RESEARCH ARTICLE

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Comparative Study of DNA Ploidy and *BRAF* Immunohistochemistry between Colonic Adenocarcinoma and Inflammatory Colonic Lesions

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Abstract

Objectives: To evaluate DNA ploidy and S-phase fraction (SPF) in non-Lynch colonic adenocarcinoma, ulcerative colitis (UC), Crohn disease (CD) which are known as risk factors, and colitis. We correlated ploidy and SPF with tumor grading, staging and *BRAF* expression. **Methods:** All studied adenocarcinomas have intact mismatch repair genes as proved by immunohistochemistry. All were assessed for ploidy by automated image-based DNA cytometry and histograms were drawn. Immunostaining by anti-*BRAF* V600E was performed. Diagnostic laparoscopy (DL) was done as a preliminary step for staging GI cancers. **Results:** there is significant difference in DNA ploidy between groups; 77.5% and 17.5% of aneuploid cases are adenocarcinoma and UC. Groups are compared in terms of 2C, 4C, above 4C DNA content and SPF and significant difference is principally found between adenocarcinoma group and others. In adenocarcinomas, DNA ploidy is significantly correlated with tumor staging and grading. Regarding *BRAF* expression, there is significant difference between groups; all adenocarcinomas, 83.33% of UC were positive, while all cases of colitis, bilharzial colitis, CD were negative. There is significant relation between *BRAF* and SPF among all diploid cases including adenocarcinoma, and among non-neoplastic diploid cases. There is direct significant relation between *BRAF* intensity and adenocarcinoma staging. There is no significant difference between *BRAF* and ploidy among UC cases, although 75% of aneuploid UC are positive. DL helps in GI cancer staging. Routine laparoscopy before laparotomy, especially in cancers which have equivocal operability helps to avoid unnecessary laparotomies. **Conclusion:** Based on significant difference in ploidy between adenocarcinoma and UC and between SPF and ploidy, assessment of ploidy by DNA cytometry for UC and other colitis could therefore predict impending malignant transformation before development of colonic dysplasia. Also measuring SPF in adenocarcinoma helps to select patients who could greatly benefit from chemotherapy. DL has vital role in staging GI cancers.

Keywords: DNA ploidy- Histogram- *BRAF*- colon adenocarcinoma

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Introduction

Colorectal cancer (CRC) is one of most common malignancy worldwide. It develops from sequential activation of oncogenes and inactivation of tumor suppressor genes. Mutation in v-Raf murine sarcoma viral oncogene homolog B (*BRAF*) occurs in approximately 10%–15% of CRC (Barras, 2015). *BRAF* oncogene encodes *BRAF* protein, stimulates mitogen-activated protein kinase (MAPK) pathway. Change from valine to glutamic acid at codon 600 (V600E mutation) represents 80% of all *BRAF* mutations (Ritterhouse and Barletta, 2015). *BRAF* mutation is as well an independent predictor of poor prognosis in CRC (Barras, 2015). *BRAF* mutations in CRC can be detected by allele-specific polymerase chain reaction (PCR) or Sanger sequencing, but both are expensive and time-consuming. Nowadays, IHC against *BRAF* V600E mutant protein becomes feasible as an alternative diagnostic tool for *BRAF* V600E mutation detection in CRC (Bledsoe et al., 2014).

DNA ploidy is a measurement of DNA content. In the histogram, the x-axis shows DNA-ploidy values (2c, 4c, and so on), where 2c are diploid cells where the nuclear DNA ploidy are in the G0/G1 phases of the cell cycle. The y-axis illustrates the number of nuclei corresponding to each interval in the histogram. A diploid histogram has a major peak corresponding to the DNA content of the 46 chromosomes (point 2c; DNA index, DI of 1), with a small proportion of cells captured at stages in which DNA is being, or had been, replicated, thus, possess

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up to double the normal amount of DNA (up to 4c on the x-axis). A tetraploid histogram shows an abnormal 'tetraploid' peak at the 4c indicating increased number of cells containing 4 sets of 23; DI 1.99 which represents either cells at G2 phase, or viable cells with tetraploid genome, or both. An aneuploid peak indicates that some cells possess an abnormal DNA content that is exceeds that of diploid cells, but less than if the whole set chromosome was duplicated. Aneuploidy is an inevitable result of chromosomal instability and can be detected quantitively using automated image-based DNA cytometry. The DI is the degree of aneuploidy and vary according to the extent of chromosomal losses or gains. S phase fraction (SPF) is the percentage of cells synthesizing DNA for chromosomal replication, lies along a continuum between diploid (2c) and tetraploid (4c) before completion of mitosis (Danielsen et al., 2016).

DNA ploidy and SPF are significant independent factors for prognosis of CRC. Cases with DNA diploid tumors have a higher survival rate than those patient with aneuploid tumors. Aneuploid tumors have a higher median SPF than diploid tumors (Zhao et al., 2021).

Ulcerative colitis is associated with a significantly increased risk for CRC development that is directly related to both extent and disease duration. To detect patients at high risk, colonoscopic surveillance with mucosal biopsy to detect dysplasia is widely used nowadays. Total reliance on microscopic changes as the only marker for malignant transformation has limitations owing to the subjective nature of the dysplasia assessment and interobserver and intraobserver variability due to the influence of inflammation. In addition, sampling error may occur because of the patchy nature of dysplasia. Detection of DNA aneuploidy in mucosal biopsies may be more reliable marker for subsequent malignant transformation of the colorectal mucosa (Beaugerie and Itzkowitz, 2015). Aneuploidy as well occurs as an early genetic alteration preceding morphologic changes of neoplasia, it is not confined to dysplastic epithelium only, DNA aneuploidy in flat mucosa may constitute an additional marker in the identification of patients at increased cancer risk who could benefit from a closer surveillance (Schimmelpenning et al., 2000).

