RESEARCH ARTICLE

IMMUNOHISTOCHEMICAL EXPRESSION OF S100A4 AND CD24 IN COLORECTAL ADENOMA AND CARCINOMA

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Manuscript Info	*Abstract*
	Background:- Colorectal carcinoma (CRC) is a major cause of morbidity and mortality worldwide. Both calcium-binding protein, S100A4, and the cell adhesion molecule, CD24, have been implicated to play an important role in carcinogenesis and tumor progression in various human malignancies. **Aim:-** To evaluate the expressionof S100A4 and CD24 in colorectal tumors and their role in development and progression of CRC and correlate their expression with the clinicopathological features. **Methods:-** S100A4 and CD24 expression was analyzed by immunohistochemistry in paraffin-embedded specimens of colorectal adenomas (n=18) and CRC (n=40). **Results:-** S100A4 and CD24 expression was detected in 28/40 (70%) and 24/40 (60%) of CRC respectively.The positive expression rates of S100A4 and CD24 were significantly higher in CRC than adenomas (*P*<0.05). S100A4 was significantly expressed in tumors with high histological grades (*P*= 0.03).A statistically significant relationship was found between S100A4 and CD24 expression and advanced tumor stage (*P*= 0.009 and =0.03), lymph node metastasis (*P*=0.04 and =0.0001) and lymph-vascular space invasion (LVSI) (*P*=0.02 and =0.008) respectively, but not with the age, gender and tumor size (*P*>0.05). **Conclusion:-** S100A4 and CD24 up-regulation may be associated with the development and progression of CRC. Combined detection of S100A4 and CD24 may serve as an indicator of the aggressive behavior and poor prognosis of CRC.

Introduction
Colorectal carcinoma (CRC) is the third most common cancer and is one of the leading causes of death worldwide(1).In Egypt,according to the national cancer institute (NCI) registry, CRC contributes to 6.5% of all cancers(2).

The 5-year survival rate is approximately 85% after surgical resection in early stages of CRC. However, the rate is decreased (<50%) in stage III CRC with lymph node metastasis (3).It is well established that the most frequent cause of treatment failure and high mortality rates is distant metastasis (stage IV)(4).

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The development of CRC involved accumulated genetic alterations in cancer cells. It is of general importance to identify new molecular markers that can help early detection of tumors, improve the treatment strategies and adequate patient monitoring.To date, conventional prognostic markers for many tumors are the tumor grade, nodal status, tumor-size and tumor-type (5).

S100A4 is a calcium binding protein belonging to the members of S100 family of proteins and is directly involved in extracellular signal transduction, cell proliferation, intercellular adhesion and motility as well as tumorigenesis. S100A4 protein is up-regulated in malignant forms of gall bladder, pancreatic, breast, prostate, gastric, esophageal, lung and thyroid tumors (6).S100A4 directly interacts with cytoskeletal proteins actin and myosin and hence participate in cytoskeletal rearrangement, cell shape changes and motility. Furthermore, S100A4 protein can increase endothelial cell motility and induce angiogenesis (7).Recent researches reported that S100A4 is directly involved in cellular processes such as migration, invasion and angiogenesis that are essential for the development of metastasis (8).

CD24 is a small glycosylated cell surface protein, which is expressed in hematological malignancies and in various solid tumors eg: renal cell carcinoma, small cell carcinoma of the lung, hepatocellular carcinoma, nasopharyngeal carcinoma,glioma and breast cancer (9).CD24 is a ligand of P-selectin, an adhesion receptor on activated endothelial cells and platelets, and thus might enhance the metastatic potential of CD24-expressing tumor cells. It is also involved in cell-cell and cell-matrix interactions (10).

The aim of this study was to evaluate the expression of S100A4 and CD24 in colorectal tumors and correlate their expression with the clinicopathological features of CRC.

Materials and Methods
This retrospective study was conducted on 58 colorectal tumors: 18 adenomas and 40 CRC. The cases of CRC were obtained by surgical colectomy while adenomas were obtained by endoscopic biopsies. Paraffin-embedded **blocks were** obtained from Pathology Department, Zagazig University over theperiod between 2010 and 2014. Hematoxylin and eosin stained slides were reviewed to **confirm the** diagnosis. Tumors were graded according to WHO 2010 criteria(11) and staged according to the TNM staging system(12).

