

Immunodiagnostic Potential of Mucin (MUC2) and Thomsen-Friedenreich(TF) Antigen in Egyptian Patients with Colorectal Cancer

Ibrahim H. El-Sayed¹ and Mahmoud Moawad²

¹Molecular Biology Department, Genetic Engineering and Biotechnology Research Institute, Minufiya University, Sadat City, Minufiya, Egypt.

² Pathology Department, National Cancer Institute, Cairo University, Cairo, Egypt.

Email: ibrahimelsayed@yahoo.com

Abstract: Colorectal cancer (CRC) is more common in developed countries and is the third most common cancer among both men and women. CRC provides an attractive model of tumour biology with normal mucosa to adenoma to carcinoma sequence. The TF-antigen (Thomsen-Friedenreich) can be identified by galactose oxidase-Schiff's (GOS) reaction either on tissues or on rectal mucus samples from patients with CRC. TF antigen is expressed in the neoplastic mucosa and not expressed in colonic mucosa of normal subjects. Apomucins play important role in cell signalling and their specific pattern of expression during the different steps of tumor progression toward adenocarcinoma suggests that they play significant roles in tumorigenesis. The family of secreted mucins including MUC2 is contributing in mucus formation to protect underlying epithelia against diverse injuries. The current study was investigated the expression of MUC2 and TF antigen in patients with adenoma and CRC. MUC2 and TF antigen expressions were detected immunohistochemically in CRC biopsies using specific monoclonal antibodies. Moreover, the TF antigen was investigated using GOS reaction. The results showed that in normal colonic specimens, MUC2 expression was detected in 20% , while TF antigen was completely negative in 100% of samples as detected by GOS and immunohistochemistry using anti-TF monoclonal. Expressions of MUC2, and TF antigen as detected by GOS and anti-TF monoclonal were positive in 96%, 80%, and 60% respectively in cases with adenoma. On the other hand, in cases with adenocarcinoma, the expression of MUC2 was seen in 92% of cases, while TF antigen was detected in 84% and 60% of cases as detected by GOS and immunohistochemically respectively. Thus, it is concluded that the expression of MUC2 and TF antigens are altered during CRC carcinogenesis. Furthermore, MUC2 and TF antigens may have a diagnostic and or prognostic potential in CRC. [Nature and Science 2010;8(9):257-264]. (ISSN: 1545-0740).

Key Words: CRC, immunohistochemistry, MUC2, TF antigen, Expression.

1. Introduction

Colorectal cancer (CRC) is more common in developed countries and is the third most common cancer among both men and women. Each year more than one million people worldwide are diagnosed with colorectal cancer and approximately 150,000 of those cases are recorded in the US and 194,000 in Europe. The disease affects nearly an equal number of men and women. It is the second most common cause of cancer deaths with just over 655,000 dying of it annually. The number of diagnosed cases has been declining for the better part of the past two decades largely due to awareness, successes with screening and early detection, and improvements in treatment methods. Nearly 40% of colorectal cancers are detected at an early enough stage before

metastasis has occurred. The overall 5-year survival rate in the US is greater than 60% and, when detected at an early stage, approaches 90% with treatment (Levin, 2010)

Early detection by screening for colon cancer should be part of routine care for all adults starting at age 50 years, especially for those at high risk (those with first degree relatives with colon cancer, patients with ulcerative colitis, previous colon cancer, a family history of cancer, or a history of sporadic colon polyps) (Kim and Lance, 1997). After treatment of colon cancer, periodic follow up by laboratory studies and physical examination may lead to earlier identification and management of recurrent cancer. Primary prevention of cancer attempts to reverse precancerous lesion or in situ carcinoma to

normal or stops them from progressing to invasive carcinoma in high-risk population. Thus, for prevention and early detection, the key point is to detect the marker, which is differentially expressed in cancers and precancerous tissues but not in normal ones (Yang GY, Shamsuddin, 1996).

