ejpmr, 2017,4(11), 159-171

SJIF Impact Factor 4.161

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Research Article

ISSN 2394-3211

EJPMR

USING FLOW CYTOMETRY MARKERS TO DETECT COLORECTAL CANCER AMONG EGYPTIAN PATIENTS

Mona A. Sadek[1], Lamiaa A. A. Barakat[2], Aly Hassan Maabed[3] and Asmaa Mohamed Ali Abd Elhak*[1]

[1]Department of Biochemistry and Nutrition ,Faculty of women, Ain Shams University.
[2]Department of Biochemistry, Faculty of Science, Port Said University.
[3]Medical Oncology Department, National Center Institute, Cairo University, Cairo, Egypt.

***Corresponding Author: Dr. Asmaa Mohamed Ali Abd Elhak**

Department of Chemistry, Girls College, Ain Shams University, Cairo, Egypt.

Article Received on 15/09/2017 **Article Revised on 06/10/2017** **Article Accepted on 27/10/2017**

ABSTRACT

Colorectal cancer (CRC) is the third most frequent cancer and fourth cause of cancer related mortality around the world. Early detection and adenoma eradication contribute to decrease CRC mortality and incidence. There are a lot of extraordinary screening techniques which range from invasive and costly strategies such as flexible sigmoidoscopy, double contrast barium enema, and colonoscopy to more low-cost and non-invasive tests such as fecal occult blood test (FOBT). All these procedures have advantages and disadvantages in regards to their sensitivity, specificity, hazard, availability and cost yet they have been appeared to diminish CRC rate and mortality. There is a requirement for timely cheap and non invasive diagnosis method. Our aim in the present study was to evaluate the expression of CD133, CD44 and CD26 in Egyptian colorectal cancer patients by flow cytometry. These cell surface markers seemed to be useful in detection and distinguishing of tumor cells, in colon, rectum, and other tumors. This study included 100 CRC patient and 50 healthy individuals as control. The studied population consisted of 46 males (46%) and 54 females (54%) of patients group. The mean patients' age was 52.33 ±14.22 years. After Lymphocyte isolation and Preparation of tissue suspension, surface markers were labeled with PE-conjugated anti-CD133 and FITC-conjugated anti-CD44 antibodies, PE-conjugated Anti-CD26, and analyzed by a flow cytometer. The results of flow cytometric analysis for surface markers CD133, CD44 and CD26 obtained from patients group was highly significant increased as compared to that of healthy control group ($P \leq 0.001$). The results of the present study suggest that increased CD133, CD44 and CD26 expressions are a useful marker for determination of CRC.

KEYWORDS: Colorectal Cancer, Flow Cytometry, CD133, CD44, CD26.

INTRODUCTION

Colorectal cancer is clinical harmful tumor. More than 1.2 million new patients endure colorectal cancer and more than 0.6 million individuals passed on of the disease consistently everywhere throughout the world. Information from America national cancer institute has demonstrated that there were 143460 new colorectal cancer patients, 51690 individuals were passed away because of this illness and the morbidity and death rates are in the third place between different tumors in 2012 in America. Lately, the morbidity of colorectal cancer elevated speedily and became one of the 5 most frequent malignant tumors. The 5-year survival rate of this illness is about 60% and no considerable change in the treatment viability is obtained during last 30 years.[1] Because of the late beginning of symptoms, colorectal cancer patients are frequently suffer delayed diagnosis.[2] The identification of a non-invasive-screening test, with a remarkable analytic performance, that can achieve high patient satisfaction and that is cost-effective is an exceptional test.

Flow cytometry is an exceptionally adaptable technique with an extensive variety of assays. Many studies use flow cytometry to detect and predict for disease-free probability in patients with CRC.[3] Three of the proposed markers of flow cytometry in CRC are CD133[4], CD44[5] and CD26.[6] These markers have been reported to be helpful in detection and recognition of tumor cells, in colon, rectum, and other tumors.

CD133, a five-transmembrane glycoprotein, was found to be expressed in hematopoietic stem and progenitor cells that has a molecular weight of hundred and twenty kDa and to membrane protrusion.[7] The glycoprotein CD133 is additionally known as “Prominin-1” and is thought to be related to tumorigenicity and progression of the cancer. The CD133 has been evaluated to know the characteristics and functions of cancer cells. Various studies have explained that surface expression of CD133 having the characteristics of self renewal and proliferation in many kinds of human cancer tissues,

including colon Cancer.[8] CD133 can even be applied to predict neoplasm progression, chemoradiotherapy resistance, patient survival and another clinical parameters.[9] CD133 has been notified as examination marker for colorectal cancer[10] and it`s expression in the CRC primary tissue or liver metastases has been informed to be a considerable prediction factor.[11]

CD44 includes a group of cell adhesion and signaling molecules which exert pleiotropic effects on leading biological processes including proliferation, survival, migration, epithelial as well as mesenchymal transition (EMT) and CD44 play a role in facilitating cell–cell and cell–matrix interaction via its affinity for hyaluronic acid. It is identified in impart adhesion, and is additionally concerned with gathering of growth factors on the cell surface.[12] There is accumulating confirmations that CD44 is implicated in the initiation and progression of intestinal neoplasms and the occurrence of metastasis.[13]

CD26– also called dipeptidyl peptidase IV is a 110-kDa, cell-surface, kind II membrane glycoprotein which has an important function in neoplasm progression.[14] It is excessively expressed in almost all cell kinds asT lymphocytes, endothelial as well as epithelial cells and is found in plasma too, serum and other biological fluids in a form called soluble CD26 (sCD26).[15]

In addition to its expression on tumor cell surface, serum CD26 correlates with tumor status and behavior for many cancers. It`s levels were proposed to be used for early detection and prediction of colorectal cancer.[16] Elevated levels of circulating CD26 have been reported in CRC patients with metastatic disease.[17]

All of these studies suggested that CD133, CD44 and CD26 are potential biomarkers for CRC diagnosis.

SUBJECTS AND METHODS

A total of 100 Egyptian CRC patients (52 females and 48 males) and 50 control cases (30 females and 20 males) were included in this study. The patients ages ranged from 24 to 81 years with mean age of (52.33 ±14.22) years. The control cases are in age ranged from 24 to 83 and were free from any diseases and not taking any treatment that may affect on the result of this research. They were routinely investigated and selected after hospitalization in the Cancer Institute, Cairo University, Egypt to assess the extent of the diseases associated with colorectal cancer.

All of the patients received an examination to determine the stage of cancer, including physical examination, colonoscopy, specimens histology, complete blood count, liver function, serum carcinoembryonic antigen (CEA), serum carbohydrate antigen 19-9 (CA19-9), thorax contrast-enhanced computed tomography (CT), abdomen contrast-enhanced CT or contrast-enhanced magnetic resonance imaging (MRI). The clinical T stage, lymph node metastasis and liver or lung metastases were made by the multidisciplinary teams though the iconographic examinations. In addition, an informed consent was obtained from all participants before their enrollment into the study. Clinicopathological information was derived from hospital records, and any family history of cancer or polyps was extracted from hospital records or retrospective questionnaires.

The data collected include demographic information (age and gender), prior personal and family history of cancer, smoking, stage, tumor location. The treatment decision for the patients, including surgical resection, preoperative chemotherapy, radiochemotherapy, palliation chemotherapy, or palliation surgery was also made by the multidisciplinary teams.

The Fresh peripheral blood samples were taken from patients and control cases by clean venipuncture using plastic disposable syringes (about 6 ml of whole blood were withdrawn from each individual; patient and controls) and divided into 2 parts:
2 ml blood was collected without anticoagulant and centrifuged at 500 r.p.m for 15 min to obtain serum, which used for biochemical measurements.

2 ml on EDTA-containing tube, then used for isolation of lymphocyte by Lymphoflot for flow cytometric analysis.

Tissues samples were taken from patient during surgery and divided into fresh tumor tissue 2x2 cm from the malignant tumor and fresh normal tissue, normal tissues taken as colonoscopic biopsy specimens.

These tissue samples were washed with normal saline solution three time, surrounded fats were trimmed carefully, then were used for preparing cell suspension ready for flow cytometric analysis for this study.

Lymphocyte isolation

Lymphoflot has a higher density than that of platelets, lymphocytes or monocytes, but a lower density than that of erythrocytes and granulocytes.

