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Immunohistochemical Study of Galectin-3 Expression and Tumor Immune Response in Colorectal Tumors

[1] Azza Hassan Mohamed Zidan, [2] Nadia Mahmmoud Mokhtar, [3] Wessam Ismail Mostafa, [4] Mohammed Abd-Elrazek Nassef

[1] Professor of Pathology
Head of Pathology Department, Faculty of Medicine
Port-Said University - Egypt
[2] Professor of Pathology
National Cancer Institute and
Misr University for Science and Technology - Egypt
[3] Professor of Pathology
Head of Pathology Department, Faculty of Medicine
Beni - Suef University - Egypt
[4] Teaching Assistant of Pathology
Pathology Department, Faculty of Medicine
Misr University for Science and Technology – Egypt

Corresponding author: Mohammed Abd-Elrazek Nassef dr_moh_nassef@hotmail.com
+201007448448

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Abstract: Colorectal carcinoma is the third most commonly diagnosed malignancy and the fourth leading cause of cancer death in the world. The aim of present study is to evaluate Galectin-3 expression and tumor immune response as evaluated by CD8 expression in series of colorectal tumors obtained from Egyptian patients and correlate them with different clinicopathological variables. For this retrospective study, 50 formalin fixed paraffin embedded sample blocks of Colorectal carcinoma and 20 from normal colonic mucosa from patients subjected to colectomy in National Cancer Institute between 2011 and 2015 were enrolled. The 70 colorectal samples were stained with Hematoxylin and Eosin for routine histopathological evaluation and Immunohistochemical staining by Galectin-3 antibody and CD8 antibody. Current study showed significant association between Galectin-3 overexpression and colorectal carcinoma as it was expressed in 92% (46/50) of cases of colorectal carcinoma and in 25% (5/20) of colorectal non-tumorous lesions. Also there was statistically significant correlation between increased Galectin-3 expression and increasing tumor stage, lymphovascular invasion and metastasis. Also between increased CD8 immune cells density and low stage carcinomas. so Galectin-3 expression on tumor cells has considerable importance to predicting tumor invasiveness and CD8 immune cells density assess patient immune response against tumor cells.

Keywords: Immunohistochemical, Galectin-3, Tumor Immune Response, CD8, Colorectal Tumors. Carcinoma

Introduction

Colorectal cancer (CRC) is already the third leading cause of cancer death in the world, and its incidence is steadily rising in developing nations. [1]

Colorectal cancer (CRC) is the third most common cancer worldwide after lung and breast cancers with two-thirds of all colorectal cancers occurring in the more developed regions of the world [2].

It is predicted that about 2.4 million cases will be diagnosed annually worldwide by 2035 [3].

Within South Africa (SA), it is the fourth most commonly diagnosed cancer, being the second most common among males and the fourth most common among females and the sixth leading cause of cancer mortalities in SA [4].

Colorectal cancer ranks 7th among both genders, accounting for about 5% of all malignant tumors, according to the EgyptGlobal cancer observatory (Globocan) statistics, for exploring the cancer burden in Egypt, 2020 [5].

More than 90% of colorectal carcinomas are adenocarcinomas originating from epithelial cells of the colorectal mucosa. [6].

The mucinous colorectal cancer histological subtype of adenocarcinoma tends to represent approximately 10-15% of all colorectal carcinoma and is characterized by the predominant histological pattern of extracellular mucin [7].

Signet ring cell carcinoma (SRCC) is one of the rare histologic variants of colorectal carcinomas, occurring in less than 1% of all CRCs [8].

Multiple genes are involved in the biology of CRC, including different galectins. Galectins are glycan-binding proteins, whose importance is due to their ability to control the innate and adaptive immune system in health and disease conditions [9].

Gal-3 is the only “chimera-type” galectin, whose unique and intriguing structure is composed of a COOH-terminal domain containing one CRD linked to an extended N-terminal (NT) region [10].

Gal-3 is expressed in the normal colon in nuclear compartments, but significant changes in its expression and/or subcellular localization have been observed both in inflammatory bowel diseases and colorectal carcinoma [11].

Gal-3 overexpression increases the migration of colon cancer cells through the activation of the K-Ras–Raf–Erk1/2 pathway and that its interaction with extracellular carcinoembryonic antigen (CEA) promotes the migration of cancer cells and the appearance of distal metastases. [12].

