Medicine Updates Faculty of medicine
April 2023,volume 13, issue 13

https://muj.journals.ekb.egdean@med.psu.edu.eg
vice_dean_postgraduate@med.psu.edu.eg
DOI: 10.21608/MUJ.2023.190677.1129

ISSN : 2682-2741

Submitted: 30/01/2023
Accepted : 30/03/2023
Pages:108 - 122

" Assessment of Immunohistochemical Expression of CD44 and Osteopontin in Colorectal Carcinoma"

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Abstract:

Background: Colorectal carcinoma considered the most worldwide concern and one of the major causes of morbidity and mortality rates. The cancer originates from a types of tumor cells, called cancer stem cells, they have important in the initiation of the tumor, invasion, metastasis, and therapeutic resistance. Among CSC markers, CD44 and OPN are two of the most investigated colorectal cancer markers and they are considered as the subpopulation with a greater tumorigenicity. This study aiming assessing the immunohistochemical expression of CD44 & OPN in colorectal adenomas & CRCs. And their relation between immunohistochemical expression of CD44 & OPN with tumor differentiation (grading), lympho-vascular invasion, perineural invasion, desmoplasia and TNM stage. **Methods:** the study is a retrospective descriptive study that included forty-two paraffin embedded blocks from the pathology laboratory, Suez Canal University Hospital. Paraffin blocks included, paraffin blocks reviewed for clinicopathological prognostic factors and stained by CD44 & OPN, monoclonal antibodies by immunohistochemical method. **Results:** The CD44 protein was expressed in 80% of CRC, the expression of CD44 and lympho-vascular invasion and tumor stage. **Conclusions:** CD 44 is highly expressed in large number of CRC (80 of tumors). It is also significantly more expressed in CRC than in adenomas, suggesting a role of CD 44 in CRC tumorigenesis and progression of adenomas into carcinomas. Our study also associated CD 44 overexpression with both late TNM stage and lympho-vascular invasion.

Keyword: Immunohistochemistry, Colorectal carcinoma, CD44, Osteopontin, Cancer stem cell, Stage of tumor.

Background:

Colorectal carcinoma is the 3rd most common carcinoma in the world. It is also considered the 3rd most leading cause of death usually due to the presence of liver metastases [(1)]. In Egypt, it is the seventh among both genders [(2)].

Colorectal carcinomas are caused by special nutritional habits, genetic causes, lifestyle, and other environmental factors. More cases of colorectal cancers are of adenocarcinomas type. The remaining of colorectal cancers are neuroendocrine carcinomas, squamous cell carcinomas (SCC), lymphoma, and other very rare types [(3)].

Cancer stem cells (CSCs) are related to the tumor's initiation [(4)]. Numerous studies have shown that CSCs have an important role in colorectal carcinomas [(5) & (6)].

CD44 is a cell surface adhesion marker that is expressed widely in most body cell types, including epithelial cells, leukocytes, leukocytes and in tumor cells. The CD44 gene is located on chromosome 11 of the human genome [(7)].

CD44 is a surface adhesion protein which is also part of cell adhesion molecules that plays a role in cell-to-cell adhesion and interactions with the extracellular matrix and has a role in tumor proliferation, occurrence, and spread in which affect the prognosis and patient survival [(8) & (9)]. CD44 is a marker of stem cells in CRC especially adenocarcinoma.

Osteopontin (OPN) is considered as secreted glycophosphoprotein, encoded by the SPP1 gene, located on chromosome 4q13 [(10).] It is believed that binding of OPN to integrins regulate neovascularization, tumor cell spreading. Binding of OPN to CD44 receptors controls immune system, adhesion, transformation of normal to cancerous cells and tumor growing process [(11)]. OPN thus influences the tumor growth, maintenance of proliferative signaling, evasion of growth suppressors, invasive growth, and metastasis [(12)].

Although CD44 and OPN proteins have been tested as adhesion markers in colorectal carcinoma. Their prognostic value has not been elucidated clearly [(13)].

Aim of the study:

This study aims at assessing the immunohistochemical expression of CD44 & OPN in CRCs and its with different prognostic parameters in CRC such as: tumor differentiation (grading), lympho-vascular invasion, perineural invasion, desmoplasia and TNM stage. This helps us to use CD44 & OPN in the immunohistochemical panel of colorectal carcinomas as a or prognostic factor.

