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Evaluating The Role of Transmembrane 9 Superfamily 4 (TM9SF4) and CDX2 Expression in Preneoplastic Colonic Lesions and Colorectal Carcinoma: (An Immunohistochemical Study)

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ABSTRACT

Background: Colorectal carcinoma (CRC) is one of the most commonly diagnosed cancers and a leading cause of cancer-related mortality worldwide. Transmembrane 9 Superfamily Member 4 (TM9SF4) and caudal-type homeobox transcription factor 2 (CDX2) have been implicated in colorectal tumorigenesis.

Aim: To evaluate the immunohistochemical expression of TM9SF4 and CDX2 in preneoplastic colonic lesions and CRC, and to assess their diagnostic and prognostic significance.

Materials and methods: This retrospective study included 23 CRC cases, 16 adenomas, 10 ulcerative colitis (UC) cases, and 6 normal controls. Formalin-fixed, paraffin-embedded tissue blocks were stained immunohistochemically with TM9SF4 and CDX2 antibodies. Expression was semi-quantitatively scored, and associations with clinicopathological features were analyzed using ROC curve analysis, Spearman correlation, and Monte Carlo tests.

Results: TM9SF4 showed high expression in 95.7% of CRC cases. It distinguished CRC from controls with an AUC of 0.993, 95.7% sensitivity, and 100% specificity. CDX2 achieved perfect discrimination (AUC: 1.0) between CRC and controls. Against UC and adenoma, both markers retained good sensitivity but showed reduced specificity. High TM9SF4 expression significantly correlated with tumor grade, invasion depth, stage, and lymphovascular invasion ($P < 0.05$). CDX2 loss was significantly associated with high tumor grade, lymph node and distant metastases, and lymphovascular invasion ($P < 0.05$). A strong inverse correlation between TM9SF4 and CDX2 was observed in CRC and UC cases.

Conclusion: TM9SF4 is a promising marker for CRC aggressiveness, while CDX2 remains a reliable marker of colonic differentiation. Their inverse expression highlights their potential complementary role in CRC evaluation.

Keywords: Colorectal carcinoma; CDX2; TM9SF4; Immunohistochemistry.

INTRODUCTION

Colorectal carcinoma (CRC) is major cause of cancer–related deaths worldwide and is currently the third most common cancer among women and men in the United States [1]. In Egypt, CRC is the seventh most common cancer and the third most common male neoplasm and fifth most common female neoplasm [2].

Colorectal carcinoma is a heterogenous disease. There are several molecular pathways for its development include, adenoma-carcinoma sequence (traditional pathway), serrated pathway, alternative pathway and de novo pathway. CRC and their pathological precursors display distinct molecular signature and pathological features [3].

Transmembrane 9 superfamily 4(TM9SF4) is a transmembrane protein characterized by the presence of large variable extracellular domain and nine putative transmembrane domains [4]. CDX2 is a caudal type homeobox transcription factor involved in the proliferation and differentiation of intestinal epithelium. CDX2 is known as a specific diagnostic marker for CRC but its prognostic role remains unclear [5].

Identification of novel biomarkers for early detection and monitoring of CRC is of paramount importance. Our study was done to evaluate immunohistochemical expression of TM9SF4 and CDX2 across a range of colorectal tissues—including normal colonic mucosa, adenomas, ulcerative colitis (UC), and colorectal cancer (CRC) to assess their diagnostic and prognostic value and compare their performance.

MATERIALS AND METHOD

This retrospective study was done upon selected cases of different colorectal lesions. The cases were designed as 23 colorectal carcinoma cases (all were colectomy cases) 16 cases colonic adenomatous polyps (10 tubular, 4 villous and 2 tubulovillous) and 10 ulcerative colitis cases (8 cases were active). Six cases of normal colonic mucosa taken from viable edges of resected gangrenous colon were also included in this study. The materials of the study were archival formalin-fixed paraffin embedded blocks collected from Department of Pathology and Early Cancer Detection Unit, Faculty of Medicine, Benha University, Egypt from the years 2018 to 2024. The clinicopathological data were collected from the files of the patients.

Inclusion criteria were cases with available clinicopathological data. The cases with no available clinicopathological data, no available blocks or those who received chemotherapy were excluded.

Histopathological study:

From each formalin-fixed paraffin embedded block, 5-micron thickness sections were cut on ordinary slide and stained using hematoxylin and eosin stain. The cases were reviewed by two different pathologists. The

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colorectal carcinoma cases were graded into well differentiated (Grade I), moderately differentiated (Grade II) and grade (III) according to WHO classification [6]. TNM staging system was applied according to AJCC 8th edition [7].

Immunohistochemical study:

Two 4 microns thickness tissue sections were cut from each formalin –fixed paraffin –embedded blocks on a positively charged slides to be immunostained using TM9SF4 rabbit polyclonal antibody and CDX2. The immunostaining was done according to manufacture instructions. A standard labeled streptavidin –biotin system was used for immunodetection (Genemed, CA 94080, South San Francisco, USA). The sections were visualized with freshly prepared 0.02% diaminobenzidine (DAB) solution and finally counterstained with Mayer's hematoxylin then dehydrated and mounted. Gastric tissue and reactive lymphoid tissue were used as a positive external control for TM9SF4 and CDX2 respectively. Negative control was performed by omitting the primary antibody and replacing it with phosphate –buffered saline (PBS) (Table 2).