One plausible reason for colon cancer cells being chemotherapy unresponsiveness is the large cell number in the G0 phase (Jedema et al., 2003). In this study, we compared DNA ploidy and SPF in colonic adenocarcinoma, UC, CD, non-bilharzial colitis, bilharzial colitis and control group. We correlated DNA ploidy and SPF with tumor grading, staging and *BRAF* expression. Assessment of DNA ploidy could therefore be a main element in future surveillance programs for screening early malignant transformation. Evaluation of SPF of tumor cells could select the patients who have the greatest benefit from chemotherapy.

Diagnostic laparoscopy (DL) has several advantages over other diagnostic modalities. This is reflected on its effectiveness in visualizing abdominal and pelvic cavities and detecting minimal ascites and small peritoneal deposits that may be missed by other imaging studies. It also detects adenopathies and small hepatic metastases. Moreover, it makes it possible to evaluate the local infiltration of the structures surrounding a given lesion and to decide whether it should be resected. An important goal of this technique is to reduce the number of negative laparotomies and reduce the surgical burden in patients who appear to have resectable abdominal masses. This results in reduced perioperative morbidity and mortality, shorter hospital stays, and earlier referral to chemoradiotherapy (Yeola et al., 2018).

Materials and Methods

Ninety patients were enrolled in this study. They were admitted to the Gastroentrology and Surgery Departments at the Theodor Bilharz Research Institute (TBRI) Hospital. The study protocol was approved by the Ethics committee of Theodor Bilharz Research Institute (TBRI) according to the institutional committee for the protection of human subjects and adopted by the 18th world medical assembly, Helsinki, Finland. The patients were grouped into:

Group (1): control, 6 cases

Group (2): non-bilharzial colitis, 8 cases.

Group (3): chronic bilharzial colitis, 10 cases.

Group (4): UC, 12 cases, 6 with dysplasia, 6 without dysplasia

Group (5): CD, 10 cases.

Group (6): CRC, 44 cases, all were non-Lynch adenocarcinoma (have intact mismatch repair genes; MMR). Immunostaining for MMR genes were performed (Figure 1, 2).

The studied cases include 6 colonic biopsies with unremarkable pathological lesions as a control group. The patients of groups 2-5 underwent colonoscopy and biopsy from apparent gross lesions. Tissue samples of group 6 were obtained through partial colectomy specimens. All tissue specimens were delivered to the Pathology Department of TBRI for histopathological assessment (Figure 3).

Operation protocol

The peritoneal cavity was insufflated to 15 mm Hg and two trochars (10m, 5m) were introduced. Any suspicious lesion was biopsied and sent for frozen section, and if ascites was present, the fluid was collected and sent for cytology, if negative, we proceed for colectomy according to site of tumor.

Histopathological study

Tissues were fixed in 10% buffered formalin. A paraffin-embedded tissue biopsy is routinely processed and stained by Hematoxylin and Eosin stains. Histopathological diagnosis was done using the standard criteria for diagnosis of inflammatory bowel disease (IBD), WHO classification (Nagtegaal et al., 2020) and modified Dukes' classification (Bresalier, 2010) of colo-rectal tumors.

In the 10 patients of chronic bilharzial colitis, schistosomal infestation was diagnosed by identifying the bilharzial ova in tissue samples or detecting schistosomal antibodies in serum using ELISA technique.

Immunohistochemical evaluation of BRAF

Tissue sections from all different studied cases were immunohistochemically stained for *BRAF* V600E (ROCHE USA Tuscon) using the Dako autostainer (48 link, Dako, Denmark):

Preparation of Slides

- Three unstained Paraffin sections 5µ thickness for each case were cut by the microtome. Sections were mounted on the glass slides.

- All slides were deparaffinized according to the setup of the laboratory where the autostainer is being installed.

- After deparaffinization, we rinsed slides thoroughly under running water and placed them in a bath of autostainer wash buffer to soak for at least 5 minutes before staining. The reagent vials were labelled, the time was set on the computer to the time at the current autostainer location, and a protocol template was created using the programming as presented in DAB programming subsection. We loaded the reagents and slides onto the autostainer, then started the run.

- After the run had ended, the slides were removed from the autostainer, placed in a bath and rinsed thoroughly under running water, counterstained with Hematoxylin, and finally we coverslip the slides using DPX.

Interpretation of BRAF immunohistochemistry

The intensity of anti-*BRAF* V600E in cells was graded on a 0–3 scale. Strong cytoplasmic staining was scored as 3, medium cytoplasmic staining as 2, weak cytoplasmic staining as 1 and the absence of staining was scored as 0. The staining is considered positive if diffuse (>50% of tumor cells) with intensity ≥1 (Dvorak et al., 2019)

Evaluation of DNA Content Using Image Analysis System

In analysis of DNA ploidy, the DNA content is measured per nucleus for all nuclei of a tissue specimen to yield a histogram illustrating the DNA content in each sample. This is an objective measure of genomic instability which discriminates between nuclei of normal DNA content and nuclei of abnormal DNA content.

Principle of the Procedure

The Feulgen stain specifically and quantitatively stains the nuclear DNA blue while the cytoplasm appears transparent. The quantity of this blue colored compound formed is directly proportional to the DNA content within the nucleus of the cell. In a population of normal and abnormal cells there is an obvious difference between the nuclei in the staining intensity. Cells in the S phase appear darker than their normal counterparts because of their increased amount of DNA content (Schutte et al., 1989).