Material and Methods:

Sample collection: This study was carried out in the Pathology lab, Faculty of Medicine, Suez Canal University Hospital. It included 60 specimens divided into 42 specimens of CRCs and 18 specimens of colorectal adenomas which was chosen randomly. The sample size was calculated based on the following equation:

$$n = \left[\frac{Z_{\propto/2} + Z_{\beta}}{P_1 - P_2}\right]^2 (p_1 q_1 + p_2 q_2)$$

$\boldsymbol{n}$ = required sample size per group

$\boldsymbol{Z_{\propto/2}}$= 1.96 (The critical value that divides the central 95% of the Z distribution from the 5% in the tail).

$\boldsymbol{Z_{\beta}}$ = 0.84 (The critical value that separates the lower 20% of the Z distribution from the upper 80%.

Histopathological examination: Hematoxylin and eosin-stained sections were reviewed to confirm the first diagnosis. All sections were assessed for the prognostic factors (Grading, lympho-vascular invasion, perineural invasion, desmoplasia and TNM Stage). The grade was determined according to WHO 5th edition grading and TNM stage was determined according to AJCC 8th edition.

Immunohistochemistry:

Was manually performed according to the manufacture company in data sheet was manual, five microns sections from formalin-fixed, paraffin-embedded tissue blocks were cut, deparaffinized & rehydrated. Positive and negative controls were prepared. Antigen retrieval was performed at 98 ◦C in 10 mM sodium citrate buffer pH 6 for 40 minutes. The slides were placed in peroxidase block for twinty minutes. Primary antibodies for CD44100 µl and for OPN were incubated with the slides. Sections were counterstained with mayer's hematoxylin. Tonsillar tissue used as positive control for CD44 and normal gall bladder epithelium for OPN [(14)].

Evaluation of staining

CD44 expression was evaluated according to its intensity & extent of staining;

The intensity was divided into four grades: Grade 0: none, Grade 1: weak, Grade 2: moderate & Grade 3: strong.

The extent of staining was divided into five grades: zero: ≤ 5 %, 1 (mild): 6 –25 %, 2(moderate): 26 – 50 %, 3(strong): 51 –75 % & 4 (maximum): 76 –100 %.

The score is determined by multiplying the intensity and the extent of staining to give a range of immunostaining scores from (0 -12) as (0,1,2,3,4,6,8,9,12), score over 3 is considered positive. mild= (3 to 5), moderate (6 to 8) & strong (9 to 12) [14].

OPN expression: **was evaluated according to its intensity & extent of staining;**

The intensity of staining was scored as

(0: negative, 1: weak, 2: moderate & 3: strong). Extent of positivity of the staining was scored as: (1 mild: (< 10%), 2 moderate: (10 – 50%), 3: strong (> 50-100%) & very strong 4: (=100%).

A scoring system was determined by multiplying the intensity score by extent score, yielding a range 0,1,2,3,4,6,8,9,12. Scoring of OPN was divided into (negative (0-2), mild (3-5), moderate (6-8) strong (9-12) [17].

Results:

The cases of CRC (42) have been examined for expression of CD44 & Osteopontin (OPN). Both markers have been correlated with clinicopathological & histopathological features of CRC (grading**,** vascular invasion, perineural invasion, desmoplasia and TNM stage).

The age of patient ranged from 26 to 80 years with mean 57±10.7 (60 ±11.3 years for male and 49.5 (± 9.5) years for females). The most common age group was those between 40 and 60 years, representing 51% of the study population. Regarding sex predilection, the predominant sex was male, representing 53.7%.

Eighty-one (81%) of the studied cases of CRCs were positive for CD44. Clinicopathological features of the studied cases are demonstrated in table 1. In the current study, positive CD44 expression was more expressed in tumors with positive LVI (95.8%) and their relation was statistically significant (p = 0.049) **(table 2).** Regarding TNM stage; In the current study we grouped the four stages of the TNM staging system into two groups (stages I/II & stages III/IV). The relation between CD44 expression and clustered TNM

stage (stages I/II and III/IV) was statistically significant ($p<0.05$), **(table 3),** significantly high CD44 expression in tumors with late stages (III/IV) compared with early stage (I/II).