During the centrifugation process which follows, erythrocytes and granulocytes pass through the density gradient medium because of their higher density, whereas lymphocytes, platelets and monocytes settle above the density gradient on account of their lower. The platelets are removed by means of two successive washing procedures.

1. Mix the anticoagulant- treated (EDTA) blood sample with an equal volume of PBS (phosphate buffered saline PH 7.4).
2. Place lymphoflot (at 18-22cc) in a centrifuge tube and layer an equal volume of the diluted peripheral blood sample on top, ensuring that the blood and lymphoflot do not mix.
3. Centrifuge for 20 min. at 1500 rpm without braking.

The lymphocytes are deposited in a white band at the interface between plasma and lymphoflot. Pipette the band of cells carefully into another centrifuge table; fill up with PBS solution and mix.
4. Centrifuge for 10 mines at 1800 rpm. Decant the supernatant, resuspend the lymphocyte sediment, and fill up with PBS solution and mix.
5. Centrifuge again for 10 min. at 1200 rpm, decant the supernatant.
6. Resuspend the lymphocyte sediment, and then fixed with ice cold absolute alcohol 1 ml for each tube and preserved in +4ºc forever until analysis.

Preparation of tissue suspension
Fresh tissue specimens were transported to laboratory in isotonic saline and prepared as follow:
The material was washed with isotone tris EDTA buffer, 3.029 gm of 0.1 M tris (hydroxymethyl aminomethane (cat. No. T-1378, sigma chemical company), 1.022 gm of 0.07 M sodium chloride (ADWIC) and 0.47 gm of 0.005 M EDTA (cat. No. E-6758, sigma). They were dissolved in 250 ml of distilled water and then adjust the PH at 7.5 by using 1N HCl.

Then, the cell suspension was centrifuged at 1800 rpm for 10 min., where upon the supernatant was aspirated. If they were macroscopically contaminated with blood, it was then subjected to haemolysis with filtered tap water for 10 min.

After centrifugation and aspiration of the supernatant the cell is fixed in ice-cold 96-100% ethanol (BDH) in approximately 1 ml for each sample. These fixed cells can be stored indefinitely in a refrigerator (+4ºc) and can also be mailed without running the sample.

Quantitative determination of CD133, CD44 and CD26
Flow cytometry technique has broadly contributed to improve knowledge on the cell cycle, which can rapidly and quantitatively measure a wide variety of cellular constituents usually only by taking DNA content or surface antigens into account and was adapted for analysis of various cellular components (nucleic acids, lipids, proteins), organelles (lysosomes, mitochondria) or functions (viability, enzymatic activities) (Chantal Jayat et al.1993).

Staining procedure (Direct Staining method)
1. Prepare cells appropriately. Adjust the cell suspension to a concentration of 1x10ˆ6 cells/ml was prepared with PBS/BSA buffer (phosphate buffered saline and 1% bovine serum albumin).
2. Aliquot 100 µl of cell suspension into as many test tubes as required then add antibody at the recommended dilution (10µl for each sample) mix well and incubate at room for 30 minutes.
3. Cells were washed with 2ml PBA/BSA, centrifuged at 1500 rpm for 5 min and discarded resulting supernatant.
4. Finally resuspend cells in 0.2ml of PBS/BSA or with 0.2ml of 0.5% Para formaldehyde in PBS/BSA if required and Acquire data from analyzed by flow cytometer Acquire data by flow cytometry (CifoneMG, et al., 1994;Yoshino N. et al.,2000)

Reagent provided
Anti-human/Mouse (CD44 FITC), Clone IM7, Cat. No (11-0441).
Anti-human (CD26 PE), Clone 2A6, Cat. No (12-0269).
Anti-human (CD133 PE), Clone AC133, Cat. No (130-080-801).
Phosphate buffered solution, pH 7.2, containing 0.09% sodium azide and 0.2 % (w/v) BSA (origin USA).
Isotype: Mouse IgG1, kappa.
DAPI was used to identify the dead cells.

The collected data were statistically processed and analyzed using the Statistical Package of Social Science (SPSS, version 17.0). Power calculations were performed to give the probability of finding the differences between the gene frequencies as statistically significant, $P \leq 0.05$ was considered as significant, $P \leq 0.01$ was highly significant and $P \leq 0.001$was extremely significant. A minimum level of statistical significance was considered at a P level of ≤ 0.05.

RESULTS

Demographic parameters for studied group: As found in table (1): according to pathology, there were 4 (6.9%) patients had colon adenocarcinoma grade 1; 42 (72.4%) patients had colon adenocarcinoma grade 2;10 (17.2%) patients had colon adenocarcinoma grade3;2(3.2%) patients had colon adenocarcinoma metastasis;4(9.5%) patients had rectal adenocarcinoma grade 1; 22(52.4%) patients had rectal adenocarcinoma grade 2and 16(38.1%) patients had rectal adenocarcinoma grade 3.

According to lymph node, there were 34 patients with positive lymph node, 18(31%) colon adenocarcinoma and 16 (38%) rectal adenocarcinoma, and 66 patients with negative lymph node, 40 (69%) colon adenocarcinoma and 26 (62%) rectal adenocarcinoma.

Table (1): Demographic data of studied groups Comparison between patients and control according to tumor markers.

Age	Control 50	Patients colon 58	Patients recta l 42
Male	44.0+17.15 (N 20)	52.18+14.46 (N 28)	49.29+9.07(N 18)
Range	29-72	24-81	34-63
Female	57.66+18.9 (N 30)	53.02+12.99 (N 30)	52.86+14.24(N 24)
Range	24-83	25-73	28-79
Smoking			
Yes	0 (0%)	10 (20%)	6 (10%)
No	50 (100%)	48(80%)	36(90%)
History			
No history	0 (100%)	48 (73.3%)	34 (85%)
present	50 (0%)	10 (26.7%)	8 (15%)
Pathology			
report	0 (0%)	4(6.9%)	4(9.5%)
grade 1	0 (0%)	42 (72.4%)	22 (52.4%)
grade 2	0 (0%)	10(17.2%)	16(38.1%)
grade 3	0 (0%)	2 (3.4%)	
metastasis			
Lymph node			
positive	0 (0%)	18(31%)	16(38%)
negative	0 (0%)	40(69%)	26(62%)

As showed in table (2) Fig (1); the CEA in patients group was highly significant increased as compared to that of healthy control group ($P \leq 0.001$). In addition, the CA 19.9 in patients group was highly significant increased as compared to that of healthy control group ($P \leq 0.001$).

Table. (2): Comparison between patients and controls according to tumor markers.

Parameter	Control group	Patiens group	P value
CEA(median±S.E)	1.40 ±0.19	6.20 ±3.16	≤ 0.001
CA19.9(median±S.E)	6.55 ±0.86	43.69±11.48	≤ 0.001

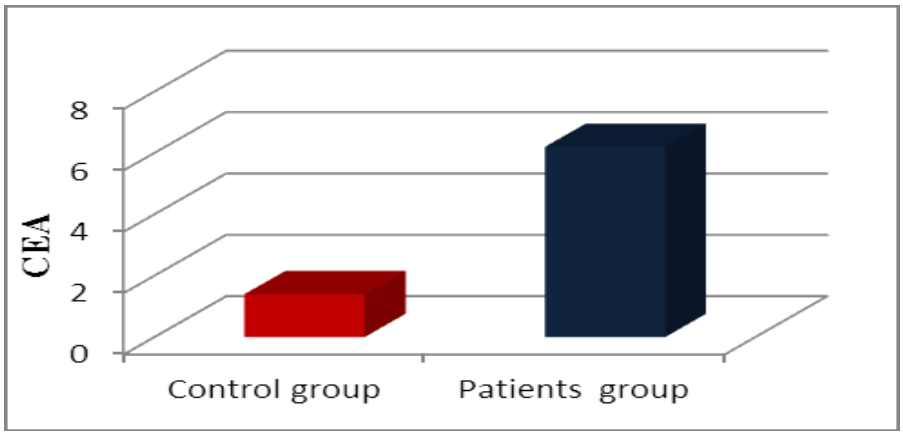

Fig. (1): CEA in patients and control groups.