Immunohistochemical staining
Sections of 3-5µm thick were cut from formalin fixed, paraffin-embedded blocks. Tissue sections were deparaffinized in xylene, and rehydrated through graded alcohol. For antigen retrieval, the slides were placed in sodium citrate buffer (0.01 M, pH 6.0) in a microwave for 20 min. Then the slides were washed in phosphate buffered saline (pH 7.4). Endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide for 30 min. The sections were incubated with primary rabbit polyclonal antibody against S100A4 (RB-9411-PO,Labvision, Fremont, CA) at 1:200 dilution and mouse monoclonal anti-CD24 (Ab-2, clone SN3b, Labvision, Fremont, CA) at 1:100 dilution overnight at 4˚C. Sections were incubated with bio tiny lated secondary antibodies for 30 minutes. This was followed by incubation with streptavidin-biotin-peroxidase complex. The slides were rinsed with phosphate buffered saline and incubated with diaminobenzidine for 15 minutes. The slides were counterstained with hematoxylin, dehydrated, cleared and mounted. Sections of melanoma and breast carcinoma were used as positive control for S100A4 and CD24 respectively. For negative control, we substituted the primary antibody with phosphate buffered saline.

Interpretation of S100A4 and CD24 immunostaining
The pattern of CD24 expression was evaluated as membranous and/or cytoplasmic staining while cytoplasmic staining was considered positive for S100A4.S100A4 and CD24 expression was scored semiquantitatively according to the percentage of positive cells as follows: 0, negative, 1+, minimal (<10%) positive cells; 2+, moderate (10–50%) positive cells; and 3+, diffuse/marked (>50%) positive cells (13). For statistical purpose, only cases with scores 2 or 3 were considered positive cases.The staining intensity was scored as follows: negative, weak, moderate, and strong. Next,each marker was compared to the clinicopathological features.

Statistical analysis
Data were analyzed using SPSS version 20. Data were represented as frequencies and percentage for categorical variables.Statistical analysis was carried out using Chi-squared ($\chi2$) or Fisher exact test.P value of less than 0.05 was considered statistically significant.

Results
Clinicopathological features
In the studied cases of adenoma, the age ranged from 40-62 years with a mean of 51±19.3.Ten adenoma patients were females and eight were males.Of 18 adenomas, 8/18 (44.4%) cases displayed low-grade dysplasia and 10/18 (55.6%) cases displayed high-grade dysplasia. All adenomas demonstrated either tubular (6/18) or tubulovillous (12/18) architecture. All the studied cases of CRC were adenocarcinoma.The clinicopathological characteristics of CRC are represented in table (3).

Immunohistochemical staining results of S100A4
In the studied cases of adenoma, S100A4 was detected in 38.9% (7/18) cases (Table 1).70% of adenomas with high grade dysplasia showed positive S100A4 expression (table 2). In the studied cases of CRC, S100A4 was detected in 28/40 (70%) and that was statistically higher than adenomas (P=0.02).S100A4 immunostaining was predominantly seen in the cytoplasm of tumor cells. However, the adjacent non-neoplastic mucosa showed negative S100A4 staining (fig.1).S100A4 was expressed significantly more in CRC with high histological grade,GIII (P= 0.03).S100A4 expression showed a statistically significant positive association with advanced tumor stage (P=0.009), lymph node metastasis (P= 0.04) and LVSI (P=0.02). However, there was no significant association between S100A4 expression and the other investigated clinicopathological features (P>0.05) (Table 3).

Immunohistochemical staining results of CD24
In the studied cases of adenoma, CD24 was detected in 5/18(27.8%) cases (table 1). 40% of adenomas with high grade dysplasia showed positive CD24 expression (table 2).In the studied cases of CRC, CD24 was detected in 24/40 (60%) of CRC and that was statistically higher than adenomas (P=0.02).CD24 showed membranous and cytoplasmic staining (fig. 2). The adjacent non-neoplastic mucosa showed weak CD24 expression in only 4 cases of CRC.Statistical analysis demonstrated a significant positive association between CD24 positive expression rates with higher tumor stage (P=0.03),lymph node metastasis (P=0.0001) and LVSI (P=0.008).However, there was no association between CD24 expression and the other clinicopathological features examined (P>0.05) (Table 3).