DNA Image Analysis

Automated image analysis assessment of nuclear DNA of hepatocytes was performed using the computer-controlled analysis system (Image Analysis System, Zeiss Germany), this essentially consists of a computer-controlled microscope (Zeiss Axioscope microscope), video camera, one monitor and a computer unit. Image analysis technique was performed using the software program Axio vision 4.8. which allowed the colored compound that develops in the stained nuclei by Feulgen to be directly proportional to DNA content within the nucleus and can be measured as quantifiable integrated optical density (IOD) (Ranaldi et al., 1992). A number of nuclei ranging from 150-200 cells were submitted for DNA analysis in each case at 400x lens magnification. Only single monolayer nuclei without overlapping were analyzed. Reference cells are necessary for DNA scaling of densometric measurements. Imprint from normal mice liver were used as standard control. Reference cells were stained exactly as the cells under analysis in the same staining bath with slides of the sample. On analyzing their DNA content, reference histograms were elaborated and considered reference control histograms for sections under study (Schutte et al., 1989).

The elaborated DNA histograms were classified into

Diploid or aneuploid histograms based on DNA index (DI) of the main peak.

Diploid

Exhibiting a significant peak in the diploid range (DI=1±10%) were further sub classified according to the percentage of proliferating cells in the S phase fraction into:

- Diploid histogram with mild increase in S phase, when the percentage of proliferating cells at S phase equal 10-20% of total number of analyzed cells.

- Diploid histogram with moderate increase in S phase, when the percentage of proliferating cells at S phase equal 21-30 of total number of analyzed cells.

- Diploid histogram with marked increase in S phase, when the percentage of proliferating cells at S phase exceeds 30 % of total number of analyzed cells (Eskelino et al., 1995; Yosef et al., 1996)

Aneuploid histogram

displaying a mass peak or multiple peaks outside the diploid or tetraploid range.

Terms used in DNA analysis

Ploidy: Analysis of DNA content

Diploid: DNA content of the normal cell or 2C.

Aneuploid: Abnormal DNA content.

S Phase: The synthesis phase during which, there is rapid replication of the DNA content of the cell, which varied from 2C to 4C.

DNA Index (DI): DI= Modal aneuploid Go/Gl DNA content /Modal diploid GO/Gl DNA content. The DI is commonly used to compare the DNA content of abnormal with that of normal cells

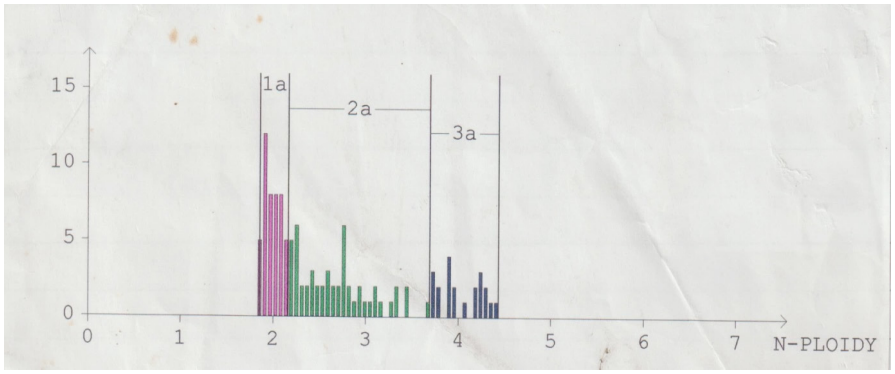
G0: Resting phase of the cell cycle.

G l: pre-synthetic phase.

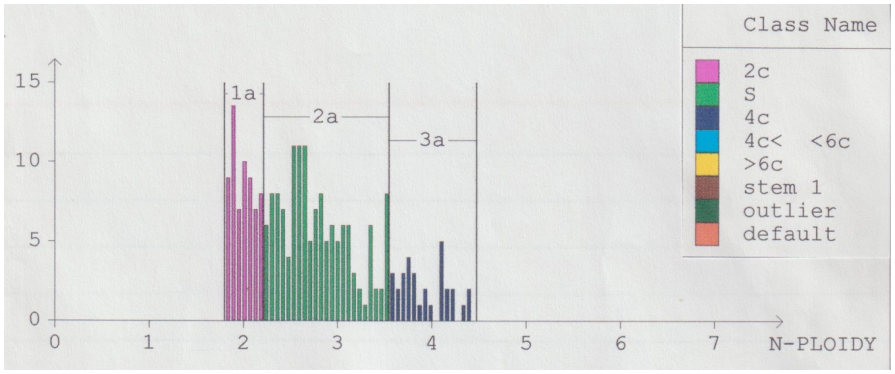
M phase: period of mitosis (Aziz et al., 1991; Borgmama et al., 1991) (Histogram 1-6).

Results

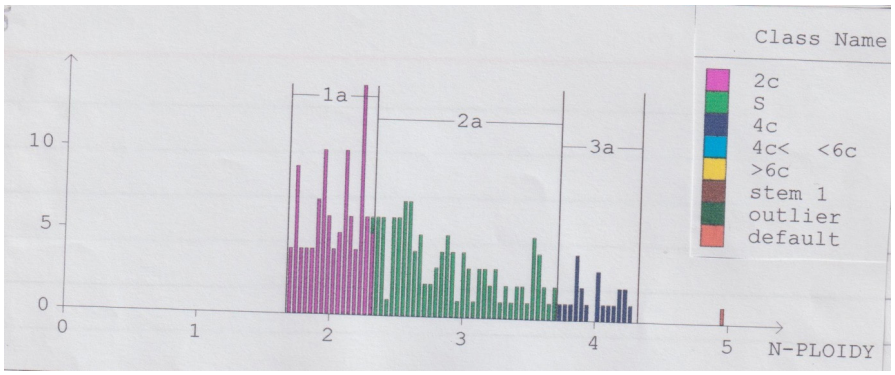
Ninety patients were enrolled in this study, they range in age from 30-86 years with mean age 58 years.



