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Stem Cell Markers Epithelial Cell Adhesion Molecule (EpCAM) & Cluster of Differentiation (CD44) Expression in Colon Cancer Patients in Relation to Prognosis and Survival

Lobna A. Abdelaziz[1] ,Ola A. Megahed[2], Ola A. Harb[3], Loay M. Gertallah[4], Ahmed M. Sallam[5]

[1]Department of Clinical Oncology and Nuclear Medicine, Faculty of Medicine, Zagazig University, Zagazig 44519, Egypt

[2, 3]Department of Pathology, Faculty of Medicine, Zagazig University, Zagazig 44519, Egypt

[4, 5]Department of General surgery, Faculty of Medicine, Zagazig University, Zagazig 44519, Egypt

Abstract: ***Background**: Worldwide, Colon cancer is the third& the second most common cancer in males& female respectively. Its recurrence, therapy resistance and metastasis remain causes of poor prognosis. The relationship between cancer stem cell (CSC) behavior and chemo-resistance to chemotherapy in patients with colon cancer is a recent point of researches that has taken attention. Identification of CSCs needed specific cell surface markers. CD44 had been the most frequently researched marker for colon CSCs. EpCAM is a cell surface molecule involved in cell to cell adhesion that is expressed in many epithelial carcinomas.Aim of the work: -was to evaluate CSC markers EPCAM & CD44 expressions in colon carcinoma correlating their expressions with clinicopathological parameters and patients outcome. Patients and Methods: The clinic-pathological correlations between CD44&EpCAM expressions were assessed in specimens of colon carcinoma embedded in paraffin blocks and were taken from 50 patients, which we followed up for survival and prognosis. Results: CD 44&EpCam positive expression was significantly correlated with the grade, stage, presence of lymph node, lymphovascular invasion (p<0.001 for all), distant metastases (p<0.001& = 0.016 respectively), T stage (p= 0.027& 0.003) respectively. Mean PFS was 27.4±1.5 , median PFS was not reached.3-y PFS was 56% which was significant with each one of CD44&EpCam (P<0.001 for both). While, Mean OS was 30.5±1.2, median OS was not reached, 3-yOS was 64% with significant association with each one of the markers (P=0.002 for both). Conclusion: We concluded that bothCD44&EpCam positive expressions would be useful poor prognostic markers for colon carcinoma patients.***

Keywords: Stem cell markers, CD44, EpCam, colon cancer, survival

1. Introduction

Worldwide, Colon cancer is the 3rdcommonest cancer in men but in female, it is the second most common cancer (1). Colon cancer mortality accounts for 8% of all cancer deaths, and colon cancer is the fourth most common cause of death from cancer (1). Colorectal cancer is the 6th commonest cancer both in Egyptian males and females and it forms 4.5% and 3.6% of all cancers, respectively (2). Although improvement of survival by diagnostic and therapeutic advances, but recurrence and metastasis is still a leading cause of poor prognosis, with about twenty – forty percent of patients hadhepatic metastases at diagnosis, this may be because of therapy resistance (3).

The relationships between colon cancer stem cells (CSCs) criteria and chemo-resistance in patients are novel points of researches that have taken attention (4). The researchers have noticed that some cancer cells in cancers of many organs like breast& colon developed CSCs criteria by the epithelial-mesenchymal transition (EMT) (5-6), which allowed colon cancer cells invasion into the basement membrane, surrounding and distant tissues (4). That sub-population of cancer cells was CSCs and formed 0.1-10% of the whole cancer cells (7, 8). These CSC was found to have no relation to cell proliferation, invasion, metastasis; but they had a slower rate of cycling which played a role in chemo-radiotherapy resistance and cancer recurrence (9, 10). Lots of biomarkers which have been detected as colon CSCs surface antigens, but it is not well known which are the best markers to identify them (11, 12) due to presence of variations between patients having the same cancer type (13).

CD44 was the most frequently studied and is found to be the most suitable marker colon CSCs (3). It is a transmembrane glycoprotein that participated in lots of cellular functions as growth, differentiation, survival and motility (12). It is an adhesion molecule that plays different roles in malignant cells migration so increasing the rate of tumor cell growth (14). Ep-CAM is a cell surface molecule, had a role in cell adhesions and its protein expression was found in many cancers (15).

Aim of the work: -was to evaluate the CSC markers EPCAM & CD44 expressions in colon carcinoma correlating their expressions with each other, clinicopathological parameters and patients outcome.

2. Patients and Methods

We carried out this retrospective study at departments of Clinical Oncology&nuclear medicine, Pathology and General surgery, Faculty of Medicine, Zagazig University, Egypt in the period from January 2014 to December 2016.We included fifty patients who were diagnosed as

colonic carcinoma by routine H&E histopathological examination which was done in the Pathology-Department, Faculty of Medicine, Zagazig University . We identified patient sex, age, tumor size, stage, grade, lymph nodes, distant metastases, neural invasion, lymphovascularinvasion, type of treatment received (surgery, chemotherapy) by retrospective examination of the patients' records at the involved departments. Local Research Ethics Committee approval of the study was obtained. The 7th edit of American-Joint-Committee-on Cancer (AJCC-7) classification & the World-Health-Organization (WHO) grading system were used for colon cancerstaging and grading respectively**(16-17).**

The technique of immunohistochemical staining:-

We used avidin-biotin complex systems (DAKO) for immunohistochemical staining **(18)**. We incubated sections with primary mouse monoclonal anti-CD44 antibody [F10-44-2] ab6124 and primary Rabbit monoclonal EpCAM[E144] ab32392a, and then we counterstained the slides with hematoxylin. We used sections from gastric adenocarcinoma and renal cell carcinoma as positive controlsfor Epcam & CD44 respectively, but the negative control was done by replacement of the primary antibodies with non-immune serum.

Evaluation of immunohistochemical expression of CD-44&EP-CAM:-

All assessments were made on the tumor region of thespecimen (×200). We evaluated each slide without previous knowledge of clinical criteria of patients.We evaluatedCD44& EPCAMexpressions by calculating both the extent& intensity of the stainin the tumorcells to reach the total immune-reactivity score (IRS)which is the result of multipliying both the extent& intensity scores. The extent of the stained tumor cells is scoredfrom 0-4 (zero, none; one, 1%–10%; two, 11%–50%; three,51%–80%; four, 81%–100%). The stain intensity was scoredfrom 0-3 (zero, no staining;one, weak; two, moderate; three, strong). The final IRS wasfrom 0 - 12**(19).** We used the cut off 6 below which is considered negative and above which is considered positive expression.

3. Statistical Analysis

The collected data were statistically analyzed using SPSS program (Statistical Package for Social Science) version 20.Data were tested for normality of distribution by usage of Kolmogorov–Smirnov test.Chi-square ($\chi 2$) and Fisher-exact testswere used to calculate differences betweenall qualitative variables.The quantitative data were expressed by mean ± SD (Standard deviation).Kaplan and Meier method used to estimate overall and progression-free survival and log rank test compared survival curves. Overall survival (OS): was calculated as the time from the date of diagnosis till date of death or date of last follow up or end of the study.of progression-freesurvival (PFS): was calculated from the treatment initiation date till the date of documented disease progression. All statistical comparisons were two tailed, with a P value of < 0.05 required for statistical significance.