Table 1:- Expression of S100A4 and CD24 in the studied cases of colorectal adenomas and carcinomas

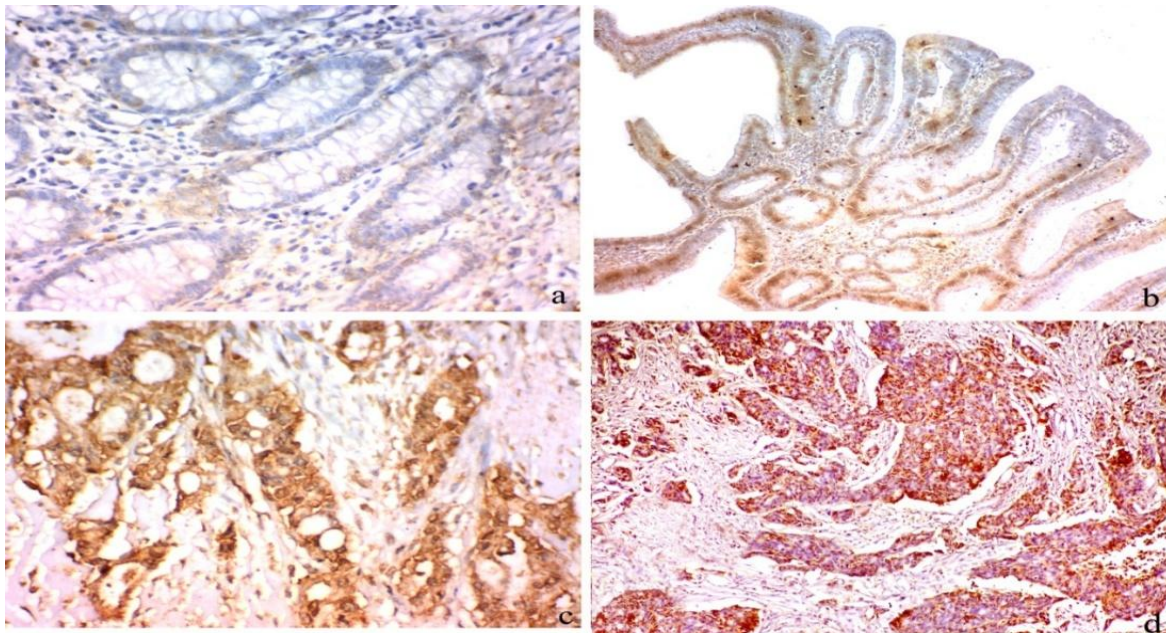|	S100A4 expression				CD24 expression		
	No.	Negative n (%)	Positive n (%)	P value	Negative n (%)	Positive n (%)	P value
Adenoma	18	11(61.1)	7(38.9)	0.02	13(72.2)	5(27.8)	0.02
CRC	40	12(30.0)	28(70.0)		16(40.0)	24(60.0)	

P value <0.05 is significant

Table 2:- Expression of S100A4 and CD24 in the studied cases of colorectal adenomas based on histotype and grades of dysplasia

