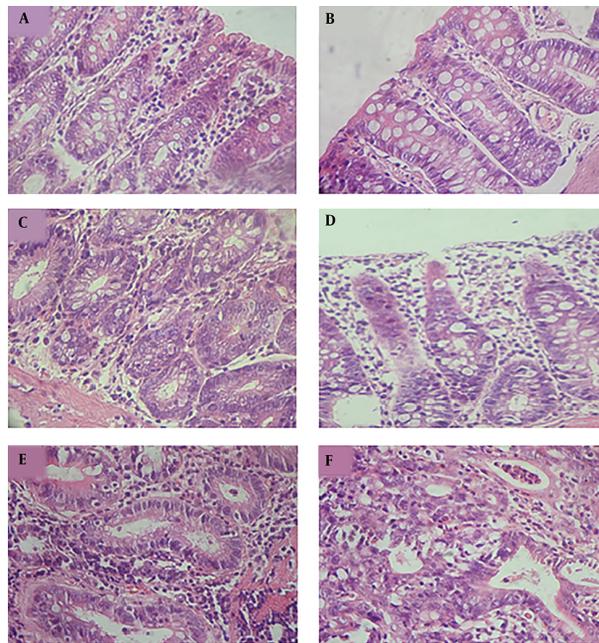
Figure 2. Microscopic images of colorectal sections in healthy and DMH-treated cancer group (magnification: × 400. H & E staining). A, healthy group shows normal crypts and goblet cells. B, DMH-treated cancer group at 10th week shows nuclear hyperchromatism, increased TILs and decreased goblet cells. C, DMH-treated cancer group at 15th week shows increased number of crypts, decreased amount of goblet cells, increased TILs and atypical mitosis. D, DMH-treated cancer group at 20th week shows structural and morphological changes of crypts and increased connective tissue among crypts. E and F, DMH treated cancer group at 25th and 30th weeks show abundant angiogenesis among abnormal crypts, destruction of crypts and atypical mitosis.
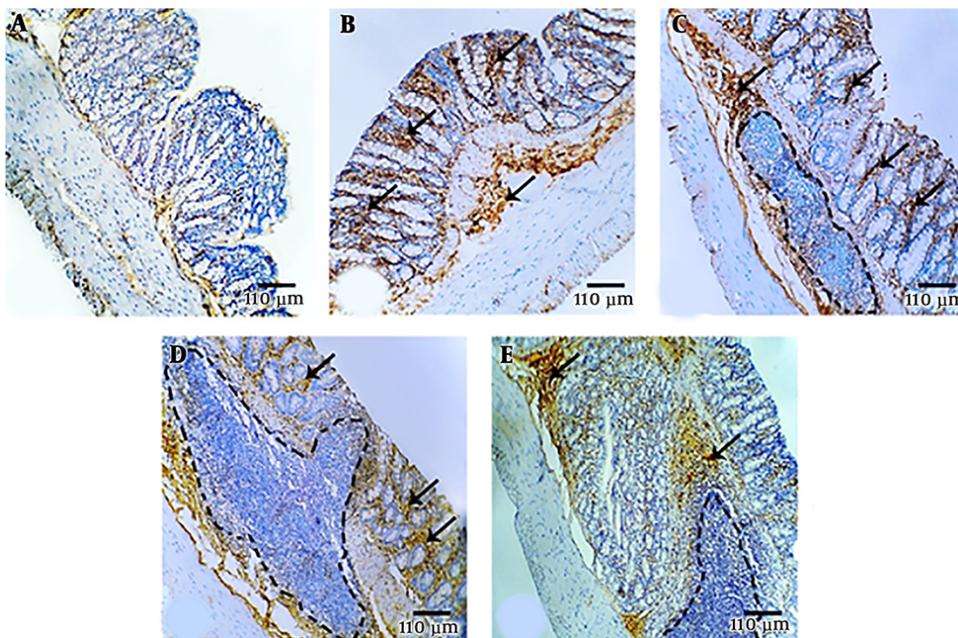
Figure 3. Cross section from DMH-treated colorectal cancer and healthy control group. A, healthy control; B, 10th weeks; C, 20th weeks; D, 30th weeks; and E, 40th weeks after DMH treatment. See time dependent increased angiogenesis (arrows) between mucosa and submucosa layers, which has been developed between weeks 30 and 40. The tumor area is marked with non-continuous line (IHC staining, × 400).