4. Results

Patient's data

The clinical data of our patients who were included in this study are detailed in **(Table 1)**

In our study, there were 28(56%) males and 22 (44%) females with age ranged from (30-68) years (Mean: 55.4 ± 6.6 years).LT colon cancer was the highest involved site which was occurredin 22 (44%) patients. The most frequent gross pattern was ulcerative and fungating types which were present in 20(40%) and 19(38%) patients respectively. Adenocarcinoma was the most commonhistopathological type which was present in 42 (84.0%) cases. Twenty four (48%) patients had grade II colon cancer which was the most frequent grade .Lymphovascularinvasion (LVI) and neural invasion were occurred in 22 (44) and 10 (20%) patients respectively. Stage III was the most common which was present in 16(32%) patients .LN metastasis and distant metastasis were present in 31(62%) and 15(30%) patients respectively.

CD44 expressions & correlations with clinic-pathological features of our patients (Table 1 and Figure 3)

CD44 had positive expression in 50% (25/50) patients and its positive expressionswere correlated with cancer stage& grade, the presence of lymph nodes&distant metastases, the presence of lymphovascular& neural invasion ($p<0.001$ for all) and T stage ($p= 0.027$), but the correlations between its expressions, age & sex of our cancer patients, site, size, gross or histopathological subtypes of the cancer were non-significant.

EpCam expressions & correlations with clinico-pathological features of our patients (Table 1,2 and Figure 4)

EpCam had positive expression in 54% (27/50) patients and its positive expression was significantly correlated with the grade, stage of the tumor, presence of lymph node metastases, lympho-vascular invasion, ($p<0.001$ for all), histopathological type of tumor($P=0.017$) , distant metastases ($p= 0.016$), and T stage ($p= 0.003$), but there is no significant correlation was found between EpCamexpression, age or sex of our patients, size, site, gross, or presence of neural invasion.

We found that there were highlysignificant positive correlations between EpCAM and CD44 expressions in our patients ($p<0.001$).

Table 1: The relation between clinicopathological characters and CD44 &EPCAM expression:-

Clinicopathological feature		All Patients (N=50)	CD44 expression		p	EpCAM expression		p
			Positive (N=25)	Negative (N=25)		Positive (N=27)	Negative (N=23)	
Age, years		55.4 ± 6.6	56.1 ± 5.8	54.6 ± 7.3	0.419	56.6 ± 6.3	54 ± 6.7	0.166
Sex	Male	28 (56.0%)	13 (52.0%)	15 (60.0%)	0.569	15 (55.6%)	13 (56.5%)	0.945
	Female	22 (44.0%)	12 (48.0%)	10 (40.0%)		12 (44.4%)	10 (43.5%)	
Tumor site	RT	19 (38.0%)	9 (36.0%)	10 (40.0%)	0.841	8 (29.6%)	11 (47.8%)	0.384
	Transverse	9 (18.0%)	4 (16.0%)	5 (20.0%)		5 (18.5%)	4 (17.4%)	
	LT	22 (44.0%)	12 (48.0%)	10 (40.0%)		14 (51.9%)	8 (34.8%)	
Gross pattern	Ulcerative	20 (40.0%)	8 (32.0%)	12 (48.0%)	0.328	9 (33.3%)	11 (47.8%)	0.523
	Fungating	19 (38.0%)	12 (48.0%)	7 (28.0%)		12 (44.4%)	7 (30.4%)	
	Annular	11 (22.0%)	5 (20.0%)	6 (24.0%)		6 (22.2%)	5 (21.7%)	
Size(cm)	<5cm	26 (52.0%)	14 (56.0%)	12 (48.0%)	0.571	15 (55.6%)	11 (47.8%)	0.586
	>5cm	24 (48.0%)	11 (44.0%)	13 (52.0%)		12 (44.4%)	12 (52.2%)	
Histological type	Adeno-carcinoma	42 (84.0%)	18 (72.0%)	24 (96.0%)	0.053	19 (70.4%)	23 (100.0%)	0.017
	Mucinous	4 (8.0%)	3 (12.0%)	1 (4.0%)		4 (14.8%)	0 (0.0%)	
	Signet ring carcinoma	4 (8.0%)	4 (16.0%)	0 (0.0%)		4 (14.8%)	0 (0.0%)	
Grading	G I	12 (24.0%)	1 (4.0%)	11 (44.0%)	<0.001	2 (7.4%)	10 (43.5%)	<0.001
	G II	24 (48.0%)	10 (40.0%)	14 (56.0%)		12 (44.4%)	12 (52.2%)	
	G III	14 (28.0%)	14 (56.0%)	0 (0.0%)		13 (48.1%)	1 (4.3%)	
LVI		22 (44.0%)	17 (68.0%)	5 (20.0%)	0.001	18 (66.7%)	4 (17.4%)	0.001
Neural invasion		10 (20.0%)	10 (40.0%)	0 (0.0%)	<0.001	7 (25.9%)	3 (13.0%)	0.256
LN	Negative	19 (38.0%)	2 (8.0%)	17 (68.0%)	<0.001	3 (11.1%)	16 (69.6%)	<0.001
	Positive	31 (62.0%)	23 (92.0%)	8 (32.0%)		24 (88.9%)	7 (30.4%)	
T	T1	7 (14.0%)	1 (4.0%)	6 (24.0%)	0.027	2 (7.4%)	5 (21.7%)	0.003
	T2	16 (32.0%)	6 (24.0%)	10 (40.0%)		4 (14.8%)	12 (52.2%)	
	T3	14 (28.0%)	11 (44.0%)	3 (12.0%)		12 (44.4%)	2 (8.7%)	
	T4	13 (26.0%)	7 (28.0%)	6 (24.0%)		9 (33.3%)	4 (17.4%)	
N	N0	19 (38.0%)	2 (8.0%)	17 (68.0%)	<0.001	3 (11.1%)	16 (69.6%)	<0.001
	N1	22 (44.0%)	18 (72.0%)	4 (16.0%)		19 (70.4%)	3 (13.0%)	
	N2	9 (18.0%)	5 (20.0%)	4 (16.0%)		5 (18.5%)	4 (17.4%)	
M	M0	35 (70.0%)	12 (48.0%)	23 (92.0%)	0.001	15 (55.6%)	20 (87.0%)	0.016
	M1	15 (30.0%)	13 (52.0%)	2 (8.0%)		12 (44.4%)	3 (13.0%)	
Stage	Stage I	7 (14.0%)	0 (0.0%)	7 (28.0%)	<0.001	0 (0.0%)	7 (30.4%)	<0.001
	Stage II	12 (24.0%)	2 (8.0%)	10 (40.0%)		3 (11.1%)	9 (39.1%)	
	Stage III	16 (32.0%)	10 (40.0%)	6 (24.0%)		12 (44.4%)	4 (17.4%)	
	Stage IV	15 (30.0%)	13 (52.0%)	2 (8.0%)		12 (44.4%)	3 (13.0%)	