Thomsen-Friedenreich antigen or TF antigen also called, the disaccharide moiety D-galactose β -(1-3)-N-acetyl-D-galactosamine (Gal-GalNAc), is a tumour-associated carbohydrate that has been used as a biomarker of colonic cancer and other adenocarcinomas (Carter et al. 1997). Enzymatic oxidation of Gal-GalNAc by the enzyme D-galactose oxidase followed by Schiff's reagent to produce a magenta coloration provided a new test for mass screening of colorectal cancer (Shamsuddin and Elsayed, 1988). The test was used in rectal mucus samples of patients with colorectal neoplasms. Studies that were done mostly in China and Japan confirmed the sensitivity and specificity of the test for detecting colorectal neoplasms and precancerous conditions (Shamsuddin, 1996). Other methods of detection of the T antigen have also been reported by using an antibody directed against the antigen in an immunoperoxidase reaction (Hanisch and Baldus, 1997)

Mucins are high molecular weight glycoproteins consisting of a mucin core protein (apomucin) and O-linked oligosaccharides synthesized by a broad range of epithelial tissues and are coded by MUC genes (Gendler and Spicer, 1995). Epithelial mucins in the gastrointestinal tract (GIT) were classified into two distinct families: secretory-gel forming (MUC2, MUC5AC, MUC5B, and MUC6) and membrane bound mucins. Members of each family possess common structural characteristics and at least some common functions (Wang and Fang, 2003; Lau et al., 2004). In general, mucins have the unique function of protecting and lubricating epithelial surfaces, but nowadays they have also been implicated in additional diverse roles, such as growth, fetal development, inflammation, epithelial renewal and differentiation, epithelial integrity, carcinogenesis, and metastasis (Roessler et al., 2005; Babu et al., 2006; Kim et al., 2008; Altered expression of mucin epitopes have been described in colon and stomach cancers and correlated with decreased survival (Baladas et al., 1988; Wada et al., 2005; in the current study, the TF antigen expression was investigated by two techniques, as well as MUC2 expression on formalin fixed, paraffin embedded

tissue sections of colorectal cancers, precancerous lesions and normal colon specimens for evaluation of their diagnostic potential.

2. Material and Methods

Sampling was performed after informed consent was obtained from each patient included in the current study to use the samples and clinical data for research purposes after being informed about the nature of the study. The study protocol conforms to the most recent ethical guidelines of the Declaration of Helsinki as reflected in a priori approval by local ethical committee. It was carried out during surgical intervention or endoscopy. The current study was pursued on those patients prior to treatment including neo-adjuvant, radiotherapy and or chemotherapy. The cohort comprised of 25 cases of adenoma, and 25 cases of colorectal adenocarcinoma. The cases were compared against ten of normal colonic specimens. The tissues were fixed in 10% buffered formalin and embedded in paraffin. All sections were cut into four μ m, stained routinely by haematoxylin and eosin and then examined to confirm the final diagnosis.

Galactose oxidase Schiff's reaction

Paraffin tissue sections were deparaffinized in xylene, rehydrated in graded alcohols according to standard procedure, and then transferred to PBS (pH 7) for ten minutes. Slides were then flooded with D-galactose oxidase solution (100 units/ml) (Sigma) for one hour at room temperature. The sections were then rinsed in distilled water for ten minutes and stained with 2% Schiff's reagent (Sigma) for ten minutes at room temperature. The sections were consequently rinsed in running tap water for ten minutes, counterstained with haematoxylin for two seconds, rinsed with running tap water, dehydrated, cleared and mounted.