Table (1): Antibodies used in the study

Antibody	Source	Cat. No.	Dilution	Incubation period
TM9SF4	**Hansa BioMed OU, Tallin Estonia**	A15704	1:50	Overnight
CDX2	**Genetex, USA**	GTX31231	1:200	20 minutes

Immunohistochemical interpretation of TM9SF4:

Positivity was considered as brownish homogenous cytoplasmic staining. The immunohistochemical scores (scored from 0-3) were obtained by light microscopy as the score of the staining intensity multiplied by the score of the percentage area of positive immunostaining within the visual field (the percentage of positive cells within 5 high power fields in hot areas). The intensity of TM9SF4 protein expression was scored as: 0 (no staining); 1 (weak staining); 2 (moderate staining); or 3 (strong staining). The percentage area of positive immunostaining was scored as: 0 (0%); 1 (1-10%); 2 (11-50%); 3 (>51%) [4].

Immunohistochemical interpretation of CDX2:

Positivity was considered as brownish nuclear staining in more than 5% of the stained cells. The immunohistochemical scores were assessed by light microscope (scored from 0 to 3) by multiplication the staining intensity score by the staining extent score. Extent of positivity was scored 0 (less than 5 % positivity), score 1 (5-25% positivity), score 2 (26-75% positivity), score 3 (more than 75% positivity). The intensity of staining was scored 0 (absent or negative staining), score 1 (weak intensity), score 2 (moderate intensity), score 3 (strong intensity) [8].

Ethical considerations:

The collection of the blocks and clinicopathological information was ethically approved from the Ethics Committee of Faculty of Medicine, Benha University (RC 4-12-2024). The Helsinki Declaration was followed throughout the study's conduct.

Statistical analysis and data interpretation

Data analysis was performed by SPSS software, version 26 (SPSS Inc., PASW statistics for windows version 26. Chicago: SPSS Inc.). Qualitative data were described using number and percent. Quantitative data were described using mean±standard deviation. Significance of the obtained results was judged at the (0.05) level. Monte Carlo test was used to compare qualitative data between groups as appropriate. The Spearman's rank-order correlation was used to determine the strength and direction of a linear relationship between two non-normally distributed continuous variables and / or ordinal variables. Receiver operating characteristics curve (ROC curve) was used to calculate validity (sensitivity and specificity) of continuous variables with calculation of best cut off point. Predictive values and accuracy were assessed using cross tabulation.

RESULTS

A-Demographic characters of studied groups:

The demographic analysis showed that the colorectal carcinoma group was the oldest ranged from 38 to 72 years with mean±SD (55.30 ± 11.09 years), followed by the adenoma group (43.56 ± 9.71 years), while the ulcerative colitis and control groups were younger; ranged from 27 to 64 years with mean±SD (31.30 ± 4.25 years and 33.17 ± 10.07 years, respectively). Regarding sex (49%) of cases were males.

B-Immunohistochemical results:

1-Comparison between TM9Sf4 and CDX2 immunohistochemical expression in studied groups:

Immunohistochemical analysis of TM9SF4 and CDX2 expression revealed significant differences across the groups. For TM9SF4, 95.6% of colorectal carcinoma cases showed high expression, with 65.2% (15/23) scoring 3. In contrast, ulcerative colitis cases showed a more balanced distribution, with 40% (4/10) scoring 0 and no cases scoring 3. The adenoma group had 37.4% (6/16) with score 2, while 66.7% of control cases showed score 0.

For CDX2, 43.5% (10/23) of colorectal carcinoma cases scored 0, and no cases showed score 3. Conversely, 70% (7/10) of ulcerative colitis cases exhibited score 3. The adenoma and normal colon groups displayed more varied distributions, with 43.8% (7/16) of adenoma cases scoring 2, while all control samples scored 3 (Table 2 and Figures 1, 2).

Table 2: Comparison between TM9Sf4 and CDX2 immunohistochemical expression among studied groups:

	Colorectal carcinoma N=23	Ulcerative colitis cases N=10	Adenoma N=16	Control N=5	Test of significance
TM9Sf4 score:					
Score 0	**0**	**4(40) A**	**4(25)**	**4(66.7) A**	**MC=32.18**
Score 1	**1(4.3)**	**3(30)**	**3(18.8)**	**2(33.3)**	**P<0.001***
Score2	**7(30.4)**	**3(30)**	**6(37.4)**	**0**	
Score3	**15(65.3)**	**0**	**3(18.8)**	**0**	
CDX2 score:					
Score 0	**10(43.5)**	**0 A**	**0**	**0 A**	**MC=40.8**
Score 1	**6(26.1)**	**0**	**4(25)**	**0**	**P<0.001***
Score 2	**7(30.4)**	**3(30)**	**7(43.8)**	**0**	
Score 3	**0**	**7(70)**	**5(31.2)**	**6(100)**	

CDX2, Caudal Type Homeobox 2; TM9Sf4, Transmembrane 9 Superfamily Member 4; MC: Monte Carlo test; *Statistically significant, Data expressed as number (%). A: Similar superscripted letters in same row denote non-significant difference between studied groups (no significant relation between normal colon and ulcerative colitis cases).