Table 2: The relation between CD44 and EPCAM expression:-

		CD44 expression		P
		Positive (N=25)	Negative (N=25)	
EpCAM expression	Negative	3 (12.0%)	20 (80.0%)	<0.001
	Positive	22 (88.0%)	5 (20.0%)	

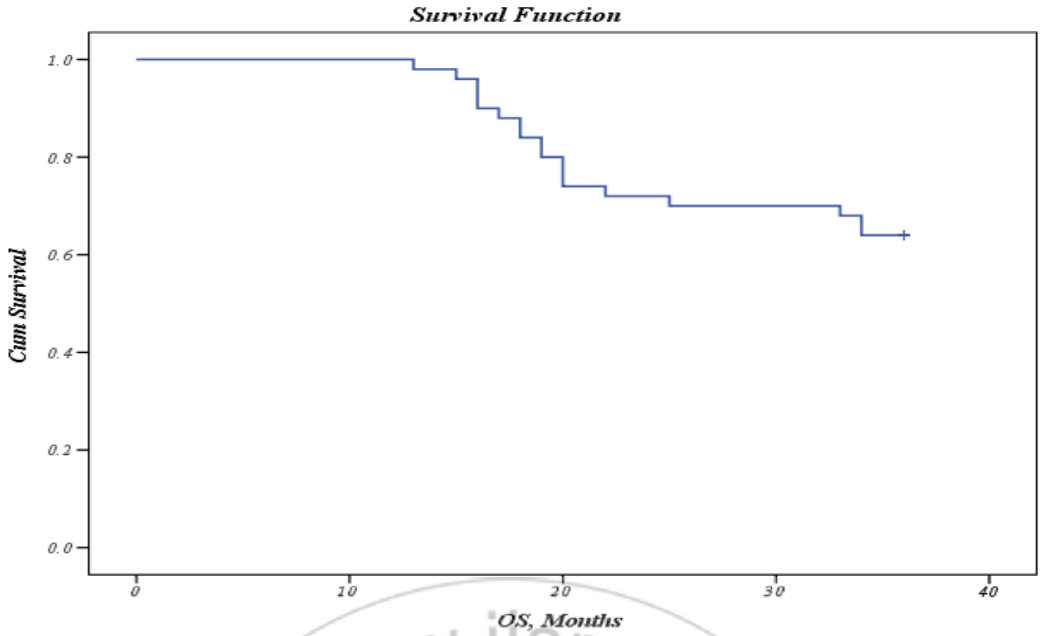
The association between the CD44&EpCam and the patient's outcome (Table3,figure 1,2):-

Twenty two out of sixty (44%) patients progressed with significant relation with each one of the markers (P<0.001). Mean PFS was 27.4±1.5, median PFS was not reached.3-y PFS was 56% which was significant with CD44 &EpCam (P<0.001) .While 18 (36%) patients died which was significantly correlated withCD44 & EpCam (P=0.003 and 0.002 respectively).Mean OS was 30.5±1.2 ,median OSwas not reached .3-yOS was 64% with significant association with each one of the markers (P=0.002).

Table 3: The overall Survival and progression free survival in Relation to Expression of EpCAM& CD44

Variable		*3-year overall survival Rate (%)*	*p-value*	*year3-PFS Rate (%)*	*P-value*
EpCAM	Negative	87%	0.002	87%	<0.001
	Positive	44.40%		29.60%	
CD44	Negative	84%	0.002	84%	<0.001
	Positive	44%		28%	
Expression Pattern	No Expression	100%	<0.001	100%	<0.001
	Co-expression	50%		31.80%	
	EpCAM Only	20%		20%	
	CD44 Only	0.00%		0.00%	

NR, Not reached



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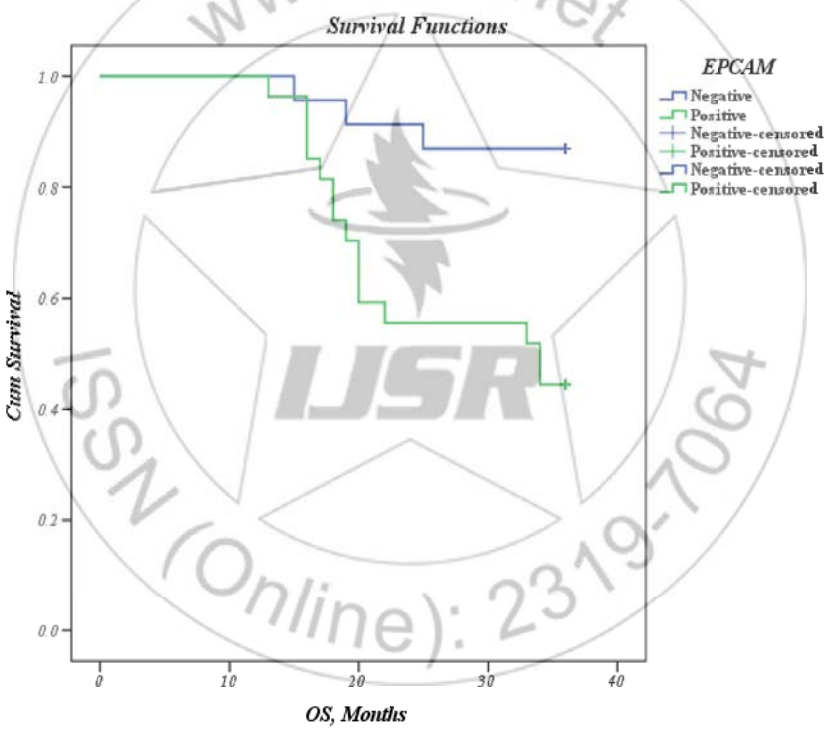

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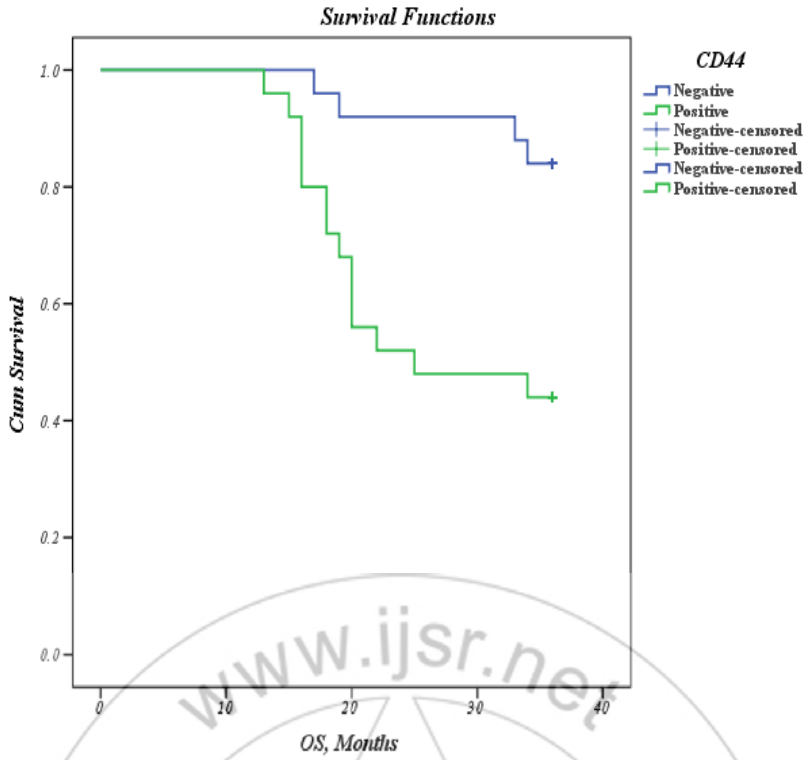