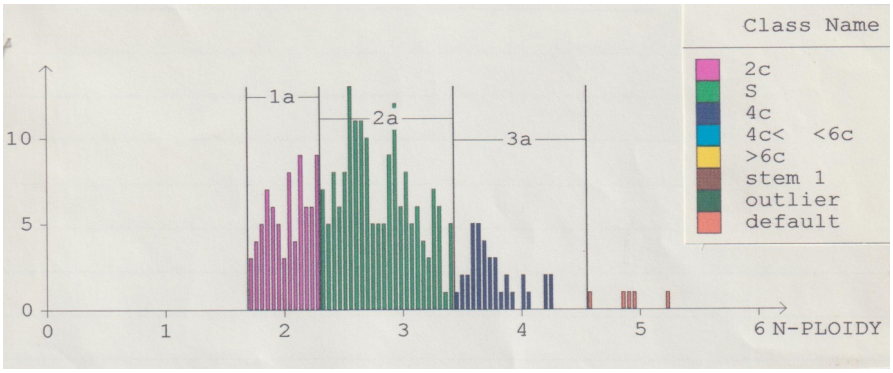
Histogram 1. A histogram of control mucosa (control case), the majority of the nuclei (70%) are diploid at 2C with S phase (20.73%), tetraploid at 4c (5.61) and DI peak 0.99.



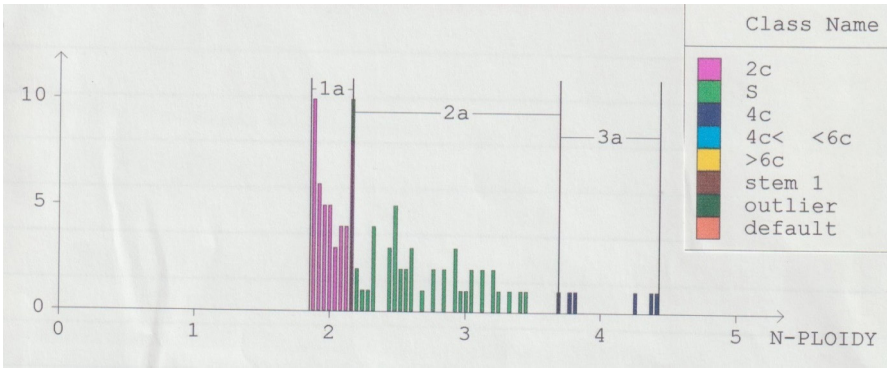
Histogram 2. A histogram of bilharzial colitis case, the nuclei (27.78%) are diploid at 2C with S phase (58.97%), tetraploid at 4c (13.25) and DI peak 0.94.



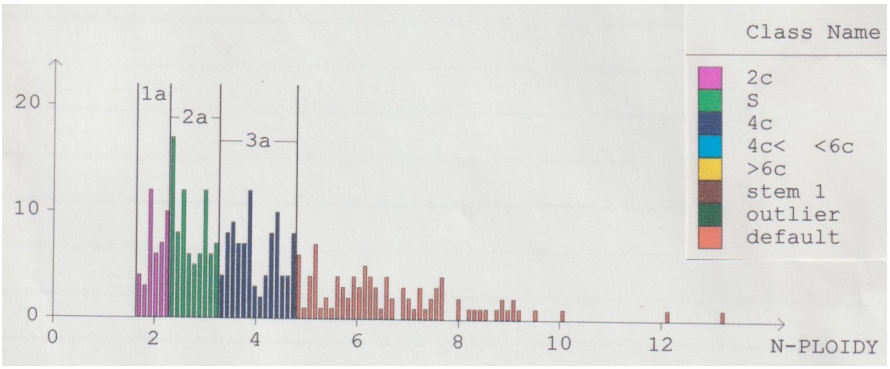
Histogram 3. A histogram of non-bilharzial colitis case, the nuclei (43.22%) are diploid at 2C with S phase (47.46%), tetraploid at 4c (8.90), the nuclei above 5C (0.42%) and DI peak 1.11.



Histogram 4. A histogram of UC case, the nuclei (26.60%) are diploid at 2C with S phase (58.87%), tetraploid at 4c (12.77), the nuclei above 5C (1.77%) and DI peak 1.28.



Histogram 5. A histogram of Crohn's case, the nuclei (46.88%) are diploid at 2C with S phase (46.88%), tetraploid at 4c (6.25), and DI peak 0.94



Histogram 6. A histogram of adenocarcinoma case, the majority of the nuclei are aneuploid (27.63 %) above 4C, S phase (25.99%), tetraploid at 4c (29.61), diploid at 2c (13.82%) and DI peak 1.17.

Regarding the DNA content, there was significant difference between the studied groups. 77.5% of the aneuploid cases are adenocarcinoma, 17.5% are ulcerative colitis and 5% are Crohn's disease. Regarding the diploid histogram, all the control cases (6/6), colitis (8/8), and bilharzial colitis (10/10) are diploid. Five out of the 12 cases of UC and 8 out of the 10 cases of CD are diploid. The variation between the different groups is statistically significant ($P < 0.01$). In analysis the relation between histogram of adenocarcinoma cases and Duke staging, there was significant difference between DNA content of adenocarcinoma cases and Duke stage ($P= 0.01$). 51.6% and 38.7% of the adenocarcinoma with aneuploid histogram are Duke B and C respectively. 37.5% and 50% of the adenocarcinoma with diploid histogram are Duke A and B respectively.

Regarding the relation between histogram of adenocarcinoma cases and histological grading, there was significant difference between DNA content of adenocarcinoma cases and histological grade ($P<0.01$). 71% and 19.4% of the adenocarcinoma with aneuploid histogram are grade II and III respectively. 62.5% and 37.5% of the adenocarcinoma with diploid histogram are grade I and II respectively. Regard to the 2C DNA content, each of the control, colitis, bilharzial colitis, CD is significantly different from the adenocarcinoma group and dysplastic UC group. Non dysplastic UC is as well significantly different from the adenocarcinoma group. However no significant difference between the dysplastic UC group and adenocarcinoma group. Regarding the SPF, there is significant difference between the dysplastic UC and each of control, colitis and malignancy group.