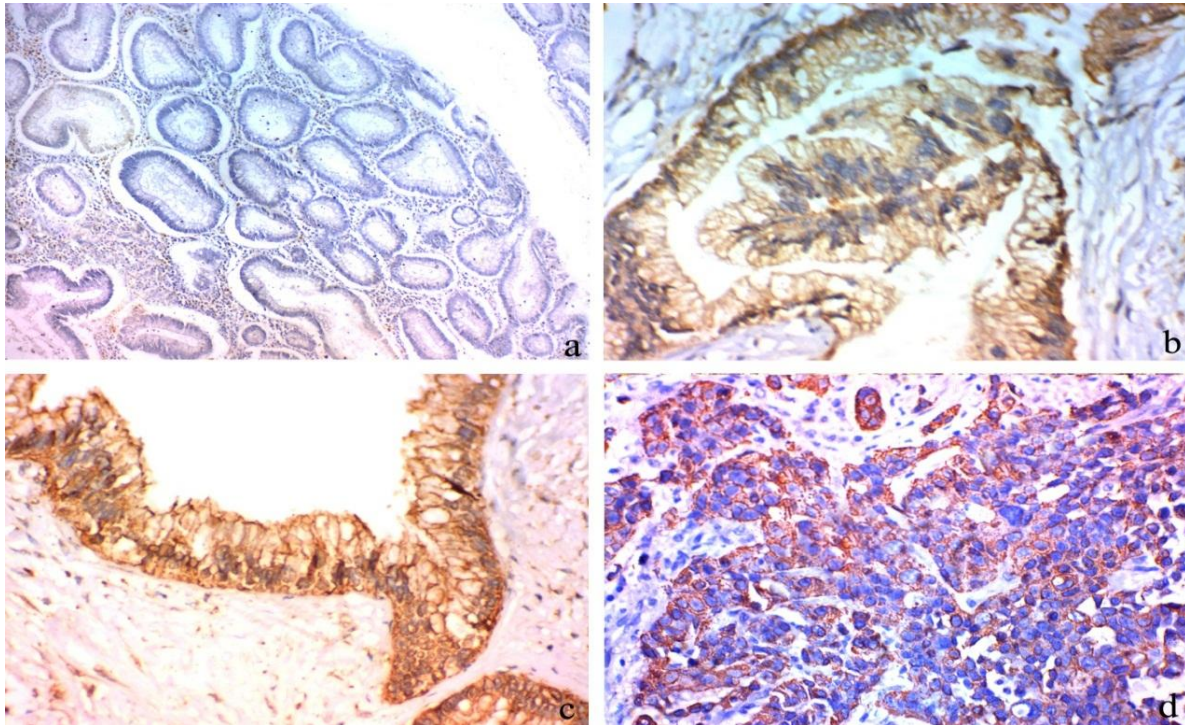|	No.	S100A4 expression		CD24 expression	
		Negative n(%)	Positive n(%)	Negative n(%)	Positive n (%)
Histotype					
Tubular	6	3(50.0)	3(50.0)	4(66.7)	2(33.3)
Tubulovillous	12	8(66.7)	4(33.3)	9(75.0)	3(25.0)
Grade of dysplasia					
Low grade	8	8(100)	0(0.0)	7(87.5)	1(12.5)
High grade	10	3(30.0)	7(70.0)	6(60.0)	4(40.0)
Total	18	11(61.1)	7(38.9)	13(72.2)	5(27.8)

Table 3:- The relation of S100A4 and CD24 expression with the clinicopathological characteristics in the examined CRC

features	n(%)	S100A4 expression			CD24 expression		
		Negative n(%)	Positive n(%)	P value	Negative n(%)	Positive n(%)	P value
Age							
≤50	19 (47.5)	8(42.1)	11(57.9)	0.1	8(42.1)	11(57.9)	0.7
>50	21 (52.5)	4(19.0)	17(81.0)		8(38.1)	13(61.9)	
Gendre							
Male	25 (62.5)	7(28.0)	18(72.0)	0.7	10(40.0)	15(60.0)	1.0
Female	15 (37.5)	5(33.3)	10(66.7)		6(40.0)	9(60.0)	
Tumor size							
≤5cm	14 (35.0)	6(42.8)	8(57.2)	0.2	7(50.0)	7(50.0)	0.3
>5cm	26 (65.0)	6(23.1)	20(76.9)		9(34.6)	17(65.4)	
Grade							
GI	15 (37.5)	8(53.3)	7(46.7)	0.03	7(46.7)	8(53.3)	0.09
GII	14 (35.0)	3(21.4)	11(78.6)		5(35.7)	9(64.3)	
GIII	11 (27.5)	1(9.1)	10(90.9)		4(36.4)	7(63.6)	
Pathological staging							
pT1	8 (20.0)	4(50.0)	4(50.0)	0.009	6(75.0)	2(25.0)	0.03
pT2	14 (35.0)	7(50.0)	7(50.0)		6 42.8)	8(57.2)	
pT3	18 (45.0)	1(5.5)	17(94.5)		4(22.2)	14(77.8)	
Nodal status							
Negative	17 (42.5)	8(47.1)	9(52.9)	0.04	14(82.3)	3(17.7)	0.0001
Positive	23 (57.5)	4(17.4)	19(82.6)		2(8.7)	21(91.3)	
LVSI							
Negative	13 (32.5)	7(53.8)	6(46.2)	0.02	9(69.2)	4(30.8)	0.008
Positive	27 (67.5)	5(18.5)	22(81.5)		7(25.9)	20(74.1)	