No statistical relation was found between CD44 expression in CRCs & the histologic grade ($p=0.488$), perineural invasion ($p = 0.669$). and stromal desmoplasia ($p = 0.469$).

Table 1: The clinicopathological characteristics of the studied colorectal carcinoma specimens:

Features	Number of cases (42)	(%)
Grading		
low	39	93%
High	3	7%
Vascular invasion		
Positive LVI	27	64.3%
Negative LVI	15	35.7%
Perineural invasion		
Free	35	83.4%
Involved	7	16.6%
Desmoplasia		
Mild	2	4.7%
Moderate	25	59.5%
Sever	15	35.8%
T stage		
T1	8	19%
T2	21	50%
T3	8	19%
T4	5	12%
N stage		
N0	28	66.6%
N1	9	21.4%
N2	5	11.9%
Distant metastasis		
No	36	85.7%
Yes	6	14.3%
Tumor stage		
Stage 1	1	2.3%
Stage 2	24	57.3%
Stage 3	12	28.5%
Stage 4	5	11.9%

Table 2: The relation between CD44 expression and lymphovascular invasion by tumor cells of CRC cases. (N=42)

Lympho-vascular Invasion (LVI)	Number	CD44 expression		P-value
		Negative	Positive	
Negative	15	7 (41.6%)	11 (59.4%)	*0.049
Positive	27	1 (4.2%)	23 (95.8%)	

Fisher's exact test *significant p-value

Table 3: The relation between CD 44 expression with TNM stage of CRC patients. (N=42)

Clustered TNM stage			CD44 expression		P-value
			Negative	Positive	
	I/II	25 (59.5%)	6 (24%)	19 (76%)	*0.05
	III/IV	17 (40.5%)	2 (11.9%)	15 (88.1%)	

Fisher's exact test * Significant p-value

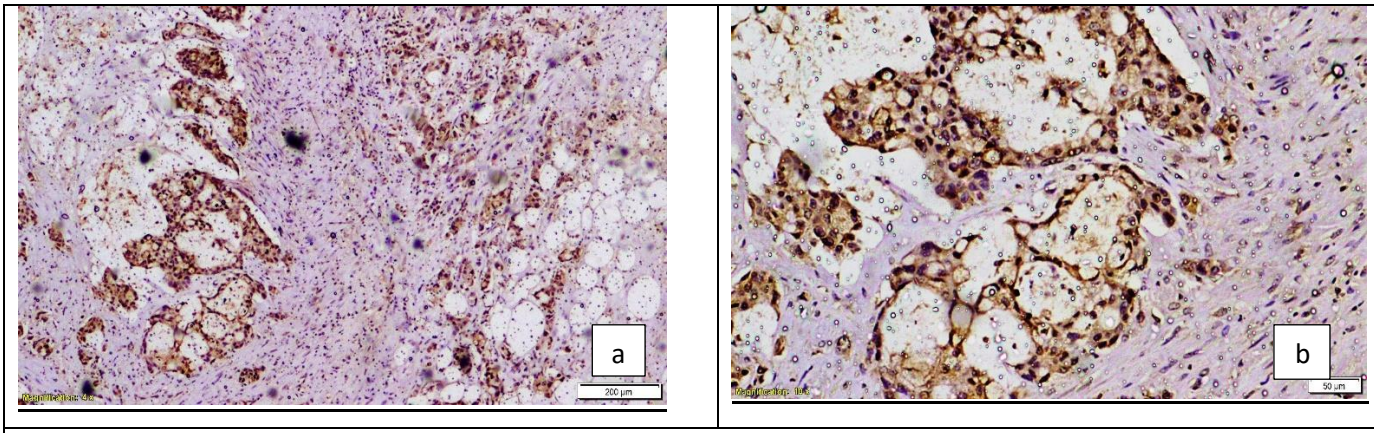

Figure 1: Moderately differentiated invasive adenocarcinoma, positive cytoplasmic & membranous expression of CD44 (score 2): (a) (DAB x100) l (b) The same tumor (DAB x200).