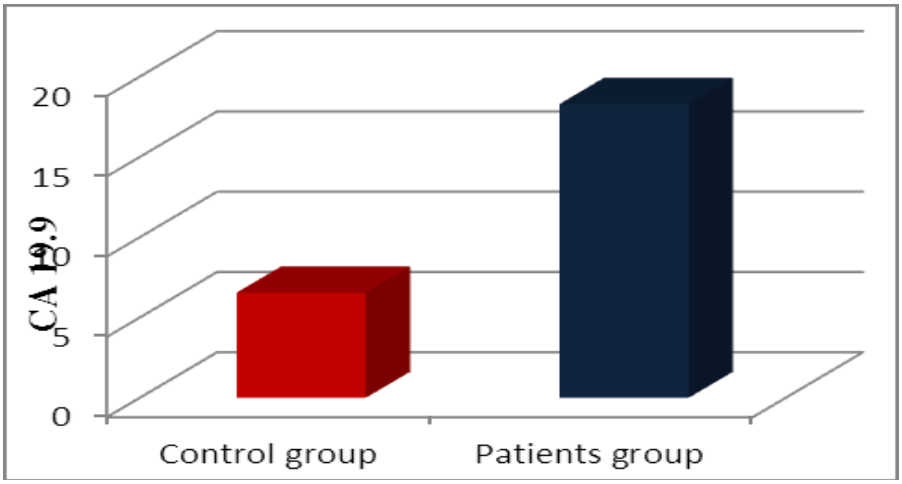

Fig. (2): CA19.9 in patients and control groups.

Table. (3): Comparison between patients and control according to flow cytometer markers in blood samples.

Parameter (mean± S.D)	Control group	Patient group	P value
CD44+	**4.17±3.42**	**21.72±5.51**	≤0.001
CD133+	6.84±2.64	**21.5**±4.95	P;≤0.001
CD26+	19.40±2.58	**73.48±11.54**	≤0.001
CD44+CD133+	3.64±3.65	**19.49±5.67**	≤0.001

Table. (4): Comparison between different groups of patients according to flow cytometer markers.

Parameter (mean± S.D)	Control group	Colon adenocarcinoma	Rectal adenocarcinoma
CD44+	4.17±3.42	21.85 ±4.4***	21.78±7.07***
CD133+	6.84±2.641	22.22±4.86***	20.63±5.15***
CD26+	19.40±2.58	72.42±11.90***	74.94±11.36***
CD44+CD133+	3.64±3.65	19.53±5.4***	19.57±6.3***

CD44+

As found in table (3&4) Fig (3&4); the results of flow cytometric analysis for surface markerCD44+ obtained from patients group was highly significant increased as compared to that of healthy control group (P ≤ 0.001). Also, the results of flow cytometric analysis for surface marker CD44+ obtained from colon adenocarcinoma patients group was highly significant increased as compared to that of healthy control group (P ≤ 0.001). Moreover, the results of flow cytometric analysis for surface marker CD44+ obtained from rectal adenocarcinoma patients group was highly significant increased as compared to that of healthy control group (P ≤ 0.001). On the other hand, there was insignificant difference in the results of flow cytometric analysis for surface marker CD44+ obtained from rectal adenocarcinoma patients group as compared to that colon adenocarcinoma patients group (P > 0.05).

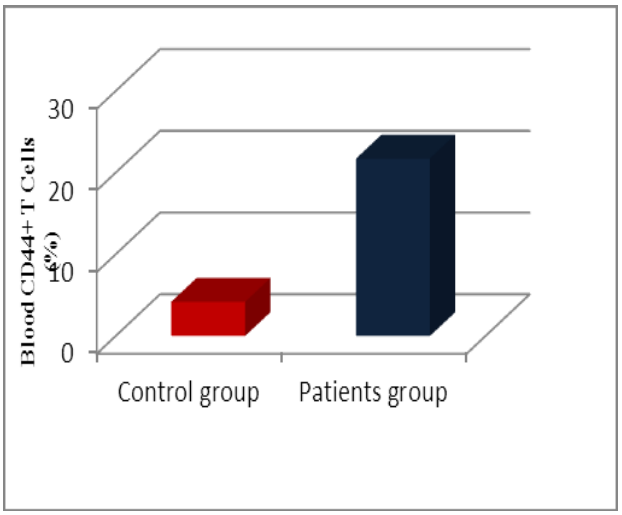

Fig. (3): The mean of Blood CD44+ T Cells (%) in patients group compared to healthy control group.

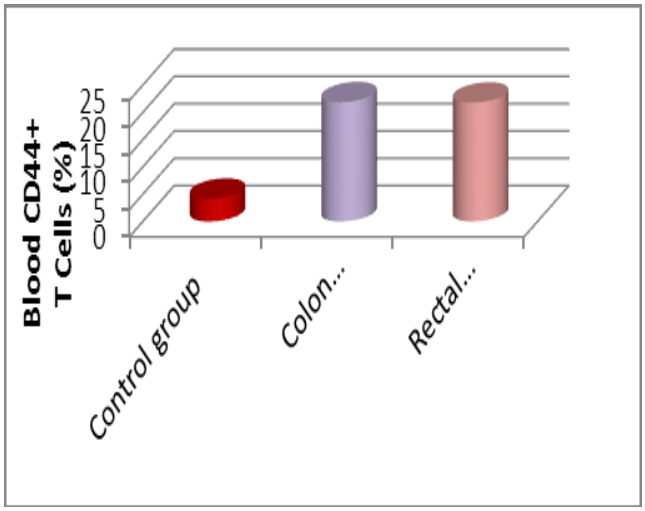

Fig. (4): The mean of Blood CD44+ T Cells (%) in different studied groups.

CD133+Tcells

As shown in tables (3&4) Fig (5&6), the results of flow cytometric analysis for surface markers CD133+Tcells in blood samples obtained from patients group was significantly increased as compared to healthy control group ($p<0.001$).In addition, the results of flow cytometric analysis for surface markerCD133+Tcells in blood samples obtained from colon adenocarcinoma patients group was highly significant increased as compared to that of healthy control group ($P \leq 0.001$). Also, the results of flow cytometric analysis for surface markerCD133+Tcells in blood samples obtained from rectal adenocarcinoma patients group was highly significant increased as compared to that of healthy control group ($P \leq 0.001$). On the other hand, there was insignificant difference in the results of flow kilometric analysis for surface markerCD133+Tcells in blood samples obtained from rectal adenocarcinoma patients group as compared to that colon adenocarcinoma patients group ($P > 0.05$).

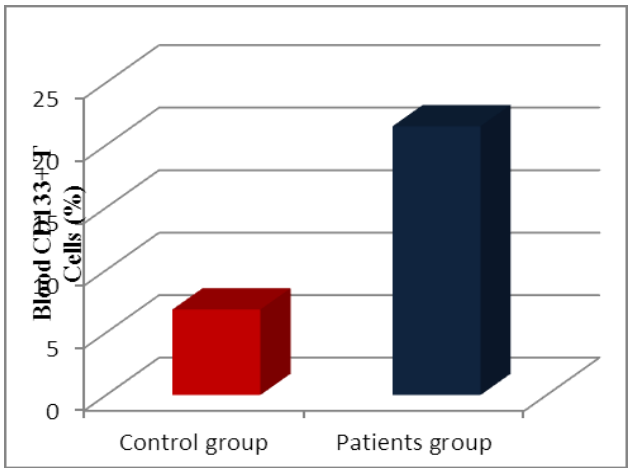

Fig. (5): The mean of Blood CD133+ T Cells (%) in patients group compared to healthy control group.

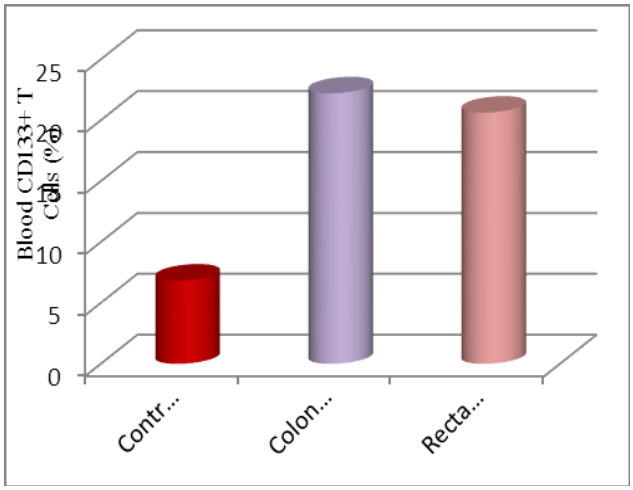

Fig. (6): The mean of Blood CD133+ T Cells (%) in different studied groups.