*P*value< 0.05is significant.

Figure 1: S100A4 immunostaining: **a**, Negative S100A4 expression in non-neoplastic colorectal mucosa (×100); **b,** S100A4 expression in tubulovillous adenoma showing high grade dysplasia (×100); **c,** Strong and diffuse staining of S100A4 in moderately differentiated **CRC (×400);d,** S100A4 expression in poorly differentiated CRC (×100).

Figure 2: CD24 immunostaining: **a,** Negative CD24 expression in tubular adenoma (×100); **b,** Well differentiated CRC showing weak membranous CD24 expression (×400);**c,**Well differentiated CRC showing strong membranous and cytoplasmic CD24 expression (×400)**;d,**Poorly differentiated CRC showing membranous and cytoplasmic CD24 expression (×400).

Discussion

To date, surgical resection remains the preferred treatment strategy for CRC patients.However, not all CRC patients derive clinical benefit from such a treatment (14).During the past few years, many molecular markers, such as TP53, KRAS, and BRAF have been investigated but still of doubted accuracy (15).Recently, There has been much interest in identifying new prognostic markers to guide the clinical management of CRC patients (16).

The calcium-binding protein S100A4, one of the members of the S100 protein family, has been shown to be implicated in tumorigenesis and associated with tumor progression and invasion and decreased patient survival(6).

In the present study, we observed that 70% of CRC showed positive S100A4 expression. However, the adjacent non-neoplastic mucosa showed negative S100A4 expression. These results are consistent with previous studies that reported S100A4 up-regulation in CRC (17,18). However, they reported different rates of S100A4 expression.Huang et al (17) and Niu et al (18) reported positive S100A4 expression in 51% and 54% of CRC respectively while kwak et al (19) reported S100A4 expression in 35.4%of their cases. Kim et al (20) investigated the expression of S100A4 in CRC using immunohistochemistry and RT-PCR.They observed increased expression of S100A4 mRNA in (82.6%) of CRC specimens and in (54.7%) of the studied cases by immunohistochemistry as compared with non-neoplastic mucosal tissue.

In our work, S100A4 was only observed in high grade adenomas.Baumhoer et al (13) reported positive S100A4 expression in 11% of the studied cases of adenomas. Zhao et al (7) reported S100A4 expression in 23.3% of the studied cases of gastric dysplasia.

As regard tumor grade, higher grades were associated with S100A4 expression. This finding was in contrast to that of Kwak et al (19).In the present study,There was a significant relationship between S100A4 expression and advanced tumor stage, lymph node metastasis and LVSI. These findings support the previous results ofLee et al (16), Huang et al (17)and Niu et al (18) and but not in line with Kwak et al (19). In agreement, Kim et al (20) also

reported that up- regulation of S100A4 was significantly related to the depth of invasion, nodal status and distant metastasis.

In the present research, no statistical association was detected between S100A4 expression and the patients' age, gender and tumor size. Similar findings were reported by Huang et al (17) and Niu et al (18).

The role of S100A4 in tumor progression and metastasis via induction of epithelial to mesenchymal transition (EMT) has been confirmed in many types of cancer including CRC (21).The positive relationship between S100A4 expression and aggressiveness was also observed in advanced-stage endometrial cancer (22) and bladder cancer (23). These findings suggest that S100A4 overexpression is involved in the growth, invasion, and metastasis of CRC and may serve as an indicator of the aggressiveness of CRC in the clinicopathological practice.