When the cytotoxic T cells are combined with CD8 surface protein, produce the CD8+ T cells and play a vital role in antigen recognition, proved that the infiltration of tumors with CD8+ T-cells has a beneficial prognostic influence in CRC. Since then, a substantial number of studies have also examined the role of CD8+ T-cells, and nowadays, the scientific society has accepted its positive prognostic impact on colorectal carcinoma [13].

Grade is made according to the degree of tubule formation and cellular array in tumor tissue. 15-20% of patients grade I or well-differentiated, 60-70% of grade II or moderate differentiated, while 15-20% of them grade III or less differentiated [14].

Modified Dukes staging:

Stage A: Tumor limited to mucosa.

Stage B1: Tumor limited to the submucosa, no lymph node invasion.

Stage B2: Tumors confined to the muscle layer, no lymph node invasion.

Stage C1: The tumor did not exceed the bowel wall, lymph node metastasis.

Stage C2: Tumor exceeded the intestinal wall and lymph node metastasis. [15].

Material and methods

- We used formalin fixed paraffin embedded tissue samples from 50 patients with colorectal carcinoma and 20 from non-neoplastic colonic mucosa (normal or inflamed)
- For Gal-3:
    - The slides was incubated with monoclonal Gal-3 mouse antibody
    - Galectin-3 Cellular Localization: Cytoplasmic Positive
    - Tissue Control: Papillary thyroid carcinoma
- For CD8
    - For CD8 T cells their numbers and distribution in samples with IHS staining using monoclonal anti-human mouse IgG
    - The CD8 co reseptor Location: on the surface of cytotoxic T lymphocytes
    - Tissue Control: Tonsil as a +ve control
- Gal-3 expressions scoring:
    - Evaluating cell staining density with the following scores:
        - No coloration → 0 points.
        - Light brown → 1 point
        - Pale brown → 2 points
        - Brown → 3 points
    - Evaluating the quantity of stained cells in one visual field with the following scores:
        - Negative → 0 points
        - <10% → 1 point
        - 11% to 50% → 2 points
        - 51% to 75% → 3 points
        - >75% → 4 points
    - The final result is achieved by multiplying the scores from the two categories:
        - 0 to 3 points → negative expression (−):
        - 4 to 6 points → weakly positive expression (+):
        - 7 to 9 points → fairly strong positive expression (++):
        - 9 to 12 points → strong positive expression (+++).

- CD8 expression scoring
    - The CD8 positively stained T lymphocytes was evaluated under light microscope from three different areas of the tumor
    - They were assessed as following:
        - No/sporadic → Score 1
        - Moderate → Score 2
        - Abundant → Score 3
        - Highly abundant infiltration → Score 4
    - A total score was obtained for each tumor by adding together the three scores from each sub-site:
        - 3–4 → Low expression (-)
        - 5–6 → Moderate expression (+)
        - 7–12 → Abundant expression (++, +++) (as an extra step we divided it)
- Statistical Analysis
    - We used statistical package for social science (SPSS) for analyzing the data.
    - P value less than 0.05 was considered statistically significant.
    - Categorical data was described in terms of frequencies and percentages.
    - Numerical data was described in terms of mean and standard deviations
    - if normally distributed. Kolmogrov-semornov test was used to test the normality of distribution of numerical variables.
    - Chi square test was used to test the association between categorical variables.
    - Fissure exact test was used in case of violation of the assumptions.
    - Independent sample test was used to test the difference between 2 groups concerning numerical variables.

Results

- 90% of colorectal cancers was adenocarcinoma
    - 66% of adenocarcinomas was pure adenocarcinoma
    - 20% of adenocarcinomas had focal mucin activity
    - 4% of adenocarcinomas had focal neuroendocrine cells
- 6% colorectal cancers was Mucoid carcinoma
- 6% colorectal cancers was Signet ring carcinoma

Table 1; showing number of subtypes of colorectal carcinomas and normal mucosa (n=70).

Sample	NO	% from total	% from CRC	
Adenocarcinoma	33	47	66	90
Adenocarcinoma with focal mucin activity	10	14	20	
Adenocarcinoma with focal neuroendocrine	2	3	4	
Mucoid carcinoma	3	4	6	
Signet ring carcinoma	2	3	4	
Normal mucosa	20	29	----------	

Galectin-3 expression

- The relation between Galectin-3 expression and pathological types of colorectal carcinomas is significant in comparison to normal mucosa
    - We also found that patients with the Mucoid and Signet ring carcinoma achieved highest positive Galectin-3 expression 100%, followed by adenocarcinoma 93.9%, adenocarcinoma with focal mucin activity 90% and adenocarcinoma with focal neuroendocrine cells 50%. On the other hand, Normal mucosa achieved the lowest Galectin-3 expression 25%. This was statistically insignificant ($p<0.001$)

Table 2; showing the association difference between cases and normal mucosa concerning Galectin-3 expression (n=70).