Regarding OPN expression, 31 cases (74%) were positive, and 11 cases (26%) were negative. **(Figure 2).**

In this study, there was 39 low grades including 71% had positive OPN staining and there are 100% of high-grade tumors were OPN positive (**figure 2),** unfortunately**,** the difference was statistically insignificant (p = 0.619). Eighty percent of tumors with LVI were positive for OPN. While 66.7% of tumor with no LVI were positive for OPN **(table 4).** However, the relation between OPN expression and LVI was statistically insignificant (P = 0.177). Regarding perineural invasion all specimens of positive perineural invasion (100%) had positive staining for OPN. While 68.7% of negative perineural invasion had positive OPN staining. The most common degree of desmoplasia in this study was the moderate one (25/42 cases) representing 60% of all study population. 19 cases (≈ 68%) of them having positive expression for OPN. While 2/42 cases showed mild degree of desmoplasia 100% of them were OPN positive. The relation between OPN and desmoplasia was statistically insignificance (p = 0.723). **Table (5)** showed that there was no statistical significance between low stage (1/2) and high stage tumors (3/4) regarding OPN expression (p = 0.591).

Table 4: The relation between OPN and lympho-vascular invasion parameter of CRCs. (N=42)

Lympho-vascular invasion (LVI)	Number	OPN expression		P-value
		Negative	Positive	
Negative	8	8 (33.3%)	19 (66.7%)	0.177
Positive	3	3 (20.0%)	12 (80.0%)	

Fisher's exact test

Table 5: The relation between OPN expression and TNM stage of CRC patients. (N=42)

Clustered TNM stage	OPN expression		P-value
	Negative	Positive	
I/II (N=25) (59.5%)	6 28.0%	19 72.0%	0.591
III/IV (N=17) (40.5%)	5 29.4	12 70.6	

chi square test

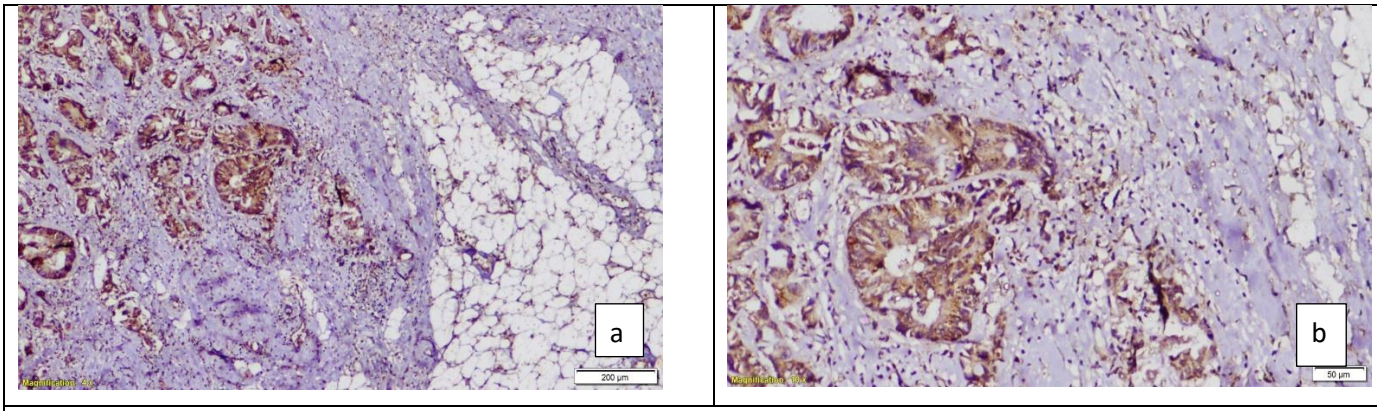

Figure 2: Moderately differentiated invasive adenocarcinoma, Positive cytoplasmic expression of OPN (score 2): (a) (DAB x40), (b) The same tumor (DAB x100).

Discussion:

Colorectal carcinoma is one of the major causes of morbidity and mortality rates in both developing and developed countries [18]. It is the third most common cancer in adult after lung cancer and breast cancer. Its incidence peak is at age 55-60 years [19]. In Egypt, CRC ranges the sixth, representing about 4% of total cancers in both genders [20]. The incidence rate of colorectal carcinomas is 5.1% in men and 4.7% in women [21].

A lot of cancer stem cells (CSC) markers have been considered in CRC. Among them, CD44 and OPN are two of the most colorectal carcinoma markers CD44+ /OPN+ cells are introduced as the cells with a greater tumor initiation potential and tumor colongenic ability [22].