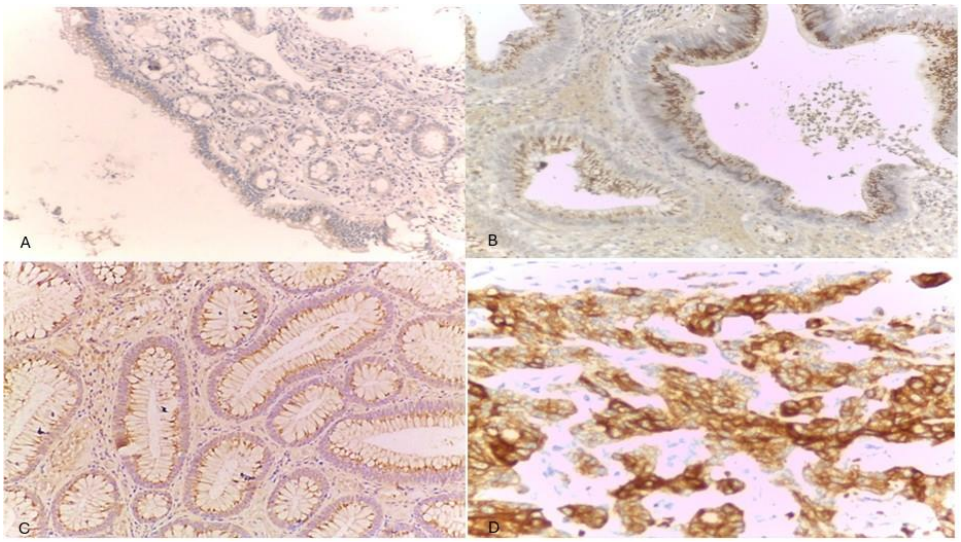

Figure 1: TM9Sf4 IHC: **A)** Negative expression in normal colonic mucosa (ABC x200), **B)** Score 1 cytoplasmic expression in ulcerative colitis (ABC x400), **C)** Score 2 cytoplasmic expression in well differentiated CRC, **D)** Score 3 in poorly differentiated CRC (ABCx400)

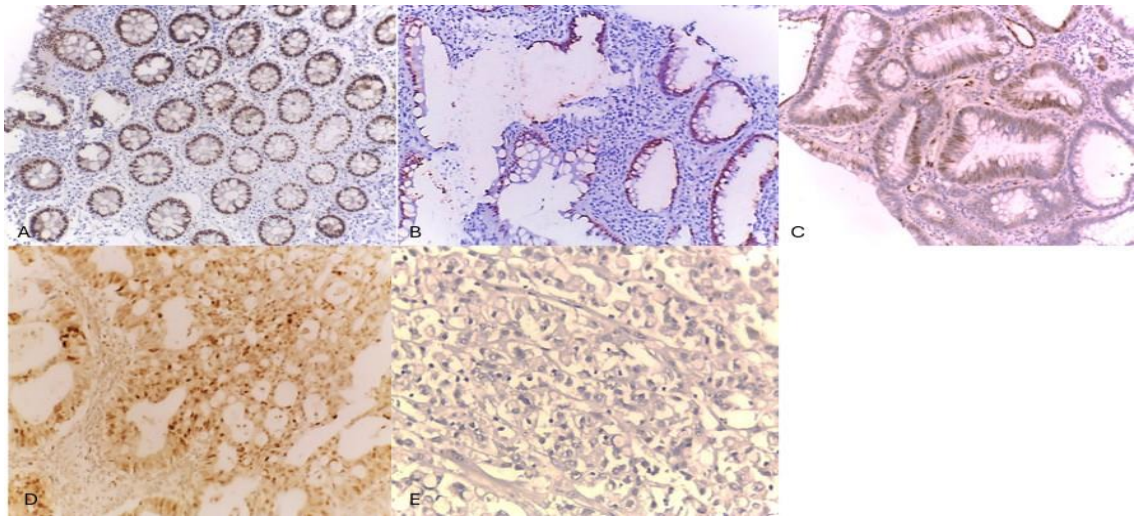

Figure 2: CDX2 immunohistochemistry: A) Score 3 nuclear expression in normal colon mucosa (ABC x200), B) Score 2 nuclear expression in UC (ABC x200), C) Score 1 nuclear expression in colonic adenoma; D) Score 2 nuclear expression in well differentiated carcinoma (ABCx200) E) negative expression in poorly differentiated carcinoma (ABC x 200).

2- Validity of TM9SF4 and CDX2 in differentiating between studied groups:

ROC curve analysis comparing TM9SF4 and CDX2 revealed key insights into their diagnostic

accuracy for distinguishing colorectal carcinoma, adenoma, ulcerative colitis (UC), and control group. For TM9SF4, the marker demonstrated excellent diagnostic performance in distinguishing colorectal carcinoma from control group, with an area under the curve (AUC) of 0.993, sensitivity of 95.7%, and perfect specificity (100%) resulting in an overall accuracy of 96.6%. In the cancer versus ulcerative colitis (UC) comparison, the AUC remains high at 0.935, with maintained sensitivity (95.7%) but reduced specificity (70%), yielding an accuracy of 87.9%. However, in differentiating cancer from adenoma, the AUC decreases to 0.796, with specificity dropping to 43.7%, although sensitivity remains consistent at 95.7%, leading to an accuracy of 87.9% (Table 3 and Figure 3).