Pathological types	Positive	Negative	P value
Adenocarcinoma	31 (93.9)	2 (6.1)	
Adenocarcinoma with focal mucin activity	9 (90)	1 (10)	
Adenocarcinoma with focal neuroendocrine	1 (50)	1 (50)	<0.001 *
Mucoid carcinoma	3 (100)	0	
Signet ring carcinoma	2 (100)	0	
Normal mucosa	5 (25)	15 (75)	
* : fissure exact test			

- Although we found an increase in scoring of Galactin-3 expression in colorectal carcinomas with high grades yet it was insignificant
    - Despite that the highest percentage of patients who tested positive for Galectin-3 were in the stage of G3 100% followed by G2 93.3% and G1 75%, this was statistically insignificant as shown in the table below ($p=0.154$)

Table 3; The relation between tumor grading and Galactin-3 expression.

Tumor Grade	No	Negative Galactin-3	Positive I Galactin-3	Positive II Galactin-3	Positive III Galactin-3	P value
G1	8	2 (25%)	3 (37.5%)	2 (25%)	1 (12.5%)	0.154
G2	30	2 (6.67%)	7 (23.3%)	10 (33.3%)	11(36.7%)	
G3	12	0	0	2 (16.67%)	10 (83.33%)	

- There is significant association between Galectin-3 expression and Duke staging
  - We also found a significant association between Duke staging and Galectin-3 expression (p=0.002). all patients with B1 stage showed negative Galectin-3 expression. On the other hand, most patients with B2 stage showed positive 1 galactin expression. However, most patients with C2 stage showed positive 3 Galectin-3 expression

Table 4; The relation between Dukes Stage and Galactin-3 expression.

Dukes Stage	No	Negative Galectin-3	Positive I Galactin-3	Positive II Galactin-3	Positive III Galactin-3	P value
A	0	0	0	0	0	0.002
B1	2	2 (100%)	0	0	0	
B2	17	2 (11.8%)	10 (58.8%)	5 (29.4%)	0	
C1	3	0	0	1 (33.3%)	2 (66.7%)	
C2	28	0	0	8 (28.6%)	20 (71.4%)	

- Higher tumor size in TMN staging had significantly elevated Galectin-3 expression

Table 6; The relation between tumor size and Galactin-3 expression.

TNM staging	Positive (n=46)	Negative (n=4)	P value
T2	3 (60)	2 (40)	0.037 *
T3	32 (94.1)	2 (5.9)	
T4a	9 (100)	0	
T4b	2 (100)	0	
* : fissure exact test			

- There is significant association between Galectin-3 expression and lymph node invasion
  - We also tested the association between LN invasion and Galectin-3 expression and found this was a significant one (p=0.037). we found most patients with no LN Mets showed positive 1 Galectin-3 expression. On the other hand, those with N2b stage showed positive 3 Galectin-3 expression as shown in the table below.

Table 7; The relation between Lymph node invasion and Galactin-3 expression.

Lymph node Invasion	No	Negative Galectin-3	Positive I Galectin-3	Positive II Galectin-3	Positive III Galectin-3	P value
N0	20	4 (20%)	10 (50%)	6 (30%)	0	0.037
N1	17	0	0	6 (35.29%)	11 (64.7%)	
N2 a	5	0	0	2 (40%)	3 (60%)	
N2 b	8	0	0	0	8 (100%)	

CD8 expression

- There is a statistically significant high CD8 expression in low grade tumors
  - We found statistically significant association between those experiencing CD8 expression and tumor grading (p=0.007). most patients with G1 tumor staging showed positive Galectin-3 expression. On the other hand, those with G3 staging showed negative CD8 expression

Table 8; The relation between tumor grading and CD8 expression.

Tumor Grade	No	Negative CD 8	Positive I CD 8	Positive II CD 8	Positive III CD 8	P value
G1	8	2 (25%)	2 (25%)	1 (12.5%)	3 (37.5%)	0.007
G2	30	7 (23.3)	11 (36.7%)	6 (20%)	6 (20%)	
G3	12	9 (75%)	2 (16.67%)	1 (8.33%)	0	

- There is a statistically significant high CD8 expression in tumors with low Duke staging
  - We also studied the relation between those experiencing CD8 expression and Duke staging and found that they were significantly associated (p=0.013). patients with B1 stage showed positive 1 CD8 expression. On the other hand, most of those with B2 stage showed positive III expression. Neither C1 stage patients nor C2 showed positive III CD8 expression as shown in the table below.