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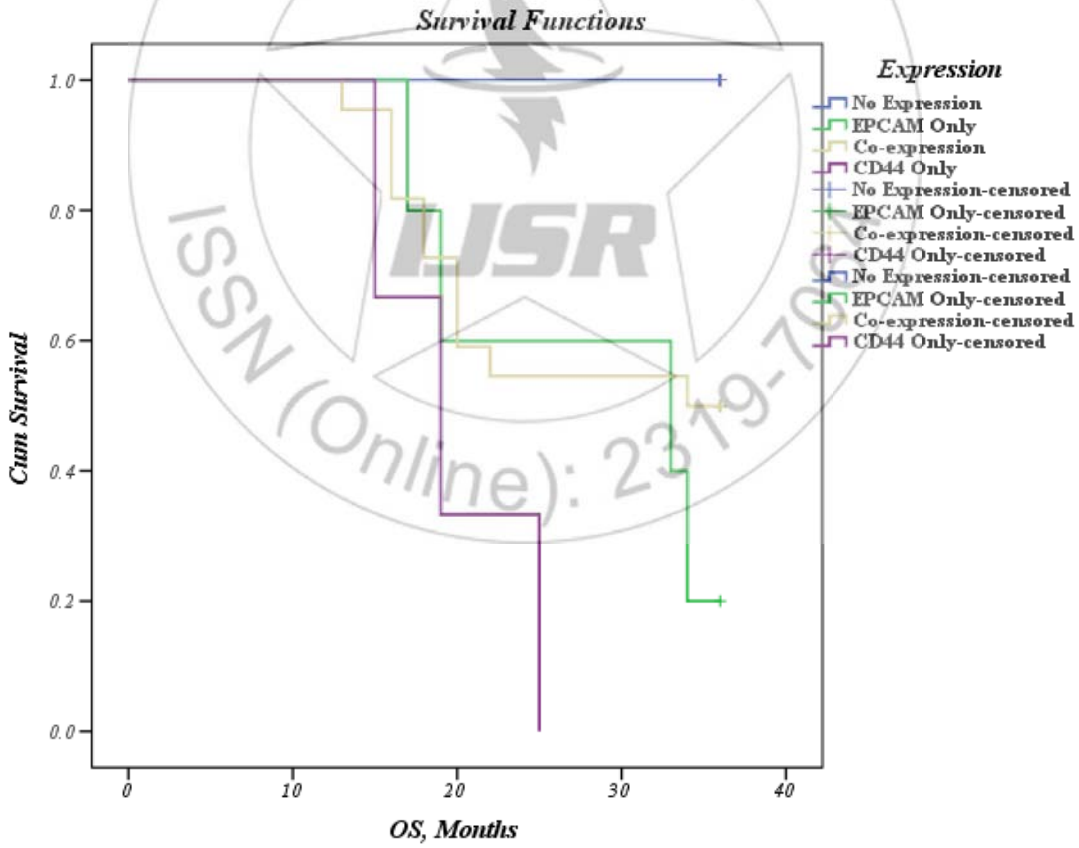
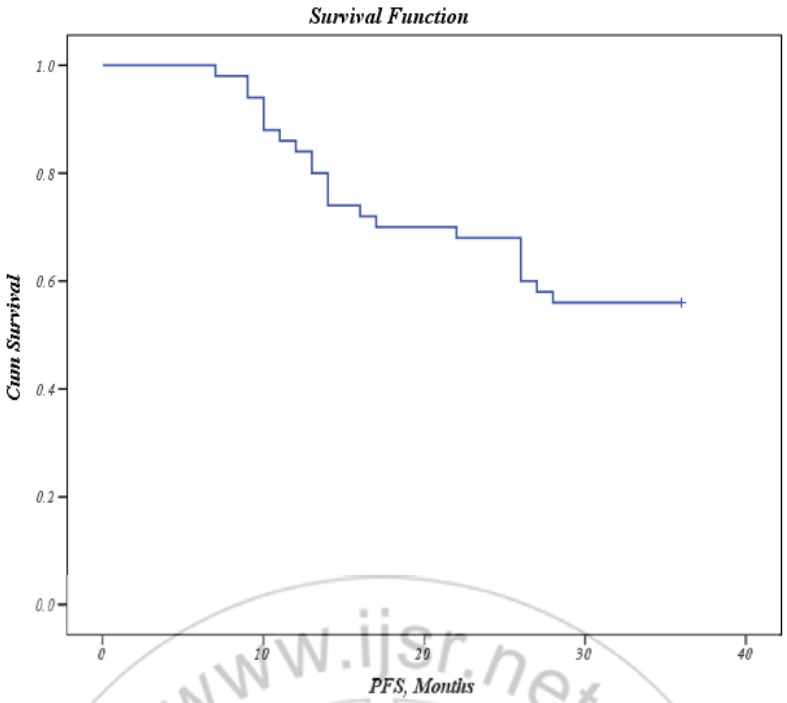
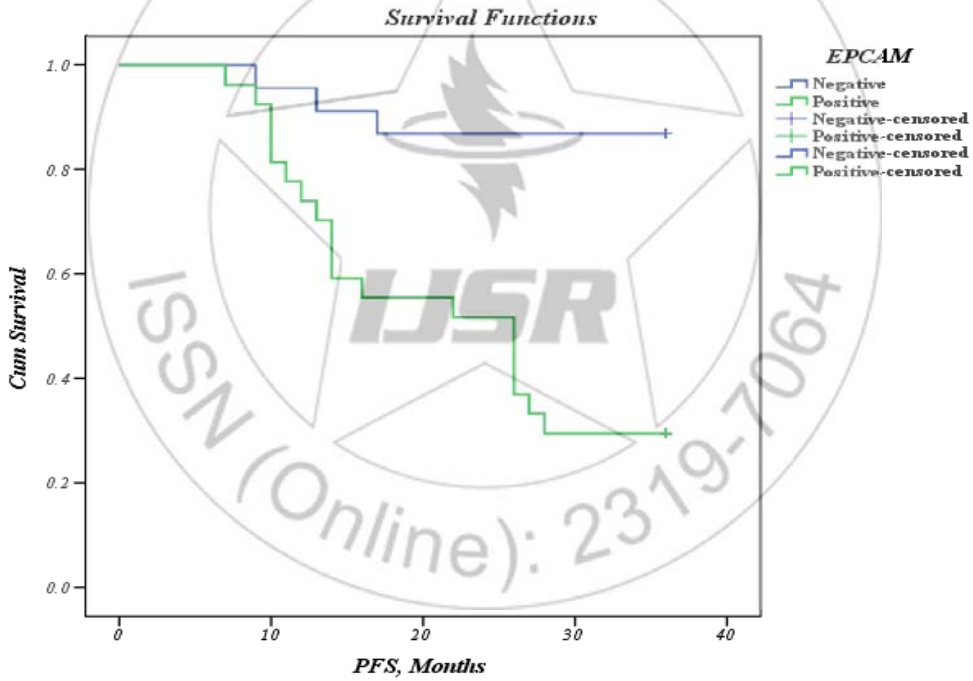