CD26+Tcells

As shown in tables (4&5) Fig (7&8), the results of flow cytometric analysis for surface markers CD26+Tcells in blood samples obtained from patients group was significantly increased as compared to healthy control group ($p<0.001$). Also, the results of flow cytometric analysis for surface markerCD26+Tcells in blood samples obtained from colon adenocarcinoma patients group was highly significant increased as compared to that of healthy control group ($P \leq 0.001$). Also, the results of flow cytometric analysis for surface markerCD26+Tcells in blood samples obtained from rectal adenocarcinoma patients group was highly significant increased as compared to that of healthy control group ($P \leq 0.001$). However, there was insignificant difference in the results of flow cytometric analysis for surface markerCD26+Tcells in blood samples obtained from rectal adenocarcinoma patients group as compared to that colon adenocarcinoma patients group ($P > 0.05$).

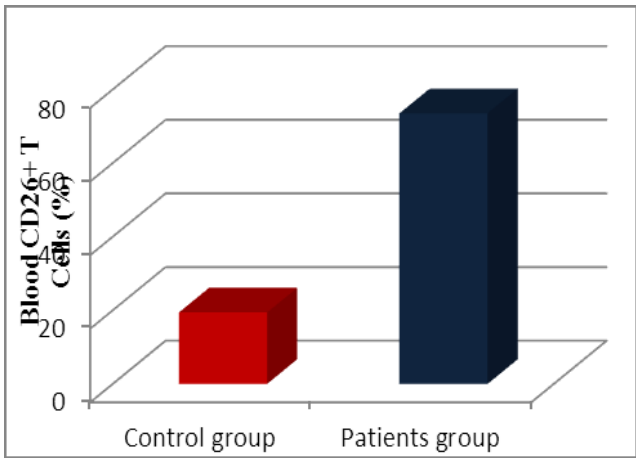

Fig. (7): The mean + S.D of Blood CD26+ T Cells (%) in patients group compared to healthy control group.

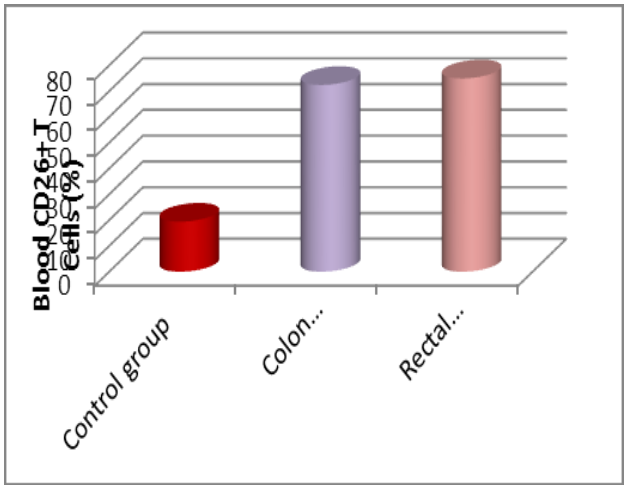

Fig. (8): The mean of Blood CD26+ T Cells (%) in different studied groups.

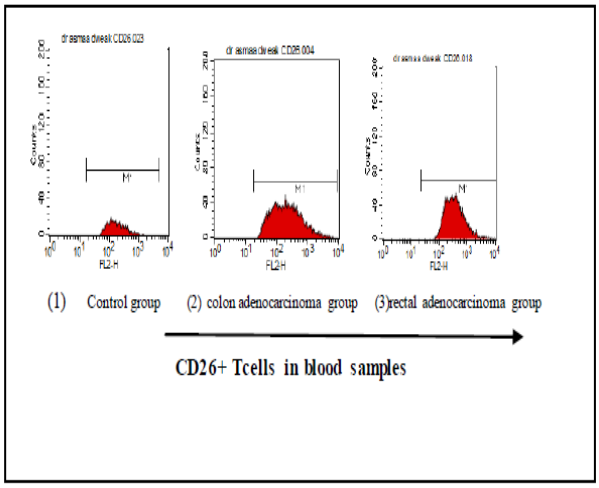

Fig. (9): The % of +ve population from total mononuclear cells in patients (colon adenocarcinoma and rectal adenocarcinoma) and control stained with CD26 in blood samples as a monoclonal antibody.

CD44+ CD133+T cells(%)in blood samples

As displayed in table (4&5) and Fig.(10&11&12).The results of flow cytometric analysis for surface markers CD44+ CD133+T cells(%)in blood samples obtained from patients was significantly increased as compared to healthy control group ($p \leq 0.001$). Also, a significant increase was obtained in CD44+ CD133+T cells (%) in blood samples of colon adenocarcinoma group as compared to healthy control group ($p \leq 0.001$). In addition, a significant increase was found in CD44+ CD133+T cells (%) in blood samples of rectal adenocarcinoma group as compared to healthy control group ($p \leq 0.001$). On the other hand, The results of flow cytometric analysis for surface markers CD44+ CD133+T cells(%)in blood samples obtained from rectal adenocarcinoma patients group as compared to that colon adenocarcinoma patients group ($P > 0.05$).

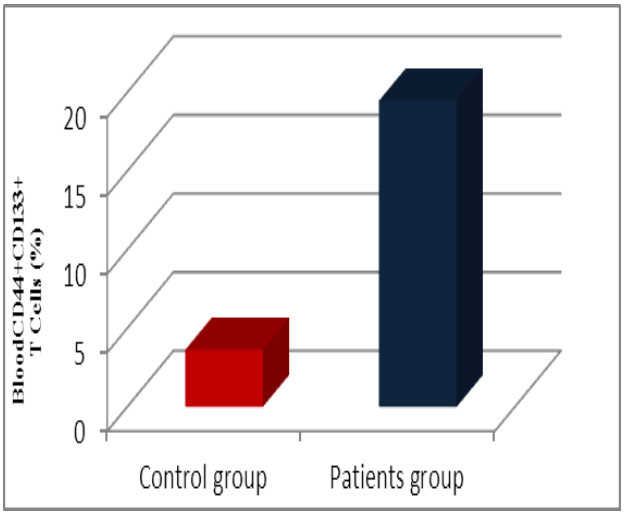

Fig. (10): The mean of Blood CD44+CD133+ T Cells (%) in patients group compared to healthy control group.

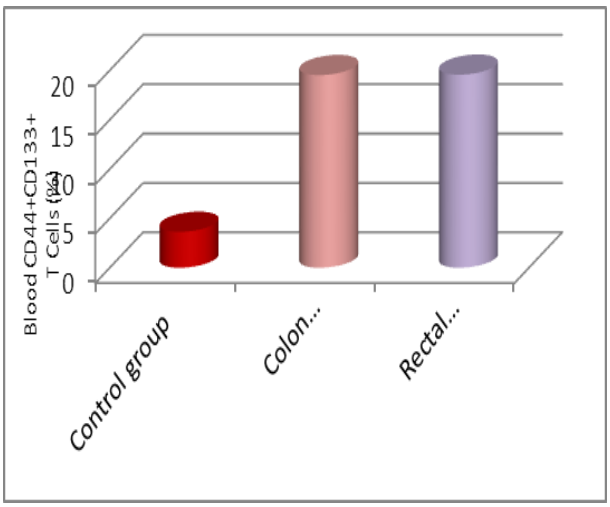

Fig. (11): The mean of Blood CD44+CD133+ T Cells (%) in different studied groups.