Table 1. Nuclear DNA Content, in Colonic Lesions

Group	2C (Mean ±SD)	4C (Mean ±SD)	SPF (Mean ±SD)	above 4C (Mean ±SD)
Control (6)	69.70±13.72@$	5.01±0.89@	21.32±8.83$	00.00±0.00@
Colitis (8)	63.75±6.43@$	11.59±3.36@	24.56±7.04$	00.00±0.00@
Bilhcolitis (10)	57.40±7.88@$	8.20±1.75@	36.30±7.87	00.0 ±0.00@
Non-dysplastic UC (6)	50.79±8.87@	12.63±6.17	39.37±10.19	00.0 ±0.00@
DysUC (6)	29.50±10.68	12.35±4.41	49.80±14.70**#@	00.58±0.93@
CD (10)	62.06±8.65@$	7.75±4.04@	35.12±19.20	0.36 ±00.58@
CRC (44)	28.41±15.25	21.89±11.09	31.91±10.74$	12.83±7.79

** P<0.05, Significant difference from control group; # P<0.01 Significant difference from colitis group; @ P<0.01 Significant difference from adenocarcinoma group; $ P<0.01 Significant difference from Dysplastic UC group.

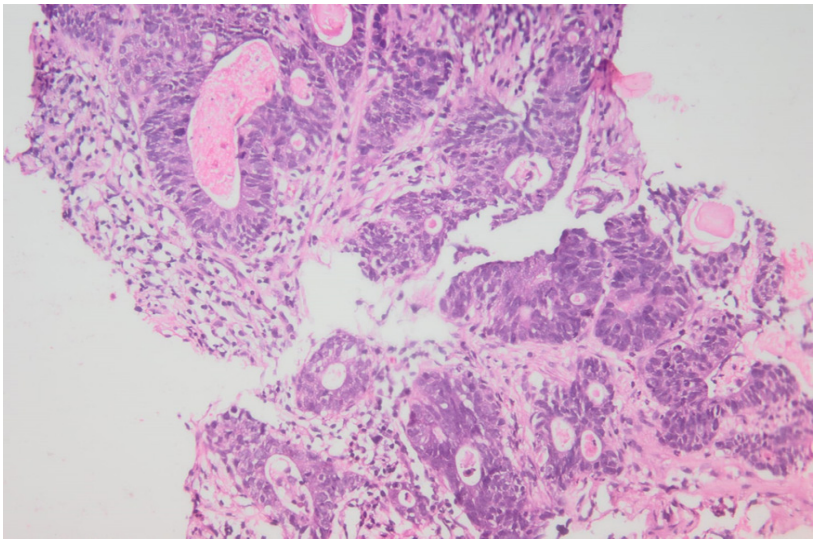


Figure 1. Colorectal Adenocarcinoma Hematoxylin & Eosin (Original Magnification, x200).

Regarding the above 4C DNA content, each of the control, colitis, bilharzial colitis, CD, non-dysplastic UC and dysplastic UC is significantly different from the adenocarcinoma group (Table 1).

Regard to the 2C DNA content, Duke A is significantly different from that of B and C. In the SPF, there is significant difference between Duke B and C. No significant difference is found between Duke A, B or C in the above 4C DNA content (Table 2). Regard to the percentage of cells expressing *BRAF* and intensity of expression, there was significant difference between the groups (P<0.01). All adenocarcinoma cases show >50% *BRAF* expression and 72.7% show marked intensity. 83.33% of UC are positive for *BRAF* showing >50%

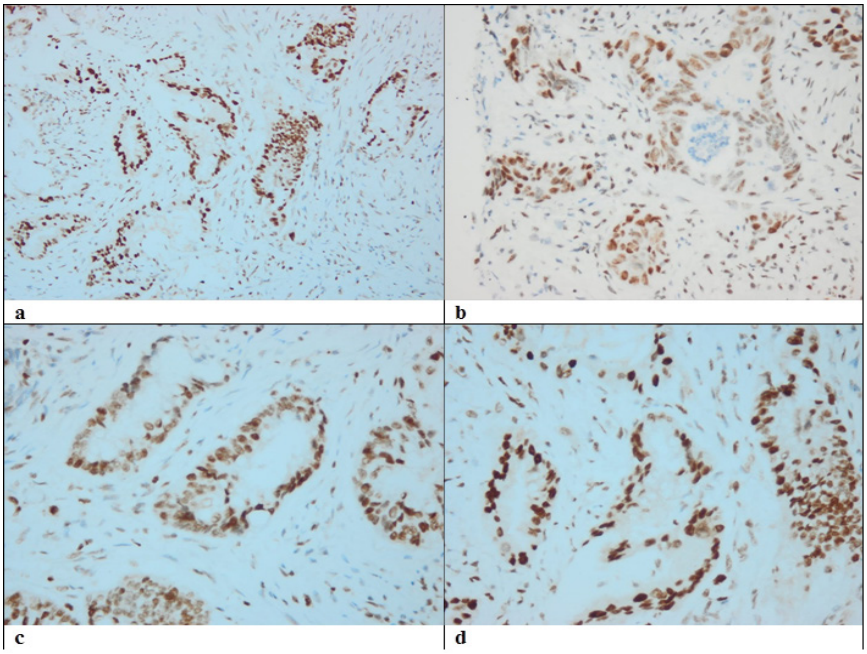


Figure 2. Colorectal Adenocarcinoma. Cases Showing Intact MMR Genes Immunostaining (Original Magnification, (a) 100x, (b) 200x, (c, d) 400x)

Table 2. Nuclear DNA Content, in Adenocarcinoma

Group	2C (Mean ±SD)	4C (Mean ±SD)	SPF (Mean ±SD)	Above 4C (Mean ±SD)
Duke A (6)	46.72±9.10	21.06±10.15	29.38±9.01	13.30±13.11
Duke B (20)	29.27±14.27*	17.13±6.51	37.99±10.13	11.76±8.34
Duke C (18)	21.35±12.83*	27.46±13.17#	26.01±8.39#	13.86±4.76

* P<0.01, Significant difference from A duck group; #P<0.01 Significant difference from B duck group.