Similarly, CDX2 demonstrated perfect diagnostic accuracy for distinguishing cancer from control group, with an AUC of 1.0 (95% CI: 1.0-1.0), achieving both 100% sensitivity and 100% specificity. When comparing cancer to ulcerative colitis (UC), the AUC is slightly reduced to 0.954 (95% CI: 0.888-1.0), with sensitivity of 69.6% and perfect specificity (100%). This results in an accuracy of 90.9%. In the cancer versus adenoma comparison, the AUC decreases further to 0.825 (95% CI: 0.698-0.951), with sensitivity remaining at 69.6% but specificity dropping to 75%. This yields an accuracy of 77.8%. (Table 4 and Figure 4).

Table (3): Validity of TM9SF4 in differentiating between studied groups

TM9SF4	AUC (95%CI)	P value	Cut off point	Sensitivity	Specificity	PPV	NPV	Accuracy
Cancer versus control group	**0.993 (0.970-1.0)**	**<0.001 ***	**≥2**	**95.7%**	**100.0%**	**100**	**85.7**	**96.6**
Cancer versus UC	**0.935 (0.853-1.0)**	**<0.001 ***	**≥2**	**95.7%**	**70.0%**	**88**	**87.5**	**87.9**
Cancer versus adenoma	**0.796 (0.647-0.945)**	**0.002***	**≥2**	**95.7%**	**43.7%**	**71**	**87.5**	**87.9**

AUC: Area under curve; **PPV:** Positive predictive value; **NPV:** Negative predictive value

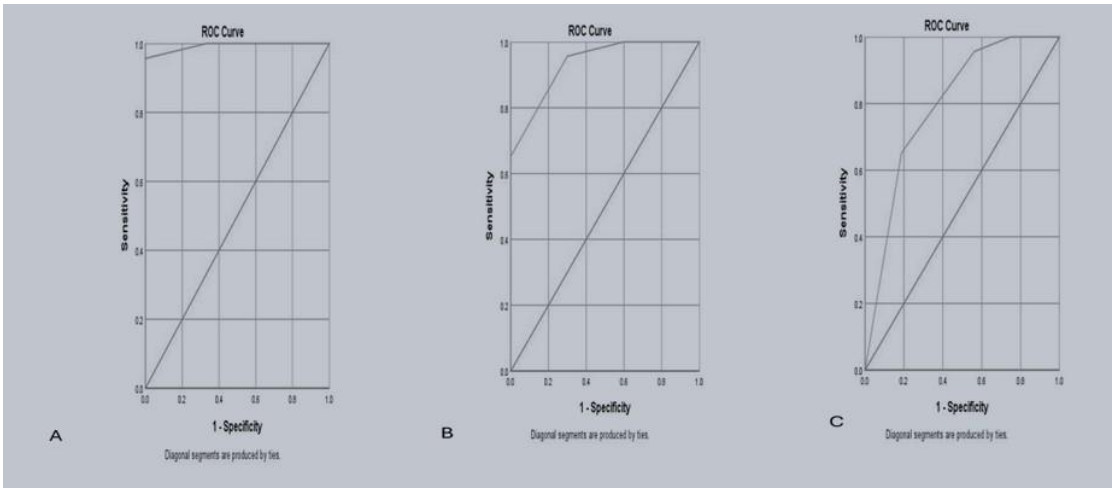

Figure (3): A) ROC curve of TM9SF4 in differentiating between cancer versus control; B) ROC curve of TM9SF4 in differentiating between cancer versus UC group; C) ROC curve of TM9SF4 in differentiating between cancer versus adenoma group.

Table (4): Validity of CDX2 in differentiating between studied groups:

CDX2	AUC (95%CI)	P value	Cutoff point	Sensitivity	Specificity	PPV	NPV	Accuracy
Cancer versus normal	1.0 (1.0-1.0)	<0.001*	≤3	100.0%	100.0%	100.0%	100.0%	100.0%
Cancer versus UC	0.954 (0.888-1.0)	<0.001*	≤2	69.6%	100.0%	100	100	90.9
Cancer versus adenoma	0.825 (0.698-0.951)	0.001*	≤2	69.6%	75.0%	80.0	63.2	77.8

AUC: Area under curve; **PPV**: Positive predictive value; **NPV**: Negative predictive value