Our study was conducted on 42 cases of CRC to correlate CD44 and OPN immunohistochemical expression with clinicopathological finding such as grade of tumor, lympho-vascular invasion, perineural invasion and TNM stage. Many earlier works studied the association between the clinicopathological characteristics of CRC and CD44 expression alone or in combination with OPN expression with controversy results. So, in this study we studied the relation between CD44 expression and OPN expression, as potential CSC markers with clinicopathological characteristics in CRC.

In our study, 80.95% of CRC had positive CD44 expression. This was in contrast with **Holah NS., et al (2017)** study that showed that 57.1% of CRC cases had a positive CD44 expression. Also, 51% had a positive CD44 expression reported by **Lugli A., et al (2015)** study. This different percentage might be attributed to different sample size in our and other studies (42 cases in this study and 250 cases in other study or may be due to

different types of colorectal carcinomas in other studies (eg: mucinous adenocarcinoma and signet ring adenocarcinoma) rather than adenocarcinoma type (NOS) used in this study [8] & [22].

Regarding lympho-vascular invasion, in this study there was a significant association between lympho-vascular invasion and expression of CD44 (p = 0.048). **Mohamed S., et al (2019)** also demonstrated a statistically significant relationship between lympho-vascular invasion and CD44 expression. This may be attributed to that tumors with high percentage of CSCs may have the capability to develop more tumor cell migration and motility. So subsequently, overexpression of the CSC markers is more expected in advanced cancers with positive LVI. However, these results were in contrast with others which showed that there was no statistically significant relation between CD44 expression and lympho-vascular invasion in CRC (p =0. 19) [26]. This may be due to the differences in number of cases [24]. According to our study we found a significant relation between CD44 and tumor staging (p = 0.05). There was an increase of CD44 expression in advanced stages than in early stages. Our findings were like **Mohamed SY., et al (2019)** who also found that there was significant relation between CD44 and the tumor stage. These results accentuate the results of many studies who also detect a significant association between CD44 scoring and stage III and IV [27] & [28]. However, in **Hong I., et al (2015)** study**,** there was no relation between CD44 expression and stage of the tumor. Also, one study showed that there was no significant relationship between CD44 and stage [8]. The controversial results of CD44 are common, it is mainly due to different isoforms of the protein and different population upon whom the study was concluded.

Osteopontin (OPN), is a phosphorylated sialic acid with non-collagenous bone matrix protein, belonging to integrin-binding ligand N-linked glycoprotein (SIBLING) family [29]. This study showed that there was no statistically significant relation between OPN expression and TNM stage of CRCs (p = 0.591. These results were similar to **Likui W., et al (2017)** who demonstrated non-significant statistical difference on estimating the IHC expression of OPN in different CRC stages (p = 0.111). The above-mentioned results were in contrast with **Mirzaei A., et al (2018)** study who showed that the expression level of OPN was significantly correlated with TNM stage (p = 0.0012). **Mirzaei A., et al**

(2018) observed high levels of OPN expression due to tumor progression and cell survival through Akt pathway activation. Moreover, OPN has been proved to regulate invasion, cell motility, and metastasis formation of tumor cells [(32)] and [(32).]

Conclusion:

We evaluated in this study the relation between expression of CD44 & Osteopontin (OPN) in adenocarcinomas (NOS) and their relationship with clinic-pathological prognostic criteria of the disease by using IHC technique. The CD44 protein was overexpressed in 80% of CRC. When we categorized the TNM stage into early and late stages, we found that there was statistical relation between positive CD44 expression and late stages (III &IV). Also, we found there was a statistical relation between CD44 expression and tumors with positive lymphovascular invasion. On the other hand, there was no relation between CD44 and Grade, perineural invasion. Also, in this study the difference between the expression OPN in CRC and adenomas was statistically insignificant. Moreover, there was no relation between OPN expression or score and the clinicopathological criteria of CRC (Grade, lymphovascular invasion, perineural invasion and TNM stage).

List of abbreviation:

CRC: Colorectal carcinoma.

CSC: Cancer stem cells.

DAB: Diaminobenzidine.

HRP: Horseraddish peroxidase.

IHC: Immunohistochemical.

LVI: Lymphovascular invasion.

OPN: Osteopontin.

SIBLING: Sialic acid–rich non-collagenous bone matrix protein, belonging to small integrin-binding ligand N-linked glycoprotein.

SPP-1: Secreted phosphoprotein 1.

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