D

Figure (1): Kaplan meier curve of overall survival (A) for all patients (B) in relation with EPCAM (C) in relation with CD44 (D) in relation with both markers



A



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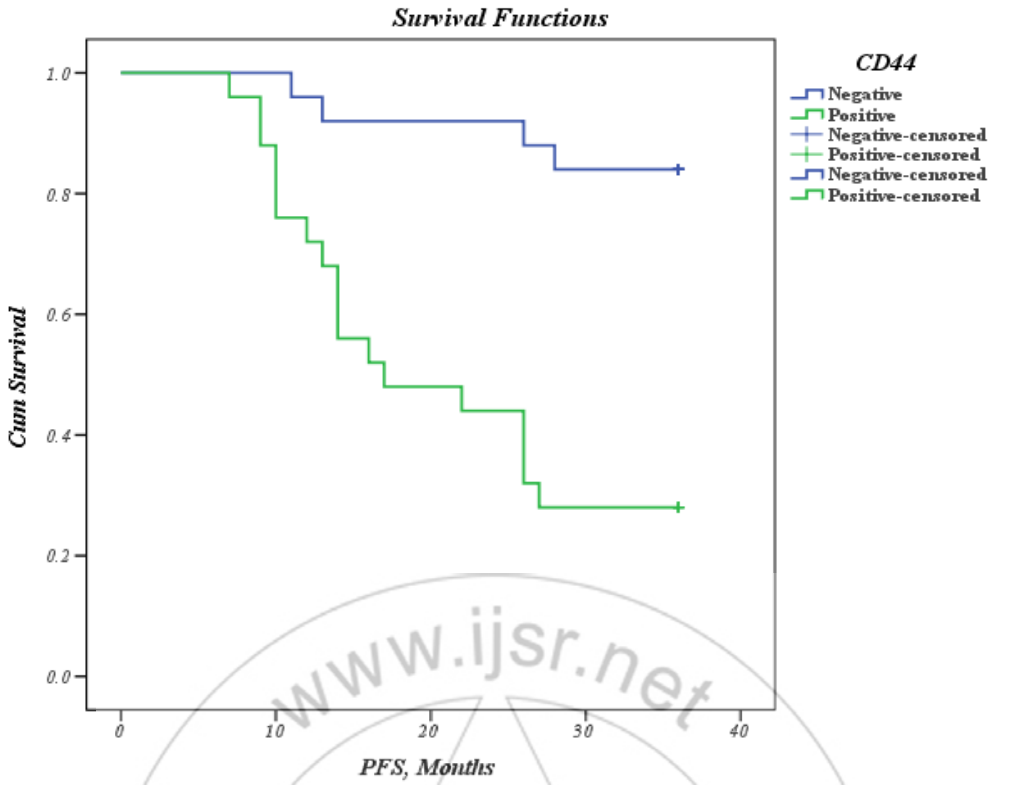

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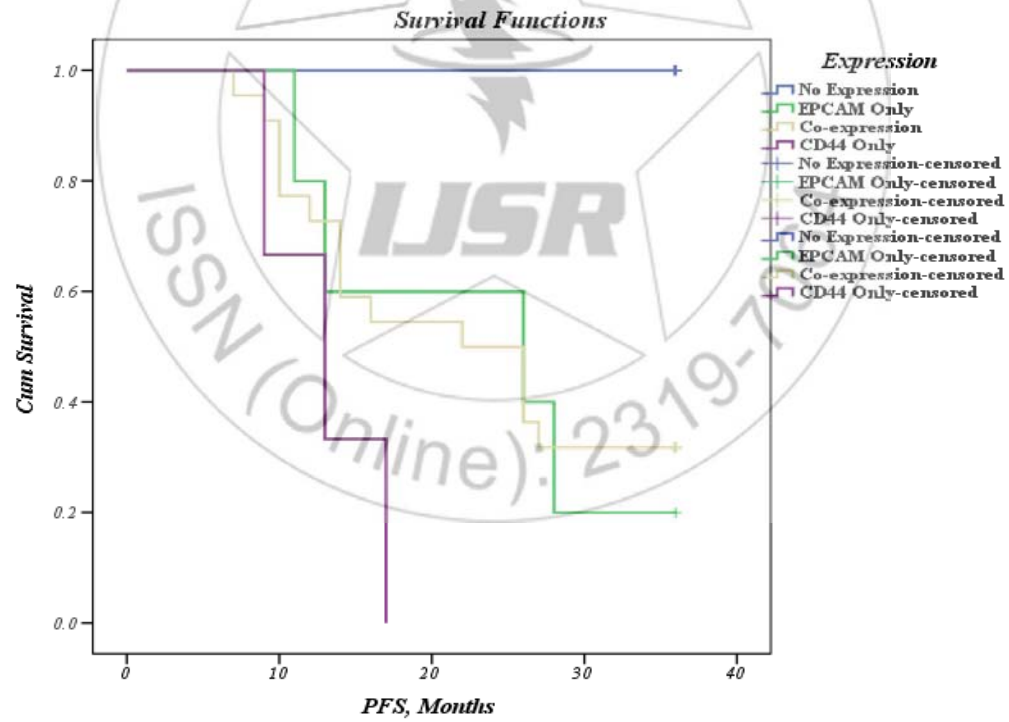


D

Figure (2): Kaplan meier curve of progression free survival (A) for all patients (B) in relation with EPCAM (C) in relation with CD44 (D) in relation with both markers