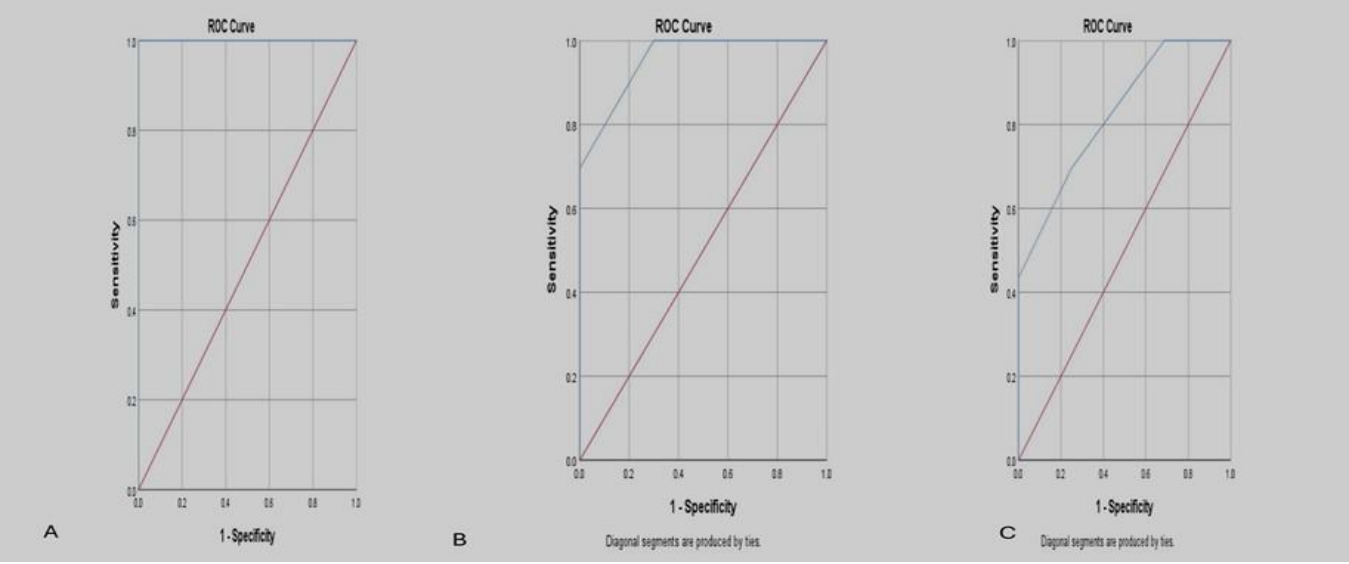

Figure (4): A): ROC curve of CDX2 in differentiating between cancer versus control group; **B)** ROC curve of CDX2 in differentiating between Cancer versus UC group; **C):** ROC curve of CDX2 in differentiating between cancer versus adenoma group.

3-Relation between TM9SF4 and CDX2 and histological criteria among adenoma cases:

As regards the association between TM9SF4 expression and histological features in adenoma cases, higher levels of TM9SF4 expression (scores 2 and 3) were more frequently observed in adenomas with higher-grade dysplasia and in the villous type. Specifically, 60% of high-grade dysplasia cases exhibited high TM9SF4 expression (score 2), and 100% of villous adenomas displayed elevated expression (scores 2 and 3). However, statistical analysis revealed no significant correlation between TM9SF4 expression and dysplasia grade or adenoma type, indicating that while a trend was observed, the relationship did not reach statistical significance (Table 5).

In contrast, the data showed a significant association between CDX2 expression and adenoma type. Specifically, a higher proportion of villous adenomas (75%) (3/4) exhibited score 1 for CDX2 expression, while none of the villous adenomas exhibited score 3. Regarding dysplasia grade, a trend toward higher CDX2 expression was observed in low-grade dysplasia, with 45.5% (5/11) of low-grade cases showing score 3. However, high-grade dysplasia cases exhibited more varied scores, and this trend did not reach statistical significance (Table 5).

Table (5): Relation between TM9SF4 and histological criteria among adenoma cases:

	Total number	TM9SF4				Test of significance	CDX2			Test of significance
		Score 0	Score 1	Score 2	Score 3		Score1	Score2	Score3	
Type of adenoma: **Tubular adenoma** **Tubulovillous adenoma** **Villous adenoma**	**10(62.5)** **2(12.5)** **4(25)**	**4(40)** **0** **0**	**3(30)** **0** **0**	**2(20)** **1(50)** **3(75)**	**1(10)** **1(50)** **1(25)**	**MC=8.13** **P=0.228**	**1(10)** **0** **3(75)**	**4(40)** **2(100)** **1(25)**	**5(50)** **0** **0**	**MC=10.2** **P=0.037***
Grade of dysplasia: **Low grade** **High grade**	**11(68.8)** **5(31.2)**	**4(36.4)** **0**	**3(27.3)** **0**	**3(27.3)** **3(60)**	**1(9.1)** **2(40)**	**MC=5.92** **P=0.116**	**1(9.1)** **3(60)**	**5(45.5)** **2(40)**	**5(45.5)** **0**	**MC=5.86** **P=0.053**

MC: Monte Carlo test, *Statistically significant, Data expressed as number (%).

4-Relation between TM9SF4 and CDX2 IHC expression and histopathological features among CRC cases:

In terms of the association between TM9SF4 expression and histopathological features in CRC cases, a significant statistical difference was observed between high TM9SF4 expression and various tumor characteristics, including depth of tumor invasion (T) and lymphovascular invasion, tumor stage and grade. However, there was no significant statistical difference between TM9SF4 expression and lymph node metastasis or distant metastasis. Regarding CDX2 expression and its association with histopathological features in (CRC) cases, this study identified an inverse relationship between CDX2 expression and several aggressive pathological features. Absence or low expression of CDX2 (score 0) was significantly associated with higher tumor grades, as 75% of grade III cancer cases showed score 0.

Additionally, CDX2 loss was strongly linked to the presence of distant metastases (M1) and advanced tumor stages, with 100% of stage IV cancer cases showing score 0. Lymph node metastasis was also associated with CDX2 loss, with 100% of N2 tumors showing score 0, and 66.7% (8/12) of cases with lymphovascular invasion exhibited score 0. However, there was no significant statistical difference between CDX2 expression and depth of tumor invasion (T) (Table 6).