CD24 is agly cosylated cell surface protein molecule that has recently attracted considerable attention in tumor biology. CD24 functions in cell-cell and cell-matrix interaction and is supposed to play a critical role in cell adhesion and metastasis through P-selectin.CD24 has been identified as a cancer stem cell marker in several tumors such as breast, pancreatic, colorectal and liver cancer (24).Contradictory data have been reported in the literature concerning the prognostic value of CD24 in CRC.

In the present study,higher CD24 expression was detected in 60% in CRC.Other researchers reported different results. Weichert et al (25) and Choi et al (26) reported CD24 expression in 84% and 50.5% of their studied cases of CRC respectively.

Whereas some authors claim that normal colonic mucosa didn't express **CD 24(27).**Others were able to detect CD24 expression in normal mucosa (13, 28).In this work, the adjacent non-neoplastic mucosa displayed weak CD24 membranous staining in only four cases of CRC.

In the current work, CD24 expression was rarely observed in low-grade adenomas, however 40% of high-grade adenomas showed CD24 expression. Nearly similar observations were reported by Choi et al (26). Baumhoer et al (13) reported absent CD24 expression in dysplastic lesions. In contrast, Sagiv et al (28) reported that CD24 was highly expressed in both cases of adenoma and carcinoma.

In the present study,CD24 was predominantly membranous and cytoplasmicinCRC.The findings of Tanakaet al (24) and Weichertet al (25) were consistent with our results.Some researchers focused on the alteration of CD 24 immunolocalization from membranous to cytoplasmic.Choi et al (29) investigated a large sample of ovarian tumors and linked cytoplasmic CD24 immunoreactivity to microinvasion and that the shift from apical membranous staining in normal cells to the cytoplasmic CD24 localization in carcinoma reflects the transition of epithelial cells to a more invasive phenotype which is well-known as epithelial-mesenchymal transition of tumor cells.

The present results showed that enhanced CD24 expression in CRC was significantly correlated with aggressive clinicopathological features.A significant increase in CD24 expression was detected with higher tumor stage, nodal metastasis and LVSI.Weichert et al (25) reported that cytoplasmic CD24 immunoreactivity was significantly associated with higher tumor stages (Dukes and pT), nodal or systemic metastasis, tumor grade and poor survival.Choi et al (26) reported that CD24 cytoplasmic expression was positively correlated with higher tumor grade, advanced stage and large tumor size.Choi et al (27) observed a significant association between CD24 overexpression and tumor grade in CRC only, but they have not seen any positive correlation between CD24 expression and nodal status and patient survival.

In contrast, Sagiv et al (28) failed to find out any prognostic significance of CD24 expression level in CRC. In their study, they only reported membranous staining pattern of S100A4 in CRC. Moreover, they reported that CD24 was expressed early in the multistep process of CRC carcinogenesis, a finding consistent with its potential role as cancer stem cell marker. These results were in agreement with Ahmed et al (5).

However, Seo et al (10) reported no association between CD24 expression and the clinicopathological features, including T stage, N stage, or lymphatic invasion.

Hasan and Ilyas (30) reported the association between CD24 over expression in CRC and enhanced EMT phenotype, and enhanced invasive potential in CRC and that CD24 could be considered as a potential therapeutic target at early stages of CRC.

The potential role of CD24 in enhancing tumor invasion can be explained by increasing the ability of tumor cells to bind to platelets via P-selectin in the blood so that a stabilized platelets-tumor thrombi is formed and can protect tumor cells from destruction and can promote tumor extravasation and tissue penetration(29,31).

The heterogeneity in the results might be attributed to the different clones of the primary antibodies and the different methods of interpretation of immunostaining.

The prognostic role of S100A4 and CD24 in CRC is still controversial and it should be better elucidated by further studies on larger sample size to evaluate their role in CRC progression and for development of new therapeutic approaches.

Conclusion
Up-regulation of S100A4 and CD24 may play an important role in colorectal carcinogenesis and is positively related to growth, invasion and metastasis of CRC.

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