Table 3. Relation between BRAF Immunohistochemical Expression among the Studied Groups

		Pathological Diagnosis					
		Control	Colitis	Bilhcolitis	UC	Crohn	Malignant
BRAF percentage	0	6 100.00%	0 0.00%	0 0.00%	0 0.00%	0 0.00%	0 0.00%
	1-25%	0 0.00%	5 62.50%	0 0.00%	0 0.00%	1 10%	0 0.00%
	25-50%	0 0.00%	3 37.50%	10 100%	2 16.67%	9 90%	0 0%
	>50%	0 0.00%	0 0.00%	0 0.00%	10 83.33%	0 0.00%	44 100%
BRAF intensity	0	6 100.00%	0 0.00%	0 0.00%	0 0.00%	0 0.00%	0 0.00%
	mild	0 0.00%	7 87.50%	1 10%	0 0.00%	4 40%	2 4.50%
	moderate	0 0.00%	1 12.5	9 90%	6 50%	6 60%	10 22.70%
	marked	0 0.00%	0 0.00%	0 0.00%	6 50%	0 0.00%	32 72.70%
Total		6 100%	8 100%	10 100%	12 100%	10 100%	44 100%

BRAF expression with moderate and marked intensity (Figure 4). *BRAF* was negative in all cases of colitis, bilharzial colitis and CD (Table 3). There was significant relation between percentage of *BRAF* expression and SPF, (P=0.02) and also significant relation between intensity and SPF among the diploid cases (P=0.002). 90% of diploid cases with mild increase in S phase are negative for *BRAF*. On the other hand, 44.4% of diploid cases with marked increase in SPF show >50% *BRAF* expression and 33.3% show marked *BRAF* expression (Table 4).

There was significant relation between percentage of *BRAF* expression and SPF, (P=0.001) and also significant relation between intensity and SPF among the non-neoplastic diploid cases (P=0.01) (Table 5).

All non-neoplastic diploid cases with mild increase in SPF are negative for B raf and 33.3% show mild intensity. On the other hand, 28.6% of non-neoplastic diploid cases with marked increase in S phase show >50% *BRAF* expression, and 57.1% and 21.4% show moderate and marked *BRAF BRAF* intensity respectively (Table 5). All adenocarcinoma cases show >50% *BRAF* expression. There was significant relation between intensity of *BRAF* expression and Duke staging. All adenocarcinoma cases in Duke C stage and 70% of Duke B show marked *BRAF* intensity. On the other hand, 33.3% and 66.7% of Duke A show mild and moderate expression respectively. This difference was statistically significant (P<0.01) (Table 6).

Among the UC cases, there was no significant relation was detected between percentage of *BRAF* expression and DNA ploidy (P=1), *BRAF* although 75% of aneuploid cases show >50% *BRAF* expression. Also, no significant relation between intensity of *BRAF* expression and DNA

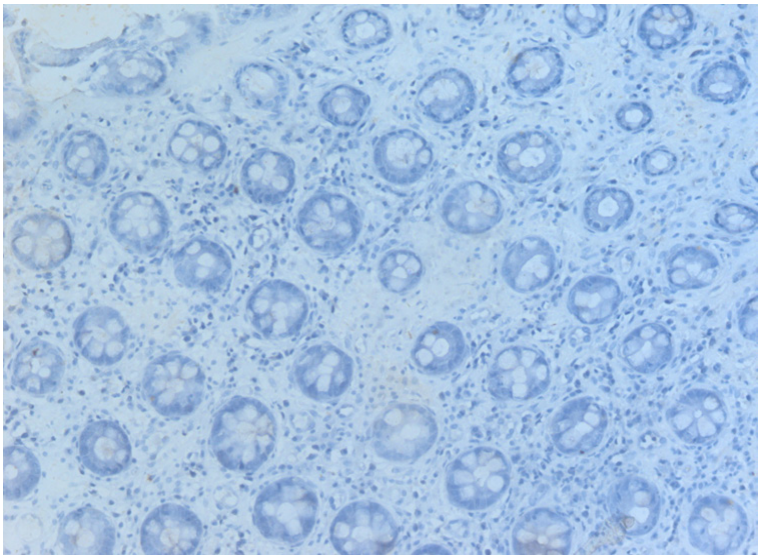


Figure 3. Control Case Immunostaining Showing Negativity for BRAF (Original Magnification, x200)