Table (6): Relation between TM9SF4 and CDX2 IHC expression and histopathological features among cancer cases:

	Total number N=23	TM9SF4			Test of significance	CDX2			Test of significance
		Score 1	Score 2	Score 3		Score 0	Score 1	Score 2	
Tumor grade:									
Grade I	**3(13)**	**1(33.3)**	**2(66.7)**	**0**	**Mc=10.41**	**0**	**0**	**3(100)**	**Mc=10.37**
Grade II	**16(69.6)**	**0**	**4(25)**	**12(75)**	**P=0.034***	**7(43.8)**	**6(37.5)**	**3(18.8)**	**P=0.035***
Grade III	**4(17.4)**	**0**	**1(25)**	**3(75)**		**3(75)**	**0**	**1(25)**	
Depth of tumor invasion (T):									
T1	**1(4.3)**	**1(100)**	**0**	**0**	**Mc=24.72**	**0**	**0**	**1(100)**	**Mc=11.02**
T2	**5(21.7)**	**0**	**2(40)**	**3(60)**	**P=0.001***	**2(40)**	**0**	**3(60)**	**P=0.08**
T3	**14(60.9)**	**0**	**5(35.7)**	**9(64.3)**		**5(35.7)**	**6(42.9)**	**3(21.4)**	
T4	**3(13)**	**0**	**0**	**3(100)**		**3(100)**	**0(0)**	**0(0)**	
Lymph node metastasis (N):									
N0	**13(56.5)**	**1(7.7)**	**7(53.8)**	**5(38.5)**		**1(7.7)**	**5(38.5)**	**7 (53.8)**	
N1	**7(30.4)**	**0**	**0**	**7(100)**	**Mc=9.44**	**6(85.7)**	**1(14.3)**	**0**	**Mc=16.21**
N2	**3(13)**	**0**	**0**	**3(100)**	**P=0.051**	**3(100)**	**0**	**0**	**P=0.003***
Distant metastasis (M):									
M1	**4(17.4)**	**0**	**0**	**4(100)**	**Mc=2.58 P=0.275**	**4(100)**	**0**	**0**	**Mc=6.29 P=0.04***
Stage:									
Stage I	**4(17.4)**	**1(25)**	**2(50)**	**1(25)**	**Mc=15.27**	**0**	**0**	**4(100)**	**Mc=21.39**
Stage II	**8(34.8)**	**0**	**5(62.5)**	**3(37.5)**	**P=0.018***	**1(12.5)**	**4(50)**	**3(37.5)**	**P=0.002***
Stage III	**7(30.4)**	**0**	**0**	**7(100)**		**5(71.4)**	**2(28.6)**	**0**	
Stage IV	**4(17.4)**	**0**	**0**	**4(100)**		**4(100)**	**0**	**0**	
Lymphovascular invasion (LV):									
Negative	**11(47.8)**	**1(9.1)**	**7(63.6)**	**3(27.3)**	**MC=13.38**	**2(18.2)**	**3(27.3)**	**6(54.5)**	**MC=7.14**
Positive	**12 (52.2)**	**0**	**0(0.0)**	**12(100)**	**p=0.001***	**8(66.7)**	**3(25)**	**1(8.3)**	**p=0.028***

M1: Presence of distant metastases, MC: Monte Carlo test, *Statistically significant, Data expressed as number (%)

5-Correlation between TM9SF4 and CDX2 among studied cases:

There was a significant negative correlation between TM9SF4 and CDX2 in the overall sample (r = -0.801), colorectal carcinoma (r = -0.711), and ulcerative colitis (r = -0.678). However, no significant correlation was found in adenoma cases (r = -0.08) (Table 7 and Figure 5).

Table (7): Correlation between TM9SF4 and CDX2 among studied cases

Correlation between 2 markers	R	P- value
All studied sample	**-0.801**	**0.001***
Colorectal carcinoma	**-0.711**	**0.0001***
Adenoma	**-0.08**	**0.825**
Ulcerative colitis	**-0.678**	**0.004***

r: Spearman correlation coefficient, *Statistically significant.

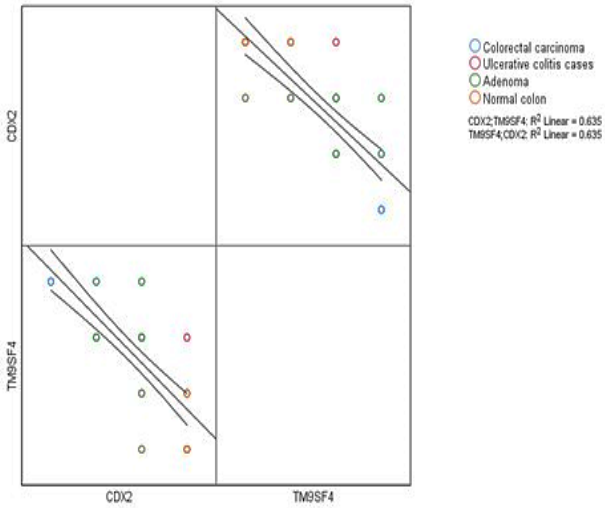

Figure (5): Scatter diagram showing correlation between TM9SF4 and CDX2

DISCUSSION

Colorectal cancer (CRC) remains one of the most commonly diagnosed malignancies and ranks as the second leading cause of cancer-related mortality worldwide [9]. Identifying novel biomarkers with both diagnostic and prognostic value is crucial for early detection, risk stratification, and therapeutic targeting. In this study, we conducted a comprehensive evaluation of TM9SF4 and CDX2 expression across a range of colorectal tissues—including normal colonic mucosa, adenomas, ulcerative colitis (UC), and colorectal cancer (CRC) to assess their diagnostic and prognostic value and compare their performance. TM9SF4 is a member of the Transmembrane 9 Protein Superfamily, characterized by nine transmembrane domains and high evolutionary conservation. Initially identified in Dictyostelium and Drosophila, it has been implicated in cell adhesion, phagocytosis, and cancer biology [10].