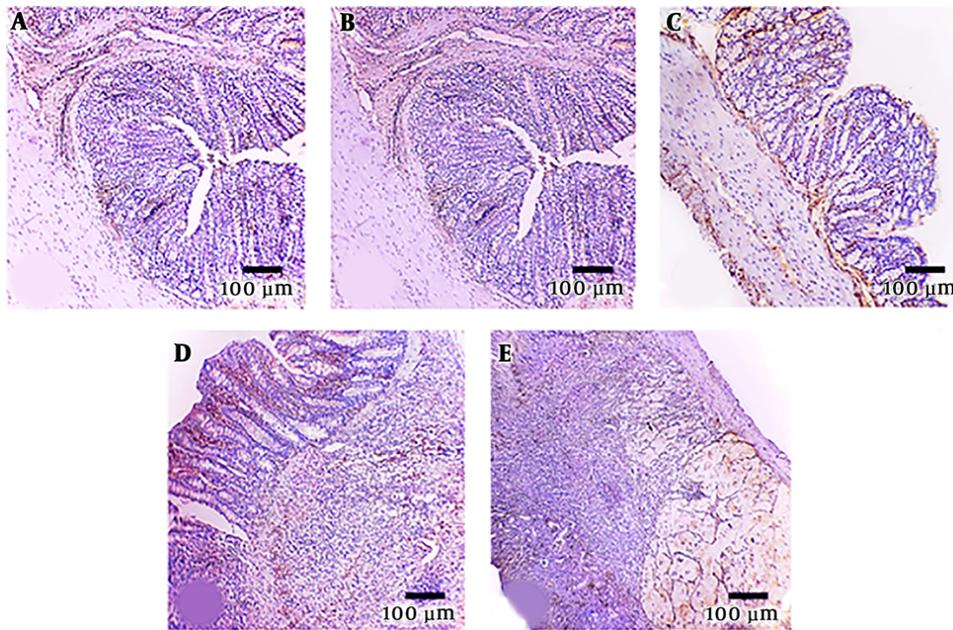
Figure 4. Cross sections from DMH-treated colorectal cancer and healthy control group. A, healthy control; B, 10th weeks; C, 20th weeks; D, 30th weeks; and E, 40th weeks after DMH treatment. Brown chromogen reactions demonstrate reduced p53 expression by the time. Note diminished p53 at week 40. (IHC staining, ×400).
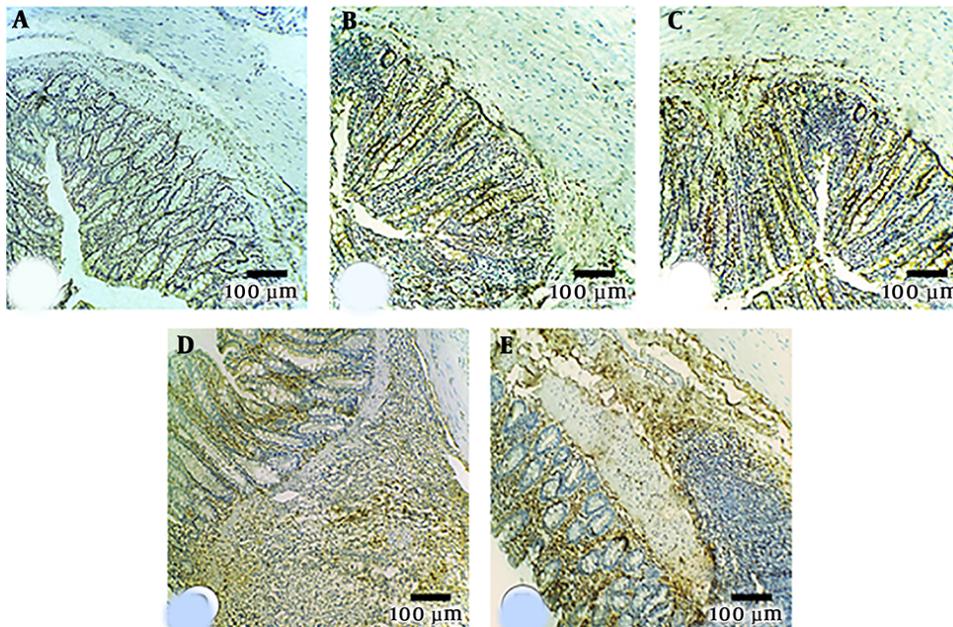
Figure 5. Cross section from DMH-treated colorectal cancer and healthy control group. A, healthy control; B, 10th weeks; C, 20th weeks; D, 30th weeks; and E, 40th weeks after DMH treatment. Brown chromogen reactions are presenting enhanced Bcl-2 expression by the time. Note intensive Bcl-2 biosynthesis between weeks 30 and 40. (IHC staining for Bcl-2, ×400).

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