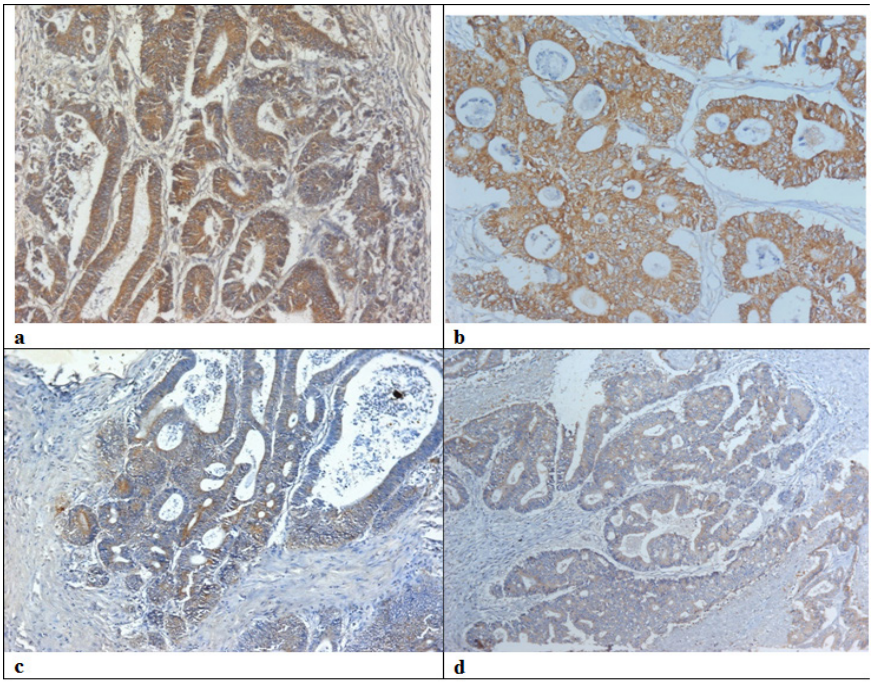


Figure 4. Colonic Adenocarcinoma Cases Showing Immunostaining Positivity for BRAF, (a, b) marked intensity, (c) moderate, (d) weak intensity (Original Magnification, 200x)

ploidy (P=0.1), however, all cases of aneuploid cases show moderate and marked expression (Table 7). Among the IBD cases, there was no significant relation was detected between percentage of *BRAF* expression and DNA ploidy (P=0.3), however, 60% of aneuploid cases show >50% *BRAF* expression. Also, no significant relation between intensity of *BRAF* expression and DNA ploidy (P=0.3), however, all cases of aneuploid cases show moderate and marked expression (Table 8). Data was analysed using the Statistical Package for the Social Science Version 22 (IBM Corp., Armonk, NY, USA). Chi square, fisher exact test and analysis of variance (ANOVA) were used for comparing the qualitative variables. P value above 0.05 was considered significant (Table 9).

Discussion

Colorectal cancer (CRC) is one of most common malignancy worldwide. Aneuploidy is an inevitable result of chromosomal instability and can be detected quantitively using automated image-based DNA cytometry (Danielsen et al., 2016). DNA ploidy and

Table 4. Relation between BRAF Expression and SPF of the Diploid Cases Including Adenocarcinoma Cases

		S phase Fraction (SPF)			
		Diploid with mild increase	Diploid with moderate increase	Diploid with marked increase	P value
BRAF percentage	0	5 50%	0 0.00%	1 5.60%	0.002
	1-25%	1 10%	4 23.50%	1 5.60%	
	25-50%	3 30%	10 58.80%	8 44.40%	
	>50%	1 10%	3 17.70%	8 44.40%	
BRAF intensity	0	5 50%	0 0.00%	1 5.60%	
	Mild	3 30%	6 35%	3 16.70%	
	Moderate	2 20%	9 52.90%	8 44.40%	0.002
	Marked	0 0.00%	2 11.80%	6 33.30%	
	Total	10 100%	17 100%	18 100%	

Table 5. Relation between BRAF Immunohistochemical Expression and SPF of the Non-neoplastic Diploid Cases

		S phase Fraction (SPF)			
		Diploid with mild increase	Diploid with moderate increase	Diploid with marked increase	P value
BRAF percentage	0	5 55.60%	0 0.00%	1 7.10%	0.001
	1-25%	1 11.10%	4 28.60%	1 7.10%	
	25-50	3 33.30%	10 71.40%	8 57.10%	
	>50	0 0.00%	0 0.00%	4 28.60%	
BRAF Intensity	0.00	5 55.60%	0 0.00%	1 7.10%	0.001
	Mild	3 33.30%	6 42.90%	2 14.20%	
	Moderate	1 11.10%	8 57.10%	8 57.10%	
	Marked	0 0.00%	0 0.00%	3 21.40%	
Total		9 100%	14 100%	14 100%	

Table 6. Relation between *BRAF* Immunohistochemical Expression and Duke Staging of Adenocarcinoma

		Duke Staging		
		Duke A	Duke B	Duke C
BRAF percentage	1-25%	0 0.00%	0 0.00%	0 0.00%
	25-50%	0 0.00%	0 0.00%	0 0.00%
	>50%	6 100%	20 100%	18 100.00%
BRAF intensity	Mild	2 33.30%	0 0.00%	0 0.00%
	Moderate	4 66.70%	6 30%	0 0.00%
	Marked	0 0.00%	14 70%	18 100.00%
	Total	6 100%	20 100%	18 100%

Table 7. Relation between *BRAF* Immunohistochemical Expression and DNA Ploidy in UC Cases

		UC	
		Diploid	Aneuploid
BRAF %	0	0 0%	0 0.00%
	1-25%	0 0%	0 %
	25-50%	1 25%	2 25%
	>50%	3 75%	6 75%
BRAF intensity	Mild	0 0%	0 0%
	Moderate	2 50%	5 62.50%
	Marked	2 50%	3 37.50%
Total		4 100%	8 100%

SPF are significant independent factors for prognosis of colorectal carcinoma (Zhao et al., 2021). Aneuploidy is an early genetic alteration preceding morphologic changes of dysplasia. Thus, measuring DNA ploidy in UC

Table 8. Relation between Immunohistochemical *BRAF* Expression and DNA Ploidy in IBD Cases

		IBD	
		Diploid	Aneuploid
BRAF %	0	0 0%	0 0%
	1-25%	1 8.30%	0 0%
	25-50%	8 66.70%	4 40%
	>50%	3 25%	6 60%
BRAF intensity	Mild	4 33.30%	0 0.00%
	Moderate	6 50%	6 60%
	Marked	2 16.70%	4 40%
Total		12 100.00%	10 100.00%

Table 9. Cases in which DL Changed the Staging of the Disease

Diagnosis	Preoperative CT staging	DL findings	Post DL staging
2 cases Sigmoid cancer	II	Liver metastasis	IV
1 case Rectal cancer	II	Liver metastasis and mimimal ascites	IV
2 cases Colonic cancer	II	Liver metastasis	IV

could be used to screen early malignant transformation. (Schimmelpenning et al., 2000).