In our study, TM9SF4 expression was significantly elevated in CRC cases compared to non-neoplastic and pre-neoplastic counterparts ($p < 0.01$). High expression (scores 2–3) was observed in 95.7% of CRC cases, while normal control and UC tissues showed weak or absent staining. This tumor-specific overexpression highlights TM9SF4 as a potential diagnostic biomarker. ROC analysis further supports its diagnostic utility, with an AUC of 0.993, high sensitivity (95.7%), and perfect specificity (100%) for distinguishing CRC from normal mucosa control. These findings align with **Guazzi *et al.*** [4], who reported TM9SF4 positivity in 85% of CRC cases and superior performance compared to conventional markers like CEA and CA19-9, underscoring its potential as a reliable diagnostic biomarker for CRC.

Also, **Paolillo *et al.*** [11], reported that TM9SF4 is significantly overexpressed in leukemic cells and acute myeloid leukemias (AMLs) compared to normal CD34+ hematopoietic progenitor cells, suggesting a potential role in tumorigenesis. This oncogenic role may be linked to TM9SF4's association with exosomes—lipid-based vesicles that mediate intercellular communication and promote cancer progression [12]. TM9SF4 contributes to the acidic tumor microenvironment, which enhances exosome release and supports the transfer of oncogenic signals, thereby facilitating malignant transformation and metastasis [4,13].

When compared with UC, TM9SF4 retained diagnostic significance (AUC = 0.935), though specificity declined to 70%, suggesting partial overlap due to inflammatory responses. Notably, TM9SF4 expression in UC was generally low, consistent with **Xie *et al.*** [14], who identified it as a protective factor in IBD through modulation of ER stress, epithelial barrier function, and macrophage polarization. Differences with studies like **Shalan *et al.*** [15], who reported high TM9SF4 expression in UC, may reflect variations in disease severity; our cohort included more active cases, suggesting expression may inversely correlate with inflammation.

In the current study, TM9SF4 expression in adenomas was intermediate, and although it showed preserved sensitivity, its specificity for CRC decreased significantly (AUC = 0.796; specificity 43.7%), limiting its ability to distinguish malignant from pre-malignant lesions. No significant association was observed between TM9SF4 expression and adenoma histological subtype or dysplasia grade, indicating the need for further study with larger cohorts

Importantly, high TM9SF4 expression in CRC was significantly associated with adverse pathological features, including higher tumor grade (P =0.034), increased depth of invasion (P=0.001), lymphovascular invasion (P=0.001), and advanced overall stage (P=0.018). Although the associations with lymph node and distant metastases did not reach statistical significance, a clear trend of elevated TM9SF4 expression in metastatic cases (N1/N2 and M1) was

observed. These results reinforce prior reports linking TM9SF4 with tumor aggressiveness and progression [4,15].

TM9SF4 promotes tumor progression by engaging multiple molecular pathways. It activates the V-ATPase proton pump, resulting in acidification of the tumor microenvironment, which enhances the activity of proteolytic enzymes like cathepsins and matrix metalloproteinases, thereby facilitating invasion and metastasis [16]. TM9SF4 also promotes tumor cell cannibalism, a survival mechanism under hypoxic and nutrient-deprived conditions that enhances immune evasion and metastatic potential [17].

CDX2, a caudal-type homeobox transcription factor, plays a key role in intestinal development, epithelial differentiation, and colonic homeostasis, functioning as a tumor suppressor [8]. Our immunohistochemical analysis revealed a significant difference in CDX2 expression across the examined groups ($P < 0.01$), with a clear trend of progressive downregulation from normal mucosa control to adenomas and carcinomas. Strong nuclear CDX2 staining was observed in all normal colonic control samples, whereas 43.5% of CRC cases exhibited complete loss of expression (score 0), and none retained strong expression (score 3). This stark contrast provided excellent diagnostic performance, with ROC curve yielding AUC of 1.0 for distinguishing CRC from normal mucosa. These results align with earlier studies reporting high sensitivity and specificity of CDX2 for colonic adenocarcinomas, **Saad *et al.*** [18], and **Bayrak *et al.*** [19], though some discrepancies have been noted in other cohorts, **Abouelkhair *et al.*** [20], likely due to methodological or population-related differences.