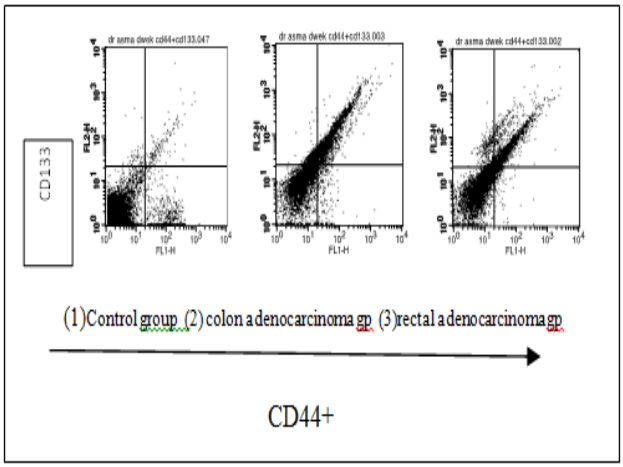

Fig. (12): Comparison of flow cytometric analysis of double stain. CD44 CD133+T-cell populations between control and patient.The peripheral blood was stained with FITC-conjugated anti-CD44, PE-

conjugated anti-CD133. Cells were gated on lymphocytes via their forward- and side-scatter properties.

Table. (5): Comparison between patients and control regarding to flow cytometer markers in tissues.

Parameter+ (mean± S.E)	Control Group (8)	Patients group (25)	P value
$CD44^+$	42.54±0.17	32.34±3.77	>0.05
$CD133^+$	23.73±0.77	13.64±1.05	≤0.001
$CD26^+$	69.61±0.95	45.00±3.17	≤0.001
$CD44^+CD133^+$	30.96±0.10	14.62±1.67	≤0.001

***highly significant compared to control group.

Table. (6): Comparison between different groups according to flow cytometer markers in tissue.

Parameter (mean± S.E)	Control group (8)	Colon adenocarcinoma (25)	Rectal adenocarcinoma (25)
CD44+	42.54±0.17	31.12±4.61	34.17±6.68
CD133+	23.73±0.77	14.26±1.41***	12.72±1.59***
CD26+	69.61±0.95	43.69±4.17***	47.18±5.02***
CD44+CD133+	30.96±0.10	15.53±2.57***	13.25±1.74***

CD44+Tcells in tissues samplesAs shown in tables (5&6) Fig (13&14), the results of flow cytometric analysis for surface markers CD44+Tcells in tissuesamples obtained from patients group was insignificantly decreased as compared to healthy control group (p>0.05).In addition, the results of flow cytometric analysis for surface markerCD44+Tcells in tissuesamples obtained from colon adenocarcinoma patients group was insignificant decreased as compared to that of healthy control group (P > 0.05). Also, the results of flow cytometric analysis for surface markerCD44+Tcells in tissue samples obtained from rectal adenocarcinoma patients group was insignificant decreased as compared to that of healthy control group (P >0.05).Moreover, there was insignificant difference in the results of flow cytometric analysis for surface markerCD44+Tcells in tissuesamples obtained from rectal adenocarcinoma patients group as compared to that colon adenocarcinoma patients group (P > 0.05).

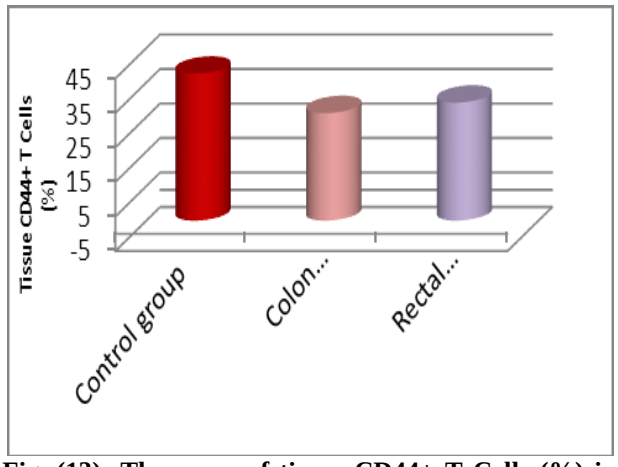

Fig. (13): The mean of tissue CD44+ T Cells (%) in patients group compared to healthy control group.

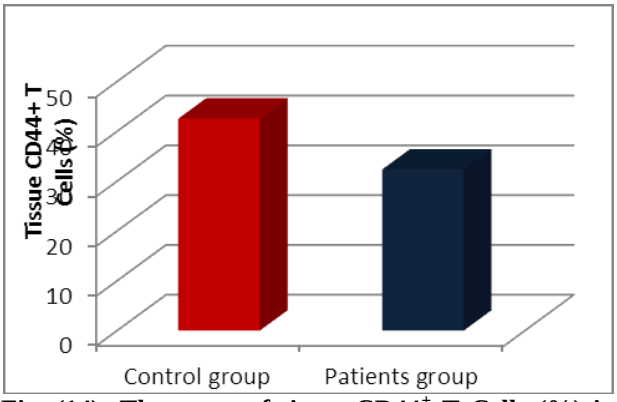

Fig. (14): The mean of tissue $CD44^+$ T Cells (%) in different studied groups.

$CD133^+$Tcells in tissue

As shown in tables (5&6) Fig (15&16), the results of flow cytometric analysis for surface markers CD133+Tcells in tissuesamples obtained from patients group was significantly decreased as compared to healthy control group (p<0.001).In addition, the results of flow cytometric analysis for surface markerCD133+Tcells intissuesamples obtained from colon adenocarcinoma patients group was highly significant decreased as compared to that of healthy control group (P ≤ 0.001). In addition, the results of flow cytometric analysis for surface markerCD133+Tcells in tissue samples obtained from rectal adenocarcinoma patients group was highly significant decreased as compared to that of healthy control group (P ≤ 0.001).On the other hand, there was insignificant difference in the results of flow cytometric analysis for surface markerCD133+Tcells in tissuesamples obtained from rectal adenocarcinoma patients group as compared to that colon adenocarcinoma patients group (P > 0.05).

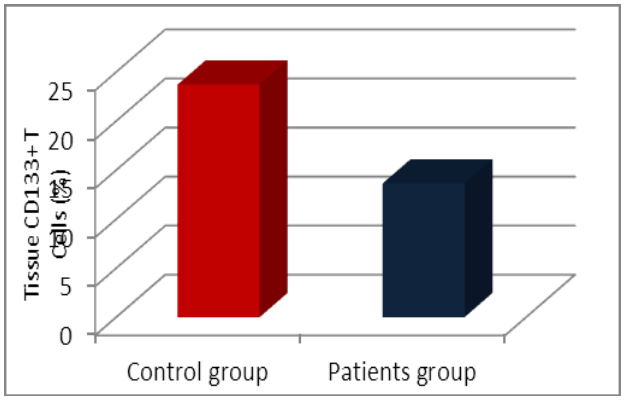

Fig. (15): The mean of tissue CD133+ T Cells (%) in patients group compared to healthy control group.

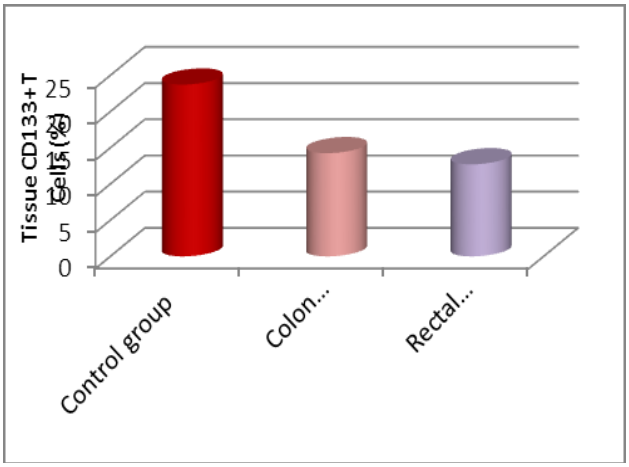

Fig. (16): The mean of tissue CD133+ T Cells (%) in different studied groups.

CD26^{+}Tcells in tissue

As shown in tables (5&6) Fig (17&18), the results of flow cytometric analysis for surface markers CD26+Tcells in tissue samples obtained from patients group was significantly decreased as compared to healthy control group ($p<0.001$).In addition, the results of flow cytometric analysis for surface markerCD26+Tcells in tissue samples obtained from colon adenocarcinoma patients group was highly significant decreased as compared to that of healthy control group ($P \leq 0.001$). In addition, the results of flow cytometric analysis for surface markerCD26+Tcells in tissue samples obtained from rectal adenocarcinoma patients group was highly significant decreased as compared to that of healthy control group ($P \leq 0.001$). On the other hand, there was insignificant difference in the results of flow cytometric analysis for surface markerCD26+Tcells in tissue samples obtained from rectal adenocarcinoma patients group as compared to that colon adenocarcinoma patients group ($P > 0.05$).