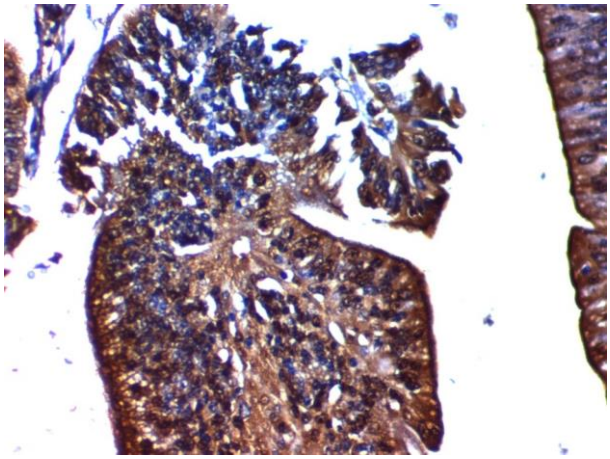
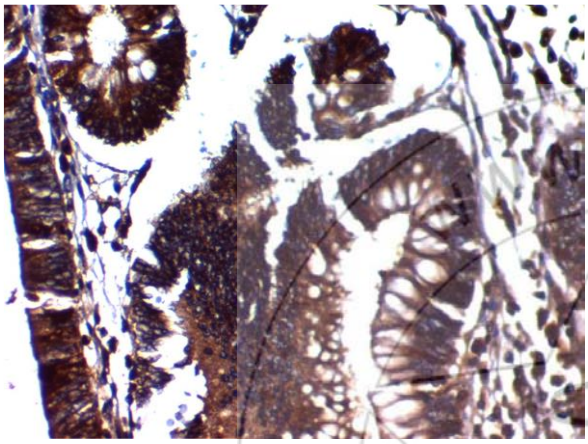


Figure 3 A



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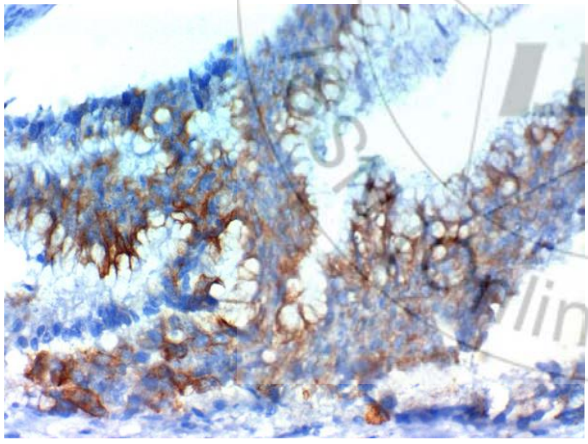

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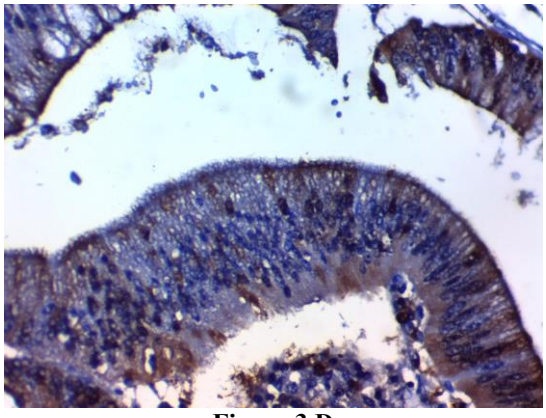

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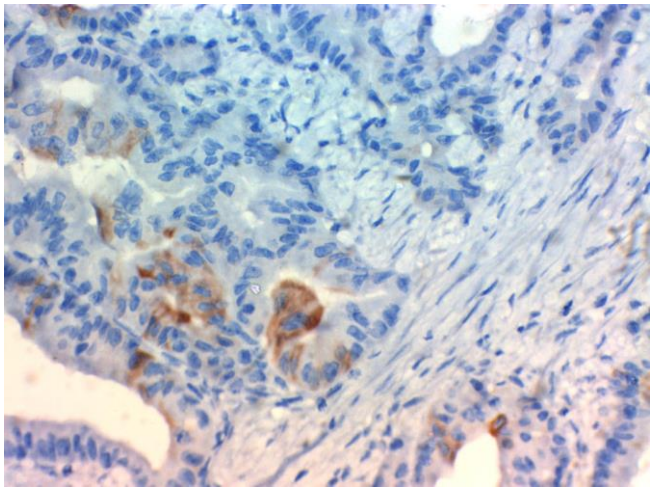

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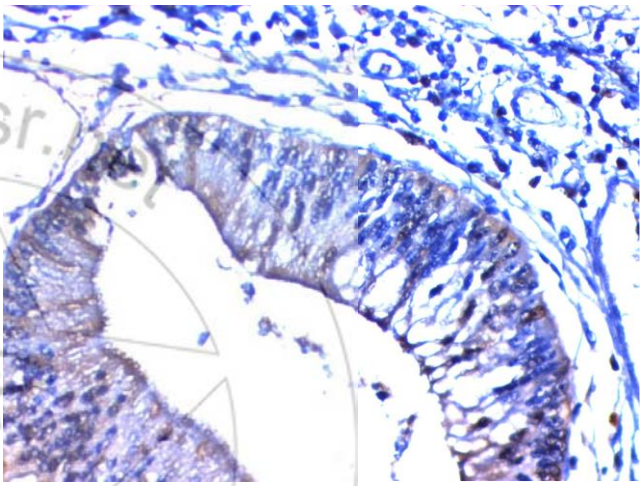

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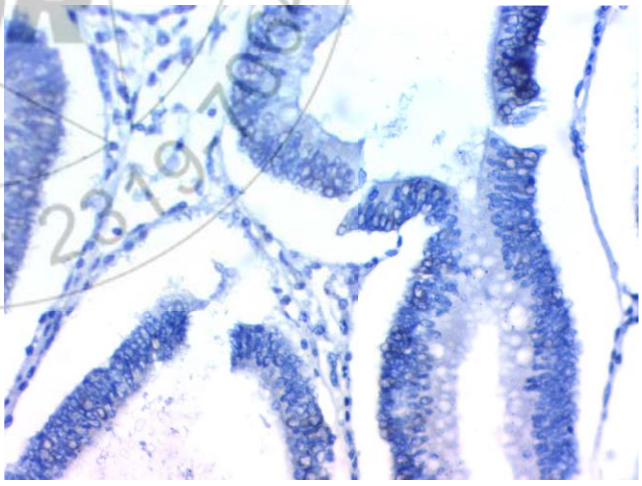


Figure 3 G

Figure 3. Immunohistochemical staining of CD44 in colon cancer(CC):**(A)**High Immunohistochemical expression in the membrane of poorly differentiated CC stage IVx400 **(B)**High Immunohistochemical expression in the membrane of moderately differentiated CC stage IIIx400. **(C)**High Immunohistochemical expression in the membrane of moderately differentiated mucinous CC stage IIIx400. **(D)** high Immunohistochemical expression in membrane of moderately differentiated CC stage IIBx400. **(E)** Low Immunohistochemical expression in the membrane of

moderately differentiated CC stage IIAx400. **(F)** Low Immunohistochemical expression in the membrane of well differentiated CC stage IBx400. **(G)** Negative Immunohistochemical expression in the membrane of moderately differentiated CC stage IAx400. **Note:** High CD44immunohistochemical expression in high grade & stage CC and low expression in low grade &stage CC:

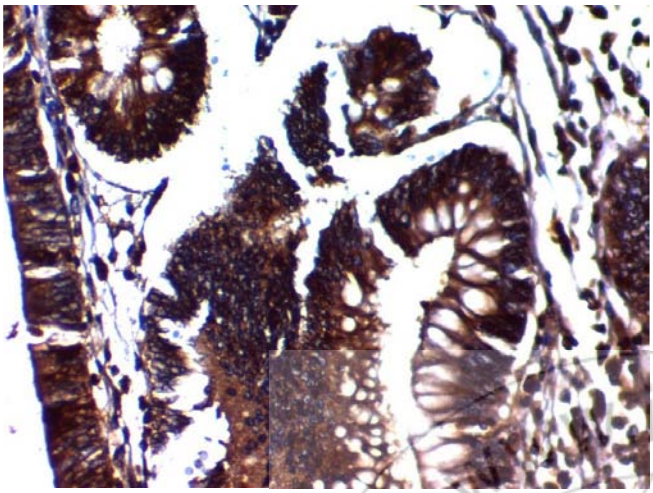
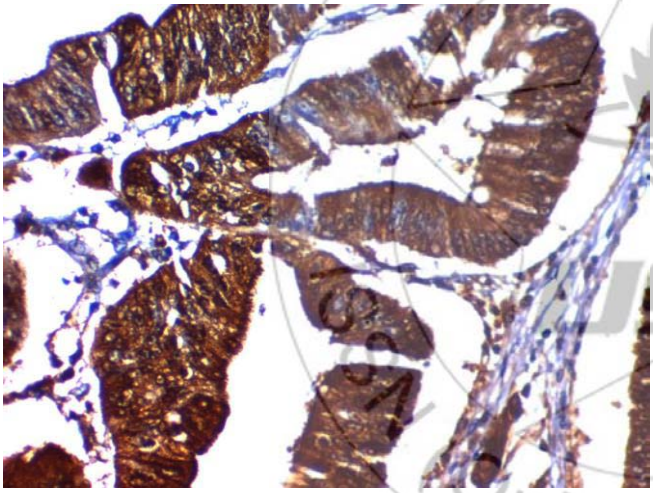


Figure 4 A



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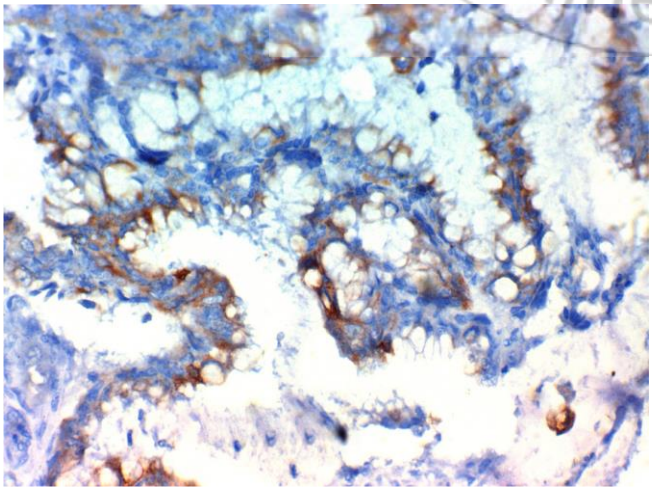

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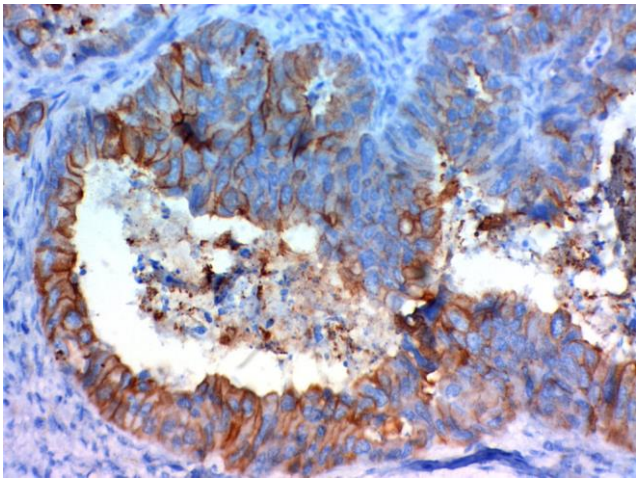

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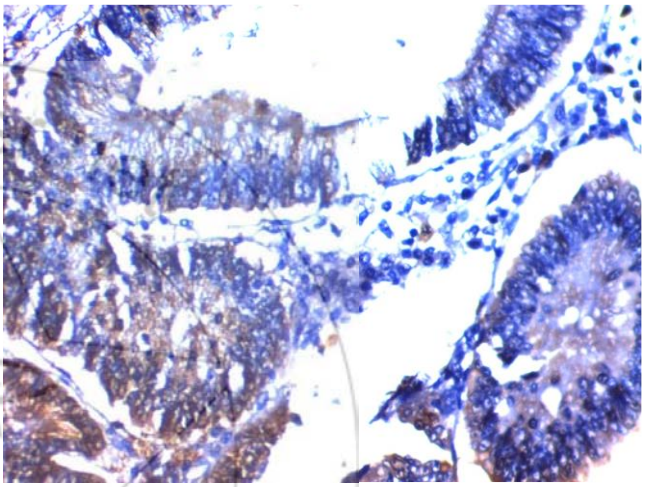

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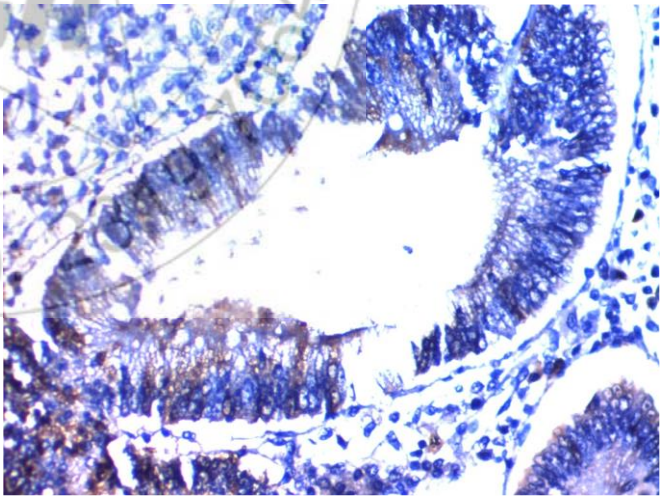