In our study, 70% of UC cases showed strong CDX2 expression, resulting in moderate sensitivity (69.6%) for differentiating UC from CRC, despite high overall diagnostic accuracy (AUC = 0.954). This relatively elevated expression may reflect a regenerative mucosal phase, where CDX2 is upregulated as part of epithelial repair mechanisms. During active UC, chronic inflammation leads to repetitive cycles of mucosal injury and repair, where epithelial cells re-enter the cell cycle and activate transcriptional programs aimed at restoring intestinal architecture and function. One of these programs includes upregulation of CDX2, a key transcription factor involved in maintaining intestinal epithelial identity and differentiation [21]. Supporting this regenerative interpretation, **Sipos *et al.*** [22] demonstrated that in active UC, CDX2 is co-expressed with HGFR in epithelial progenitor cells, suggesting a role in mucosal repair possibly through mesenchymal-to-epithelial transition

While some studies, such as **Jahan et al.** [23], have reported CDX2 downregulation during active inflammation, our findings align with others showing its preservation or upregulation during regenerative phases [5,21]. These discrepancies may reflect differences in disease activity, treatment status, biopsy timing, or sampling location. Collectively, our results support the notion that CDX2 expression in UC is dynamic and context-dependent, influenced by the balance between inflammatory damage and epithelial regeneration.

In our study, CDX2 expression in colorectal adenomas displayed a heterogeneous pattern, with nearly half of the cases exhibiting intermediate levels of nuclear staining. Notably, CDX2 expression varied significantly according to adenoma subtype: villous adenomas, which carry a higher risk of malignant transformation, showed weaker nuclear staining compared to tubular adenomas. Additionally, a trend toward reduced CDX2 expression was observed in cases with high-grade dysplasia, suggesting that CDX2 downregulation may be an early event in the adenoma–carcinoma sequence. These findings are consistent with previous reports highlighting variability in CDX2 expression across adenoma subtypes and dysplasia grades [24,25]. From a diagnostic standpoint, CDX2 demonstrated moderate utility in differentiating colorectal cancer from adenomas (AUC = 0.825), reflecting its limited specificity for malignancy. This aligns with earlier studies recognizing CDX2 as a helpful but not definitive marker for distinguishing malignant from pre-malignant lesions [25,26].

Overall, our findings reinforce CDX2 as a reliable marker for distinguishing CRC from normal mucosa. However, its reduced specificity in differentiating CRC from adenomas and UC emphasizes the need for its use alongside other diagnostic markers and histopathological criteria

Importantly, CDX2 loss was significantly associated with aggressive clinicopathological features in CRC**.** Notably, complete loss of CDX2 expression (score 0) was observed in 75% of grade III, indicating a strong correlation with poor differentiation ($P = 0.035$), in line with previous studies linking reduced CDX2 expression to poor histologic grade [9,27]. However, some studies have not confirmed this association, highlighting variability across studies [19,25,28].

Our findings also demonstrated a robust association between CDX2 loss and advanced disease stage. Specifically, 100% of stage IV and cases with N2 lymph node metastases exhibited absent CDX2 expression ($P = 0.002$ and $P = 0.003$, respectively). Furthermore, CDX2 loss was significantly correlated with lymphovascular invasion (66.7% of cases; $P = 0.028$). These observations are consistent with previous research indicating that loss of CDX2 is associated with advanced stage, poor differentiation, and presence of lymphovascular space invasion [9,26,29].

CDX2 silencing often results from epigenetic mechanisms, including promoter methylation and histone modification, rather than direct mutation [29,30]. Loss of CDX2 disrupts WNT, MAPK, and TGF-β signaling, reduces p21-mediated cell cycle control, and promotes epithelial membrane transition (EMT) and

invasion [31]. Additionally, CDX2 loss frequently co-occurs with microsatellite instability (MSI) and BRAF mutations, both markers of poor prognosis [32], further supporting its role as a biomarker for high-risk CRC.

Beyond intrinsic mechanisms, the tumor microenvironment plays a pivotal role in regulating CDX2 expression. Loss of CDX2 correlates with increased macrophage infiltration, partly through suppression of the immune-regulatory gene H2-T3 [31]. Notably, CDX2-deficient tumor cells may perpetuate a feedback loop, altering their microenvironment to suppress CDX2 in adjacent cells, promoting heterogeneity and tumor progression [33].

In our study, a significant negative correlation between CDX2 and TM9SF4 expression was observed in the overall sample, as well as in CRC and UC cases, but not in adenomas—suggesting a context-dependent regulatory relationship. In CRC, this inverse correlation may reflect a biological transition from differentiation to invasive behavior, where CDX2, a transcription factor essential for intestinal epithelial identity, is often downregulated in advanced stages and correlates with poor prognosis [34]. Conversely, TM9SF4 is frequently upregulated in CRC and associated with poor pathological features and tumor invasiveness [4,15]. In UC, although CDX2 was largely preserved, TM9SF4 expression remained low, further supporting their opposing roles. Shared inflammatory mediators such as TNF-α and oxidative stress may modulate these pathways independently, contributing to their inverse expression [14,22].

LIMITATIONS

Low number of the studied cases, single center investigation and other histopathological types of colorectal adenocarcinoma not involved in the study.

CONCLUSION

Overall, our findings underscore TM9SF4 as a novel diagnostic and prognostic marker in CRC, closely linked to tumor aggressiveness and likely contributing to the processes of invasion and metastasis. CDX2 remains a highly reliable marker for colonic differentiation, with downregulation serving as a hallmark of tumor progression. The inverse expression pattern of CDX2 and TM9SF4 underscores a potential biological interplay that warrants further investigation. Together, these markers may provide complementary insights into colorectal tumorigenesis and offer valuable tools for improved diagnosis, risk assessment, and therapeutic targeting.

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