In our study, there was significant difference in the DNA content between the studied groups. 70% of the adenocarcinoma cases (31/44) are aneuploid and 18% (8/44) are diploid. Also, Chapman et al., (1995); Jotti et al., (1995); Kouri, 1993; Flyger et al., (1999) and Hong-yi et al., (2001) found aneuploidy in 70%, 70%, 62%, 89%, 62% and 58% of colon adenocarcinoma, respectively. According to Salud et al., (1999); Karelia et al., (2001) and Çobanoğlu et al., (2009), 58.9% (44/107), 41% (32/79) and 58% of colorectal adenocarcinoma are aneuploid.

According to our results, all cases of non-bilharzial colitis (8/8), bilharzial colitis (10/10) are diploid. No other studies were found testing the DNA ploidy in these two groups. We didn't detect abnormal DNA content in any of the normal appearing colonic mucosa (control, 0/6). Also, Miyazaki et al., 1999, found no aneuploid peaks in normal appearing colonic mucosa.

In our study, 58% (7/12) of UC show aneuploidy and 42% are diploid and there was significant difference in DNA content (2c) between non-dysplastic UC (mean =50.79) and adenocarcinoma (mean=28.41). According to Meling et al., (1991), DNA aneuploidy was found in 60% of the mucosa samples of UC (dysplastic and non- dysplastic). According to Fozard et al., 1986, 42% of UC are aneuploid with higher rate of DNA aneuploidy in dysplastic tissues (21%) compared with non-dysplastic tissues (15%). Schimmelpenning et al., (2000) detected aneuploidy in 25% of UC cases and all developed adenocarcinoma within seven years follow up.

In our work, 80% (8/10) of CD are diploid and CD show less SPF (35±19)% than dysplastic UC (49.80±14.70) and non-dysplastic UC (39.37±10.19). Keeping with our study, Porschen et al., (1992), noted diploid histogram in 90% of CD with higher SPF in UC (17.8±7.7) % than in CD (13.1±4.6) %. In our study, the mean SPF of adenocarcinoma cases is 31.91±10.74. According to Karelia et al., (2001), 30 % of colorectal cancers have a high (>10%) S-phase fraction. Bazan et al., (2002) detected high SPF (>18.3%) among colonic adenocarcinomas. In our work, a significant difference was detected between DNA content of adenocarcinoma cases and histological grade and Bazan et al., (2002) documented this significant relation. Opposite to our results, Suzuki, (1988); Chapman et al., (1995); Salud et al., (1999) and Hong-yi et al., (2001) found no significant relation between DNA content of adenocarcinoma cases and histological grade.

As regarding the relationship between DNA ploidy and pathologic staging, there was significant difference between DNA content of adenocarcinoma cases and Duke stage (P= 0.01). Aneuploid tumors were identified among those with a more advanced pathologic stage in studies done by Tribukait et al., (1983); Jones et al., (1988); Halvorsen and Johannesen, (1990); Kouri et al., (1990); Salud et al., (1999); Bazan et al., (2002) and Çobanoğlu et al., (2009). In contrast to us, Armitage et al., (1985); Suzuki et al., (1988); Armitage et al., (1991); Chapman et al., (1995) found no significant relation between DNA ploidy and tumor stage.

In our work, Duke B is significantly lower from Duke C adenocarcinoma in SPF. In agreement to our finding, Pinto et al., (1997); Bazan et al., (2002) also found this significant positive relation. Chen et al., (2002) found no significant relation between SPF of adenocarcinoma and tumor staging. In our work, all adenocarcinoma cases show >50% *BRAF* expression and 72.7% show marked intensity. Similarly, González-Colunga et al., (2020) found intense and diffuse reaction against *BRAF* V600E in all cases of CRC with known *BRAF* mutation indicating that *BRAF* V600E IHC is sensitive, making it feasible as an alternative method for molecular evaluation.

According to our study, *BRAF* positivity was found in 83% (10/12) of UC cases. Yang et al., (2018) detected positivity in 51% of UC cases. In our study, *BRAF* was negative in all cases of non-bilharzial and bilharzial colitis. No corresponding studies were found. In our work, all cases of CD are negative for *BRAF*. Marcuzzi et al., (2013) studied genetic changes in CD and detected no *BRAF* expression. It is not from the susceptibility mutated genes.

We found a significant increase relation between Duke stage of adenocarcinoma and intensity of *BRAF* expression. Similarly, Luu and Timothy (2018); Ardekani et al., (2012); Barras (2015), Clarke and Kopetz, (2015) documented this significant relation. In the current study, DL altered the staging of 5 of the cancer patients (12.5%) by detecting liver deposits or minimal malignant ascites not identified by radiological investigations. This is consistent with a prospective study done on 40 patients by El Zanati et al., (2020), proved the efficacy of DL that also altered the staging in 24.3% of the cases in their study.

Author Contribution Statement

They share in putting the idea of the research, collecting the studied cases after revising their data and examining the slides. They supervised the results and their statistical analysis. The corresponding author wrote the paper and other authors read it and discussed all points.

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Recommendation of The Study

Further extended studies are recommended to prove the efficacy of DNA ploidy assessment to detect early malignant transformation in non-neoplastic colitis even development of any dysplastic morphologic change. Also, correlation between measuring SPF and chemotherapeutic response of the tumor needs more research.

Ethics approval

The research was approved by the Ethics committee of Theodor Bilharz Research Institute (TBRI) according to the institutional committee for the protection of human subjects and adopted by the 18th world medical assembly, Helsinki, Finland. The study haven't been submitted to any scientific body.

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