Table 9; The relation between Dukes Stage and CD8 expression.

Dukes Stage	No	Negative CD 8	Positive I CD 8	Positive II CD 8	Positive III CD 8	P value
A	0	0	0	0	0	0.013
B1	2	0	2 (100%)	0	0	
B2	17	2 (11.8%)	2 (11.8%)	4 (23.5%)	8 (53%)	
C1	3	1 (33.3%)	1 (33.3%)	1 (33.4%)	0	
C2	28	15 (53.6%)	10 (35.7%)	3 (10.7%)	0	

- There is a statistically significant high CD8 expression in tumors with low tumor sizes

Table 11; The relation between tumor size and CD8 expression.

TNM staging	Positive (n=32)	Negative (n=18)	P value
T2	4 (80)	1 (20)	0.144 *
T3	24 (70.6)	10 (29.4)	
T4a	4 (44.4)	5 (55.6)	
T4b	0	2 (100)	
* : fissure exact test			

- There is a statistically significant high CD8 expression in tumors with low Lymph node invasion
    - We also tested the association between LN invasion and CD8 expression and found this was a significant one (p=0.005). we found most patients with no LN Mets showed positive 3 CD8 expression. On the other hand, most of those with N2b stage showed negative CD8 expression

Table 12; The relation between Lymph node invasion and CD8 expression.

Lymph node Invasion	No	Negative CD 8	Positive I CD 8	Positive II CD 8	Positive III CD 8	P value
N0	20	2 (10%)	5 (25%)	4 (20%)	9 (45%)	0.005
N1	17	7 (41.18%)	8 (47.06%)	2 (11.76%)	0	
N2 a	5	3 (60%)	1 (20%)	1 (20%)	0	
N2 b	8	6 (75%)	1 (12.5%)	1 (12.5%)	0	

Discussion

- The relation between Galectin-3 expression and pathological types of colorectal carcinomas is significant in comparison to normal mucosa
    - On comparing between cases and normal mucosa, we found that Galectin-3 expression was significantly present among cases more than normal mucosa (92% of cases vs 25% of normal mucosa), p<0.001.
    - Liu T, et al (2017) [16] study, the positive rates of galectin-3 in cancer tissues and adjacent normal tissues were 62.5% (38/61) and 13.0% (3/23), respectively. Galectin-3 positive expression rate was significantly higher in cancer tissues than that in adjacent normal tissues (P<0.05)
    - Kazuya E et al (2005) [17] study, Galectin-3 was predominantly localized in the cytoplasm of cancer cells in 79 out of 121 patients (65%).
- Although we found an increase in scoring of Galactin-3 expression in colorectal carcinomas with high grades yet it was insignificant
    - Despite that the highest percentage of patients who tested positive for Galectin-3 were in the stage of G3 100% followed by G2 93.3% and G1 75%, this was statistically insignificant as shown in the table below (p=0.154)
    - Liu Tao et al (2017) [16] study, Correlation was found between the protein expression of galectin-3 and tumor differentiation (P<0.05)
    - Kazuya E et al (2005) [17] study, The clinicopathological factors for positive and negative galectin-3 expression groups were compared, and the positive group showed significantly in poor differentiation in histology (p=0.0037).
- There is significant association between Galectin-3 expression and Duke staging
    - Also, we found that all patients with C2 and C1 Duke staging were had positive Galectin-3 expression, followed by B2 stage (88.2%) and none of B1 stage had positive Galectin-3 expression as shown in the table below (p=0.002).
    - Liu Tao et al (2017) [16] study, Correlation was found between the protein expression of galectin-3 and Duke staging (P<0.05).
    - Kazuya E et al (2005) [17] study, Galectin-3 was predominantly localized in the cytoplasm of cancer cells. Tumor staging by Dukes' criteria was carried out. The proportion of patients classified as stage C and D in the positive group was