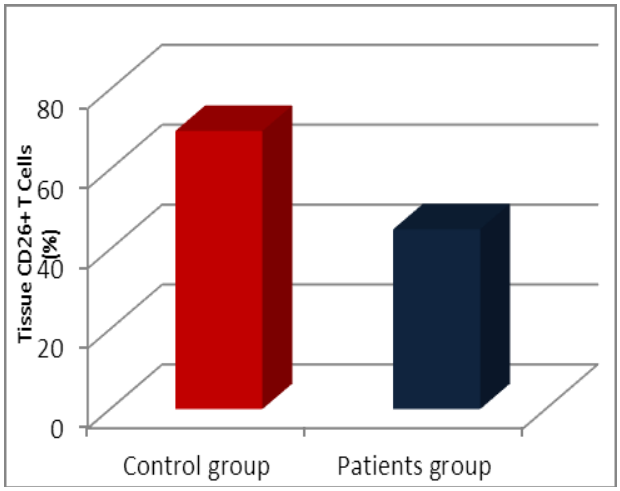

Fig. (17): The mean of tissue CD26+ T Cells (%) in patients group compared to healthy control group.

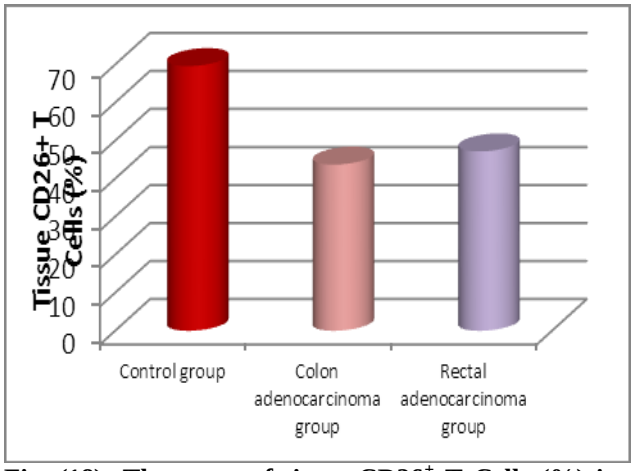

Fig. (18): The mean of tissue CD26^{+} T Cells (%) in different studied groups.

CD44^{+}CD133^{+}Tcells in tissue

As shown in tables (5&6) Fig (19&20), the results of flow cytometric analysis for surface markers CD44+CD133+Tcells in tissue samples obtained from patients group was significantly decreased as compared to healthy control group ($p<0.001$).In addition, the results of flow cytometric analysis for surface markerCD44+CD133+Tcells in tissue samples obtained from colon adenocarcinoma patients group was highly significant decreased as compared to that of healthy control group ($P \leq 0.001$). In addition, the results of flow cytometric analysis for surface markerCD44+CD133+Tcells in tissue samples obtained from rectal adenocarcinoma patients group was highly significant decreased as compared to that of healthy control group ($P \leq 0.001$). On the other hand, there was insignificant difference in the results of flow cytometric analysis for surface markerCD44+CD133+Tcells in tissue samples obtained from rectal adenocarcinoma patients group as compared to that colon adenocarcinoma patients group ($P > 0.05$).

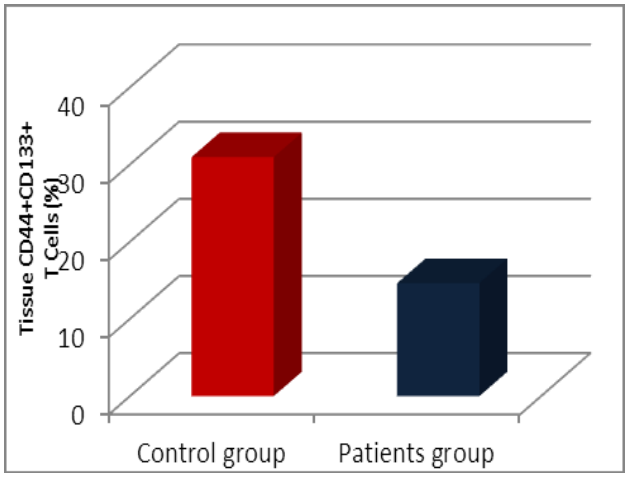

Fig. (19): The mean of tissueCD44+CD133+ T Cells (%) in patients group compared to healthy control group.

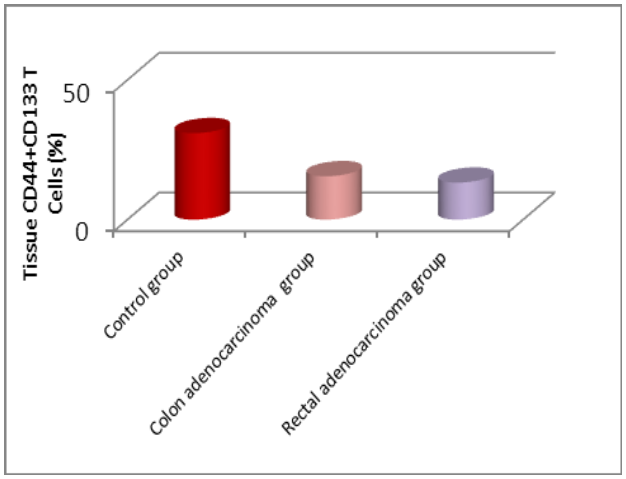

Fig. (20): The mean of tissueCD44+CD133+T Cells (%) in different studied groups.

Renal and Liver function test

Table (7): Comparison between control and patients groups according to liver function test.

(mean± S.E)	Control group (50)	Patients group (100)	P value
creatinine	0.77±0.014	0.87±0.026	0.01
Glucose	100.0±4.04	121±4.8	0.01
Total bilirubin	0.67±0.037	0.62±0.047	0.44
Albumin	4.03±0.34	3.24±0.65	0.001
sGOT	22.22±6.3	19.31±8.5	0.021
sGPT	17.79±5.7	14.77±9.3	0.038
ALP	87.9±18.8	92.6±30.8	0.325

As shown in table (7); there was significant increase in creatinine and glucose from patients group as compared from control group (P 0.01 and 0.01 respectively). Moreover,there was significant decrease in sera albumin, sGOT and sGPT in patients group as compared to control group (P≤ 0.001, 0.021 and 0.038respectively). On the othe hand, there was insignificant difference in sera total bilirubin and ALP obtained from patients group as compared to control group (P>0.05).

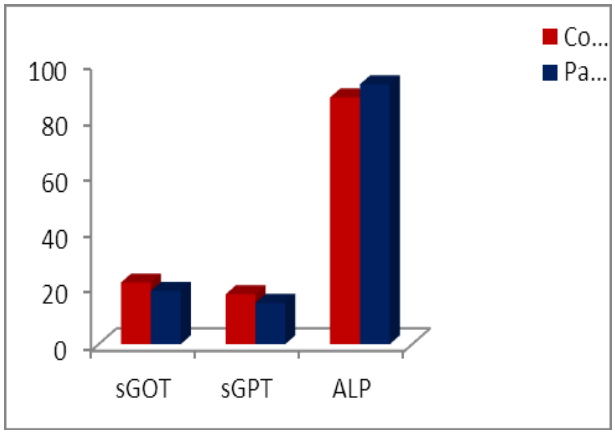

Fig. (21): Comparison between patients and control groups according to liver enzymes.

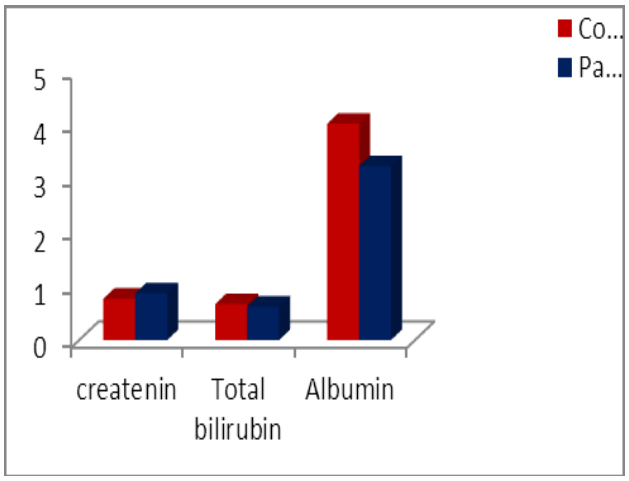

Fig. (22): Comparison between patients and control groups according to biomarkers.