Figure 4 F

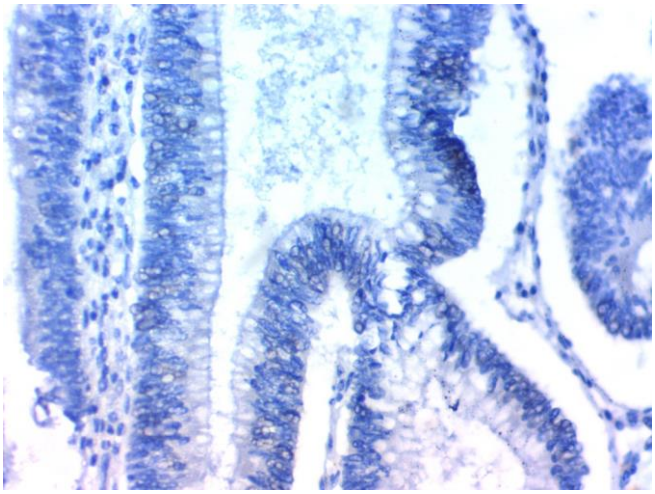

Figure 4 G

Figure 4: Immunohistochemical staining of EpCam in colon cancer(CC):**(A)**High Immunohistochemical expression in the membrane of moderately differentiated CC stage IVx400 **(B)**High Immunohistochemical expression in the membrane of moderately differentiated CC stage IIIx400. **(C)**High Immunohistochemical expression in the membrane of moderately differentiated mucinous CC stage IIIx400. **(D)** high Immunohistochemical expression in membrane of moderately differentiated CC stage IIBx400. **(E)** Low Immunohistochemical expression in the membrane of moderately differentiated CC stage IIAx400. **(F)** Low Immunohistochemical expression in the membrane of well differentiated CC stage IBx400. **(G)** Negative Immunohistochemical expression in the membrane of moderately differentiated CC stage IAx400. Note: High EpCamimmunohistochemical expression in high grade & stage CC and low expression in low grade &stage CC:

5. Discussion

The differentiated, rapidly growing cells form the major percent of most cancers which are susceptible to chemotherapy and radiotherapy, while the small part of the cancer mass is composed of CSCs, that are slowly growing and resistant to chemo- or radiotherapy and even after chemo- or radiotherapy which induced shrinkage of cancer the CSCs survived and differentiated into rapidly growing phenotypes with metastases-forming ability. So, CSCs are supposed to be a main cause of relapse of cancer and increased cancer specific mortality (20).

CD44 is a glycoprotein class I and is located on the cyto-membrane (21). It is one of cells adhesion-molecules which is involved in cell-matrix and cell-cell interactions (22). It also played vital roles in regulation of cell, growth, adhesion, differentiation, migration, angiogenesis, and has an important role in cancer progression by facilitating invasion and metastasis (21).

Schulenburg et al (23) found that CD44+ cells had criteria of CSCs which had more liability for growth and invasion than CD44−cells. So he proved that CD44 is a CSCs marker, and it can be used to differentiate between variable cancer types in addition to other surface markers. In colon cancer, there are conflicting results from a positive to negative correlations between each one of CD44, EPCAM expressions and colon cancer prognosis.

In our work we found that CD-44&Ep-Cam positive expressions were correlated with higher stage& grade of cancer, presence of lymph nodes & lympho-vascular invasion ($p<0.001$ for all), distant metastases ($p<0.001$& = 0.016 respectively), T stage ($p= 0.027$& 0.003 respectively). In addion , EpCam and CD44 had a significant association with histopathological type of tumor($P=0.017$) and neural invasion($p<0.001$) respectively.

Similar to our results, previous studies have revealed that EpCAM high and CD44+ colon cancer cells have stem cell-like features, with more liability of carcinogenesis, invasion and metastasis (24–26) , so that EpCAM high/CD44+ is the most likely markers for colon CSCs (27).

EpCAM which is an adhesion molecule of epithelial cells, its expression was localized to the epithelial lateral surface of most areas of gastrointestinal tract mucosa (23, 28). Our results could be in agreed **with Sankpal NV, 2011** (28) and **Lin CW, 2012** (29), Who explained association of Ep-Cam with aggressiveness of cancer by up-regulation of theoncogene c-myc expression which inducedcell cycle acceleration and facilitated cancer proliferation. EpCAM over-expression enhances the proliferation and invasionof cancers, but its down regulation by RNA interference inhibits these functions (30).

We assed expression of both EpCAM and CD44 together in colon cancer and found highly significant positive correlation between their expression in our patients and that was in agree with some studies , that have revealed that the structure of EpCAM and CD44 can increased colon cancer cells invasion and metastasis (31).

Marhaba et al, 2008(32) & **Dalerba et al,2007**(33) found that EpCAM high and CD44+ cells are markers of colon CSCs . So that colon cancer cells with high EpCAM and positive CD44 expression has stem cell-like features , so they proved to be effective markers of colon CSCs.

In **Liu et al, 2014** (26) study, 80 cases of colorectal cancer and their corresponding liver metastases were examined. Cells with high EpCAM and positive CD44 expression were not found in the nearby normal intestinal mucosa but these cells were present in colon cancer cells and their liver metastases.

Further researchers found that the percentage of high EpCAM and positive CD44 expressing cells in poorly -differentiated cancers were more than that in well- or-moderately- differentiated cancers, also the percentage of high EpCAM and positive CD44 expressing cells in the stage four and Dukes' D stage or in cases of distant metastases were more than lower stages or in cancers without metastases (14). This was similar to ours where, high EpCAM and positive CD44 expression was correlated with grade& clinical cancer stage, depth of invasion and metastasis, so their expressions were significantly correlated with invasion and metastasis, and confirm that EpCam high and CD44+ cells are effective markers for colon CSCs.

These findings support that CSCs is the cause of colon cancer recurrence and metastasis (14).

In our study we found, Twenty two (44%) patients progressed with significant relation with high expression of each one of the markers (P<0.001).Mean PFS was 27.4±1.5 .3-y PFS was 56% which was significant with each one of CD44 &EpCam (P<0.001 for both) . While 18 (36%) patients died which was significantly correlated with high expressed CD44 &EpCam (P=0.003 and 0.002 respectively).Mean OS was 30.5±1.2 .3-yOS was 64% with significant association with each one of the markers (P=0.002 for both).Our findings were consistent with **Huh et al,2009**(34) who reported that CD44 expression was an independent unfavorable prognostic factor for overall survival. On the other hand ,there are some trials disagree with our results and found that low CD44 expression was identified as an independent prognostic factor for shorter disease-free survival. Similarly, two studies, which evaluated the expression of CD44 by using immunohistochemical staining, demonstrated that loss of CD44 expression was related to a worse prognosis for colorectal cancer patients (35-36). In contrast, another two studies showed that CD44 expression was not significantly associated with survival (37-38).

Over-expression of EpCAM correlated with aggressiveness and poor prognosis in colon cancer patients (39), but, a reduction in surface expression of EpCAM has also been associated with aggressive cancers and poor prognosis in CRC (40). The hypothesis that loss of EpCAM expression may be involved in cancer metastasis is later on proved by the finding that EpCAM protein expression on circulating tumor cells (CTC) is reduced compared with primary and metastatic tumors (41).The limitations of our study is small sample size and short follow up duration so our recommendation is to do further studies with larger number of patients and a long follow up time to confirm the role of our markers .

6. Conclusion

BothCD44 & EpCam positive expressions would be useful poor prognostic markers for colon cancer patients, but further studies with larger number of patients and a long follow up duration are needed to confirm their roles.

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