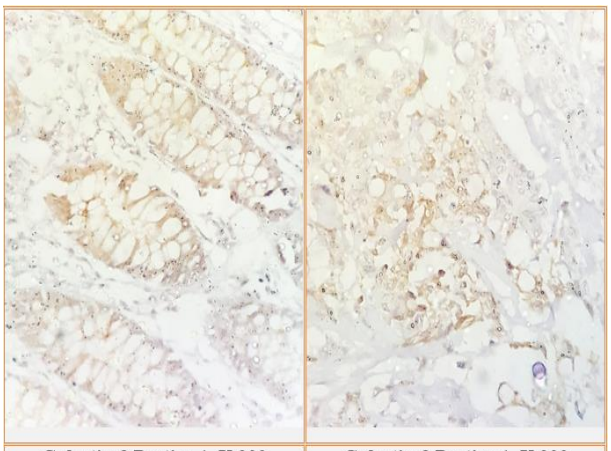
significantly higher than in the negative group (33/79 (42%) and 5/42 (12%), respectively), while the proportion of patients classified as stage A and B was significantly lower than in the negative group (46/79 (58%) and 37/42 (88%); p=0.0004).

- Higher tumor size in TMN staging had significantly elevated Galectin-3 expression
  - We found that patients with higher tumor size in TMN staging had significantly elevated Galectin-3 expression compared to others (p=0.037),
  - Liu Tao et al (2017) [16] study, Correlation was found between the protein expression of galectin-3 and the tumor size (P<0.05), tumor differentiation (P<0.05) and Duke staging (P<0.05). But it was not correlated with patient's gender(P>0.05), age (P>0.05), tumor location (P>0.05), and gross type (P>0.05),
  - Kazuya E et al (2005) [17] study, The clinicopathological factors for positive and negative galectin-3 expression groups were compared, and the positive group showed significantly larger tumor sizes (p=0.016),
- There is significant association between Galectin-3 expression and lymph node invasion
  - Also all patients with lymph-node invasion in TMN staging had positive Galectin-3 expression compared to patients with no lymph-node invasion were only 20% of them had positive Galectin-3 expression (p=0.286)
  - Kazuya E et al (2005) [17] study, Galectin-3 was predominantly localized in the cytoplasm of cancer cells. As for lymph node metastasis, the frequency was 30/79 (38%) in the positive group, significantly higher than 4/42 (9.5%) in the negative group (p=0.0007). also distant metastases, including liver and lung metastasis, were recognized in 10 patients, all of them belonging to the positive group
- There is a statistically significant high CD8 expression in low grade tumors
  - But in Jihong L et al (2005) [18] study, there was no significant correlation between the expression of both and age, gender, histological grade, histological type, and MMR proteins expression (p > 0.05).
- There is a statistically significant high CD8 expression in tumors with low tumor sizes

- There is a statistically significant high CD8 expression in tumors with low Lymph node invasion
    - We also tested the association between LN invasion and CD8 expression and found this was a significant one (p=0.005). we found most patients with no LN Mets showed positive 3 CD8 expression. On the other hand, most of those with N2b stage showed negative CD8 expression
    - Jihong L et al (2005) [18] study, The expressions of CD3 and CD8 were significantly related to lymph node metastasis and the III-IV TNM stage ($p < 0.05$), and the expression of CD8 in the right colon is higher than that in the left colon ($p < 0.05$)

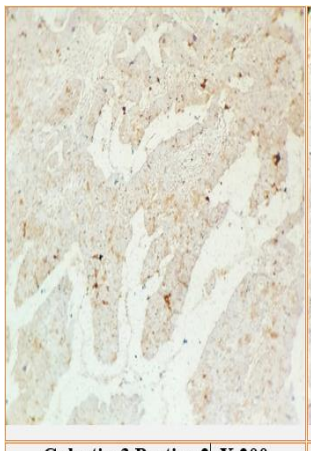
Conclusions

- The relation between Galectin-3 expression and pathological types of colorectal carcinomas is significant in comparison to normal mucosa
- Although we found an increase in scoring of Galactin-3 expression in colorectal carcinomas with high grades yet it was insignificant
- There is significant association between Galectin-3 expression and Duke staging
- Higher tumor size in TMN staging had significantly elevated Galectin-3 expression
- There is significant association between Galectin-3 expression and lymph node invasion
- There is a statistically significant high CD8 expression in low grade tumors
- There is a statistically significant high CD8 expression in tumors with low tumor sizes
- There is a statistically significant high CD8 expression in tumors with low Lymph node invasion