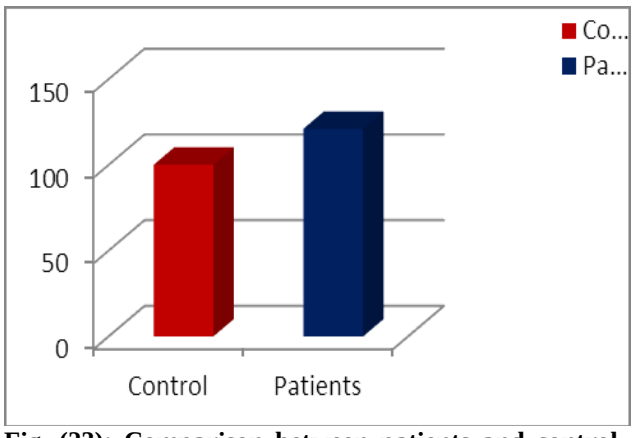

Fig. (23): Comparison between patients and control groups according to glucose.

Hematological parameters

Table (8): Comparison between control and patients groups according to hematological parameters.

Parameter+ (mean± S.E)	Control group (50)	Patients group (100)	P value
WBCs	7.06±0.20	7.81±0.369	0.076
RBCs	4.65±0.049	4.20±0.067	0.001
Hgb	12.11±0.147	10.52±0.157	0.001
Platelets	323.17±11.86	264.7±10.5	0.001

As shown in table (8); there was significant decrease in RBCs, Hgb and plateletsobtained from patients group as compared to control group ($P \leq 0.001$, 0.001 and 0.001respectively). On the other hand, there was insignificant difference in sera WBCs obtained from patients group as compared to control group ($P>0.05$).

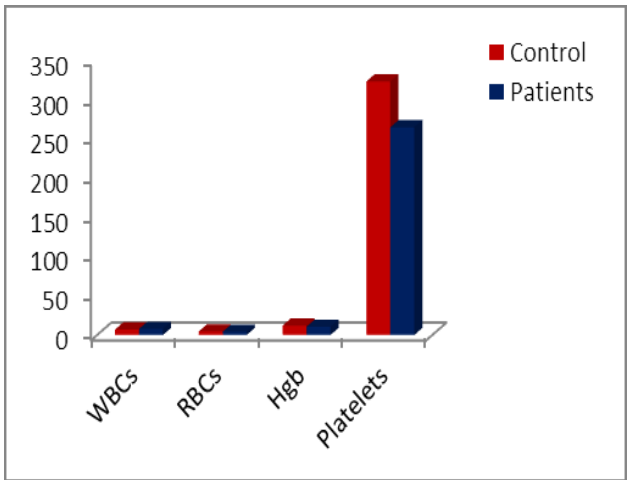

Fig. (24): Comparison between patients and control groups according to hematological parameters.

DISCUSSION

Colorectal cancer (CRC) is a major cause of cancer deaths in the world because of asymptomatic early stage and detection in advanced stages. CRC outcomes rely on the distribution and spread of the disease, and additionally early identification besides intervention.[18] The five year survival rate of CRC patients with metastasis is lower than 10%. Now many screening ways, like colonoscopy, fecal occult blood testing (FOBT), and serum biomarkers, are suggested for the detection of CRC. Restricted by the cost and bad perception, common colonoscopy screening is presently unavailable, and also the sensitivity and specificity are dissatisfying for FOBT and few biomarkers.[19] Recently, carcinoembryonic antigen (CEA) is the most common serum biomarker to be used for detection of CRC in clinical practice; ; but, new studies have proven the sensitivity of CEA to be as low as 30% to 40% for early CRC.[20] Thus, the seek for noninvasive, critical biomarkers has began to intensify. So as to guess the outcome of colorectal cancer properly, there has been major concern to develop factors and novel practical biomarkers that can help for detection or prognosis as a result survival can be dramatically enhanced at early detection and treatment of CRC.[21]

A non-invasive biological serum marker should be of incredible advantage as a result of the performance of the test, numerous additional techniques to identify CRC surface markers have been produced, including flow cytometry.[22] In this investigation, we evaluated the expression of CD133, CD44 and CD26 in 100CRC patients utilizing flow cytometry. To our knowledge, this is the first report analyzing CD133, CD44 and CD26 expressions in a subset of CRC patients in Egyptian population. Unlike the use of immunohistochemistry[23] flow cytometry enabled us to isolate only viable marked colorectal cancer cells.

The utility of surface markers CD133, CD44 and CD26 for the diagnosis of CRC suggested in preceding studies. These Markers expression were significantly higher in tumor than in non tumor colorectal cells, in accordance with the CSC model of colorectal carcinogenesis.[6,7] The results of flow cytometric analysis for surface markers CD133, CD44 and CD26 obtained from patients group was highly significant increased as compared to that of healthy control group ($P \leq 0.001$). The results of this study suggest that increased CD133, CD44 and CD26 expressions are a useful markers for determination of CRC.

CD26 could be used as the baseline in patients with CRC due to its high expression in our results. These findings will surely be of interest, considering the need of useful prognostic indicators that can accurately predict the clinical outcome of CRC patients. No significant difference was observed in our results between the patients with early stage CRC, CRC-LN patients despite CD26 expression was positively related with tumor differentiation, and metastasis. Higher CD26 expression had poorer differentiation and higher potential for developing distant.[24] Fernandez et al. discovered a critical relationship between CD44s expression and a high proliferation rate in CRC.[25]

Next, we quantified the co-expression of CD133 and CD44 to screen the potential biomarkers in CRC patients. A significant increase was obtained in CD44+ CD133+T cells (%) in blood samples of colorectal cancer patients group as compared to healthy control group ($p \leq 0.001$). Also, Gennaro Galizia et al (2012) found that CD133 and CD44 expression was significantly higher in tumor cells than in non tumor cells, and expression of one did not necessarily correlate with expression of the other.[26] Several studies have addressed the co-expression of CD133 and CD44 in CRC patients.[27]

In addition, the expression of CD133, CD44, and CD26 were also examined in the cells suspension

derived from fresh tissue of colorectal cancer. However, the results of flow cytometric analysis for surface markers CD133, CD44 and CD26 in tissue samples obtained from patients group was significantly decreased as compared to healthy control group suggesting that these surface markers may be released in circulation from malignant cells. Malignant transformation from normal to cancerous tissue is associated with cell-surface glycoprotein and glycolipid modifications.[28] These glycoconjugates can be released in the circulation through increased cell turnover, secretion or shedding from the malignant cells and have been considered as potential tumor markers for helping in screening, diagnosis, staging, prognosis and monitoring of cancer therapy.[29]

On the other hand, there was insignificant difference in the results of flow cytometric analysis for surface markers CD133, CD44 and CD26 obtained from rectal adenocarcinoma patients group as compared to that colon adenocarcinoma patients group.

However, it has to be underlined that the population studied was too small to allow a conclusive and definitive evaluation of the prognostic significance of CD133, CD44 and CD26 expression level in these patients. Thus, additional studies on a larger series of cases are warranted to confirm these results and to further elucidate the roles of CD133, CD44and CD26 in the development and progression of CRC. Moreover, CD133, CD44 and CD26 might well be a candidate molecular target for the development of new therapeutic interventions if a direct role of the molecule in the process of tumor development and progression will be demonstrated in colon tumorigenesis.

We found a higher fraction female CRC cases than that of male cases in our study (54% women vs. 46.8% men). This is in contrast to reports from developed countries, including reports on US Hispanics, where men are more often diagnosed with CRC.[30]

Moreover, there was significant decrease in serum albumin, sGOT and sGPT in patients group as compared to control group ($P \leq 0.001$, 0.021 and 0.038 respectively). On the other hand, there was insignificant difference in sera total bilirubin and ALP obtained from patients group as compared to control group ($P>0.05$).

There was significant decrease in RBCs, Hgb and platelets obtained from patients group as compared to control group ($P \leq 0.001$, 0.001 and 0.001respectively). This may because most colorectal neoplasm's bleed more than normal colonic mucosa. Chronic bleeding from colorectal cancers and adenomas leads to decreased Hemoglobin and RBC. On the other hand, there was insignificant difference in WBCs obtained from patients group as compared to control group ($P>0.05$).