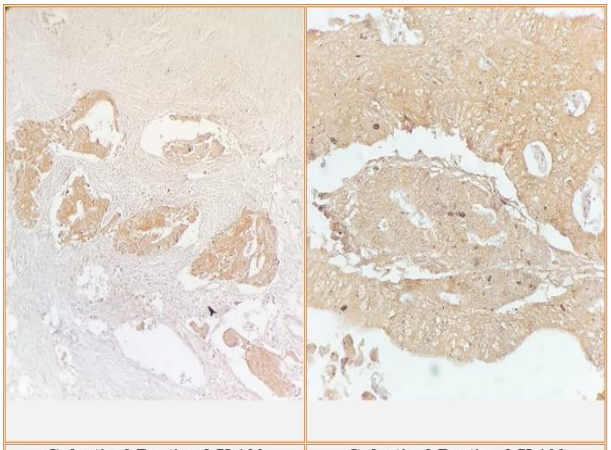
Galectin-3 Postive 1 X 200
Normal Tissues

Galectin-3 Postive 1 X 200
Adenocarcinoma (G1)



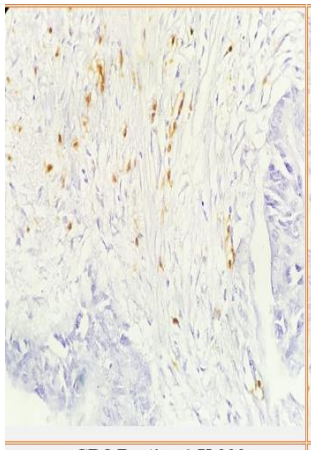
Galectin-3 Postive 2 X 200
Adenocarcinoma (G2)

Galectin-3 Postive 2 X 200
Adenocarcinoma (G2)

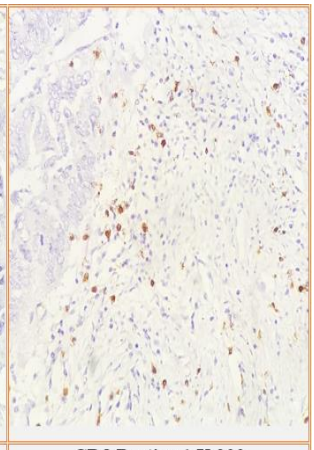


Galectin-3 Postive 3 X 100
Adenocarcinoma (G2)

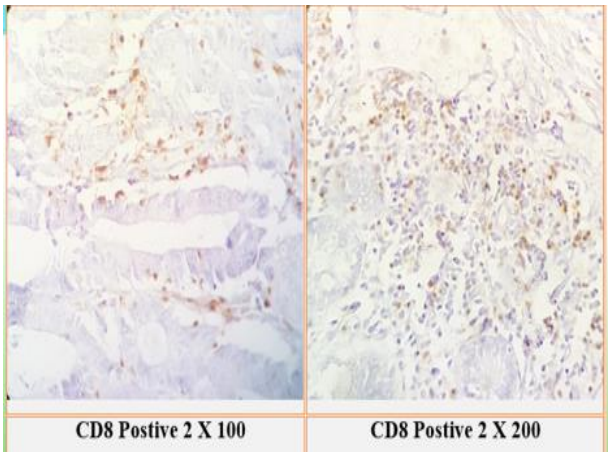
Galectin-3 Postive 3 X 100
Adenocarcinoma (G3)



CD8 Postive 1 X 200
Adenocarcinoma (G3)

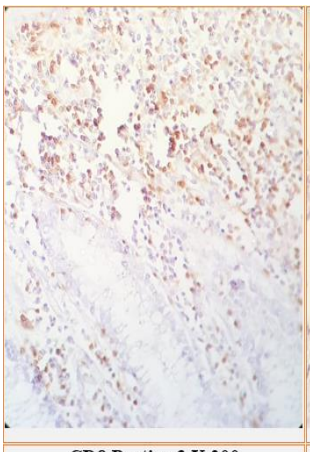


CD8 Postive 1 X 200
Signet Ring Carcinoma

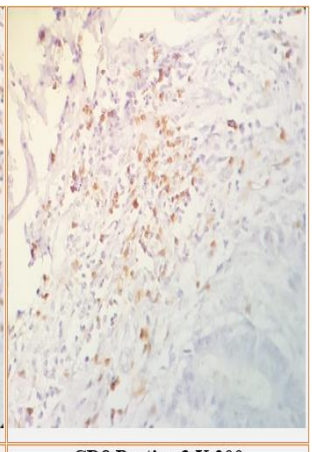


CD8 Postive 2 X 100
Adenocarcinoma with focal mucin activity

CD8 Postive 2 X 200
Adenocarcinoma (G2)



CD8 Postive 3 X 200
Adenocarcinoma (G1)



CD8 Postive 3 X 200
Adenocarcinoma (G2)

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