To summarize, the identification of a non-invasive-screening test, with an outstanding diagnostic performance, that can achieve high patient consistence and that is cost-effective is an extraordinary challenge. According to our study, The CD133, CD44, CD26 in peripheral blood may play a role in CRC detection or may serve as an auxiliary diagnosis marker. Although several studies demonstrated many diagnostic ways for CRC detection, the marker combination is capable of discriminating metastasis from control samples.

REFRENCES

1. Eli, M., Mollayup, A., Muattar, C. L., Zheng, C. & Bao, Y.-X. K-ras genetic mutation and influencing factor analysis for Han and Uygur nationality colorectal cancer patients. International journal of clinical and experimental medicine, 2015; 8(6): 10168.
2. Weekes, J. Irinotecan therapy and molecular targets in colorectal cancer: A systemic review. World Journal of Gastroenterology, 2009; 15(29): 3597.
3. Karlsson, M., Nilsson, O., Thörn, M. & Winqvist, O. Detection of metastatic colon cancer cells in sentinel nodes by flow cytometry. Journal of Immunological Methods, 2008; 334(1-2): 122-133.
4. Huang, X., Sheng, Y. & Guan, M. Co-expression of stem cell genes CD133 and CD44 in colorectal cancers with early liver metastasis. Surgical Oncology, 2012; 21(2): 103-107.
5. Du, L., Rao, G., Wang, H., Li, B., Tian, W., Cui, J., et al. CD44-Positive Cancer Stem Cells Expressing Cellular Prion Protein Contribute to Metastatic Capacity in Colorectal Cancer. Cancer Research, 2013; 73(8): 2682-2694.
6. De Chiara, L., Rodríguez-Piñeiro, A. M., Rodríguez-Berrocal, F. J., Cordero, O. J., Martínez-Ares, D. & Páez de la Cadena, M. Serum CD26 is related to histopathological polyp traits and behaves as a marker for colorectal cancer and advanced adenomas. BMC Cancer, 2010; 10(1).
7. O'Brien, C. A., Pollett, A., Gallinger, S. & Dick, J. E. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. Nature, 2006; 445(7123): 106-110.
8. Corbeil, D., Röper, K., Hellwig, A., Tavian, M., Miraglia, S., Watt, S. M., et al. The Human AC133 Hematopoietic Stem Cell Antigen Is also Expressed in Epithelial Cells and Targeted to Plasma Membrane Protrusions. Journal of Biological Chemistry, 2000; 275(8): 5512-5520.
9. Shmelkov, S. V., Butler, J. M., Hooper, A. T., Hormigo, A., Kushner, J., Milde, T., et al. 2008. CD133 expression is not restricted to stem cells, and both CD133+ and CD133– metastatic colon cancer cells initiate tumors. Journal of Clinical Investigation.

10. Yang, Z.-L., Zheng, Q., Yan, J., Pan, Y. & Wang, Z. Upregulated CD133 expression in tumorigenesis of colon cancer cells. World Journal of Gastroenterology, 2011; 17(7): 932.
11. Piscuoglio, S., Lehmann, F. S., Zlobec, I., Tornillo, L., Dietmaier, W., Hartmann, A., et al. Effect of EpCAM, CD44, CD133 and CD166 expression on patient survival in tumours of the ampulla of Vater. Journal of Clinical Pathology, 2011; 65(2): 140-145.
12. Marhaba, R. & Zöller, M. CD44 in Cancer Progression: Adhesion, Migration and Growth Regulation. Journal of Molecular Histology, 2003; 35(3): 211-231.
13. Zöller, M. CD44: can a cancer-initiating cell profit from an abundantly expressed molecule? Nature Reviews Cancer, 2011; 11(4): 254-267.
14. Pro, B. & Dang, N. CD26/dipeptidyl peptidase IV and its role in cancer. Histology and histopathology, 2004; 19(4): 1345-1351.
15. Gorrell, M. D., Gysbers, V. & McCaughan, G. W. CD26: A Multifunctional Integral Membrane and Secreted Protein of Activated Lymphocytes. Scandinavian Journal of Immunology, 2001; 54(3): 249-264.
16. Ayude, D., de la Cadena, M. P., Cordero, O. J., Nogueira, M., Ayude, J., Fernández-Briera, A., et al. Clinical Interest of the Combined Use of Serum CD26 and Alpha-L-Fucosidase in the Early Diagnosis of Colorectal Cancer. Disease Markers, 2004; 19(6): 267-272.
17. De La Haba-Rodríguez, J., Macho, A., Calzado, M., Blazquez, M., Gomez, M., Munoz, E., et al. Soluble dipeptidyl peptidase IV (CD-26) in serum of patients with colorectal carcinoma. Neoplasma, 2001; 49(5): 307-311.
18. Manne, U., Shanmugam, C., Katkoori, V. R., Bumpers, H. L. & Grizzle, W. E. Development and progression of colorectal neoplasia. Cancer Biomarkers, 2011; 9(1-6): 235-265.
19. Levin, B., Lieberman, D. A., McFarland, B., Smith, R. A., Brooks, D., Andrews, K. S., et al. Screening and Surveillance for the Early Detection of Colorectal Cancer and Adenomatous Polyps, 2008: A Joint Guideline from the American Cancer Society, the US Multi-Society Task Force on Colorectal Cancer, and the American College of Radiology. CA: A Cancer Journal for Clinicians, 2008; 58(3): 130-160.
20. Tan, E., Gouvas, N., Nicholls, R. J., Ziprin, P., Xynos, E. & Tekkis, P. P. Diagnostic precision of carcinoembryonic antigen in the detection of recurrence of colorectal cancer. Surgical Oncology, 2009; 18(1): 15-24.
21. Winawer, S. J. Screening of Colorectal Cancer. Surgical Oncology Clinics of North America, 2005; 14(4): 699-722.
22. Yu, M., Stott, S., Toner, M., Maheswaran, S. & Haber, D. A. Circulating tumor cells: approaches to isolation and characterization. The Journal of Cell Biology, 2011; 192(3): 373-382.
23. Shmelkov, S. V., Butler, J. M., Hooper, A. T., Hormigo, A., Kushner, J., Milde, T., et al. 2008. CD133 expression is not restricted to stem cells, and both CD133+ and CD133– metastatic colon cancer cells initiate tumors. Journal of Clinical Investigation.
24. Lam, C. S.-C., Cheung, A. H.-K., Wong, S. K.-M., Wan, T. M.-H., Ng, L., Chow, A. K.-M., et al. Prognostic Significance of CD26 in Patients with Colorectal Cancer. PLoS ONE, 2014; 9(5): e98582.
25. Fernández, J. C., Vizoso, F. J., Corte, M. D., Gava, R. R., Corte, M. G., Suárez, J. P., et al. CD44s Expression in Resectable Colorectal Carcinomas and Surrounding Mucosa. Cancer Investigation, 2004; 22(6): 878-885.
26. Galizia, G., Gemei, M., Del Vecchio, L., Zamboli, A., Di Noto, R., Mirabelli, P., et al. Combined CD133/CD44 expression as a prognostic indicator of disease-free survival in patients with colorectal cancer. Archives of Surgery, 2012; 147(1): 18-24.
27. Bellizzi, A., Sebastian, S., Ceglia, P., Centonze, M., Divella, R., Manzillo, E. F., et al. Co-expression of CD133+/CD44+in human colon cancer and liver metastasis. Journal of Cellular Physiology, 2012; 228(2): 408-415.
28. Hakomori, S.-i. Aberrant Glycosylation In Tumors And Tumor-Associated Carbohydrate Antigens. Advances in Cancer Research. Elsevier, 1989; 257-331.
29. Cohn, S. L., Lincoln, S. T. & Rosen, S. T. Present Status of Serum Tumor Markers in Diagnosis, Prognosis, and Evaluation of Therapy. Cancer Investigation, 1986; 4(4): 305-327.
30. iegel, R. L., Fedewa, S. A., Miller, K. D., Goding-Sauer, A., Pinheiro, P. S., Martinez-Tyson, D., et al. Cancer statistics for Hispanics/Latinos, 2015. CA: A Cancer Journal for Clinicians, 2015; 65(6): 457.