MUC2 immunohistochemistry using anti-MUC2 antibody

For MUC2 immunostaining, paraffin-embedded sections were placed on poly-L-lysine-coated glass slides and air-dried at room temperature. Deparaffinized and rehydrated sections were heated in a microwave oven for seven minutes in citrate buffer to retrieve the antigenic activity and cooled for 60 minutes at room temperature. Endogenous peroxidase activity was inhibited by incubation with 0.3% hydrogen peroxidase in methanol for twenty minutes at room temperature. After blocking the non-

specific reactions with 10% normal rabbit serum, the sections were first incubated with MUC2 antibody (mouse monoclonal antibody Ccp58, Novocastra) overnight at a dilution of 1:100. The sections were then incubated with biotinylated rabbit anti-mouse immunoglobulin for 30 minutes and next with streptavidin peroxidase complex (Histofine SAB-PO Kit, Biogenex Laboratories) for 15 minutes. The sections were carefully rinsed with several changes of phosphate-buffered saline between each step of the procedure. The colour was developed with diaminobenzidine (DAB). The sections were lightly counterstained with H & E and mounted. Negative controls were obtained by replacing the primary antibody with PBS or antibody isotype or diluted normal bovine serum. The negative control was strictly negative. Staining results were interpreted as positive or negative. Specimens were regarded as positively stained if more than 5% of the tumour area showed a reaction at a magnification of $\times 400$ (Grady et al., 2008).

TF antigen immunohistochemistry using anti-TF antibody (anti-Tag Ab)

The sections were deparaffinised, rehydrated and treated with 3% hydrogen peroxide in methanol for thirty minutes to block endogenous peroxidase. To reduce non-specific staining, the sections were incubated for twenty minutes at room temperature with normal horse non-immune serum (1:100 dilution) (Vector Laboratories, CA). Excess normal serum was blotted from the slides. The sections were then incubated with the primary mouse antibody (1:50 dilution) (DAKO, Carpinteria, CA) for one hour at room temperature, washed three times for five minutes each in PBS (pH 7.2), and incubated with biotinylated secondary (horse anti mouse IgG) antibody (1:100 dilution) (Vector Laboratories, CA) in PBS for 30 minutes at room temperature. Avidin-biotin complex (1:25 dilution) (Vector Laboratories, Burlingame, CA) was then applied for one hour. Sections were then incubated with 0.02% 3, 3-diaminobenzidine, freshly prepared in 0.05M TRIS buffer (pH 7.6) containing 0.015M hydrogen peroxide, and then counterstained with haematoxylin before dehydrating and mounting. Mucosa from colloid carcinoma patient was used as a positive control in each staining run. Negative controls were obtained by replacing the primary

antibody with PBS or the antibody isotype or diluted normal bovine serum. The negative and positive controls were strictly negative and positive. Staining results were interpreted as positive or negative. Specimens were regarded as positively stained if more than 5% of the tumour area showed a reaction at a magnification of $\times 400$ (Grady et al., 2008).

Statistical analysis

All statistical analyses were performed with PASW 18 for Windows (SPSS, Chicago, IL, USA). Comparison was performed using the statistical Chi-square test. A *P* value < 0.05 was considered statistically significant.

3. Results

The staining pattern of GOS was a magenta coloration, but in case of MUC2, and TF antigens, the antibodies reaction gave a brown staining for both. The positive status was seen at the apical cell membrane, goblet cell vacuoles extracellular mucus, intraluminal mucus, and in the cytoplasm of signet ring cells. No expression of TF antigen was detected by GOS and anti-Tag Ab (anti-TF) in the epithelial cells in ten normal colonic specimens. Weak staining was observed with the MUC2 in two cases out of these ten cases.

Expression of TF antigen was seen by GOS in 15 out of 20 (75%) in tubular adenomas with low grade dysplasia (Fig. 1) and in all five tubular adenomas with high grade dysplasia. The intensity of staining was strong in all cases and was observed in the mucus cells of normal as well as dysplastic crypts. The expression of TF-antigen was detected by anti-Tag Ab only in 12 out of 20 (60%) adenomatous polyps with low grade dysplasia (Fig. 2) and in three out of five (60%) adenomatous polyps with high grade dysplasia. Positive staining of MUC2 was found in 19 of 20 (95%) of tubular adenomas with low grade dysplasia (Fig. 3), and in all five cases (100%) of adenomatous polyps with high grade dysplasia (Table 1 & 2). Stromal cells showed a weak positive reaction.

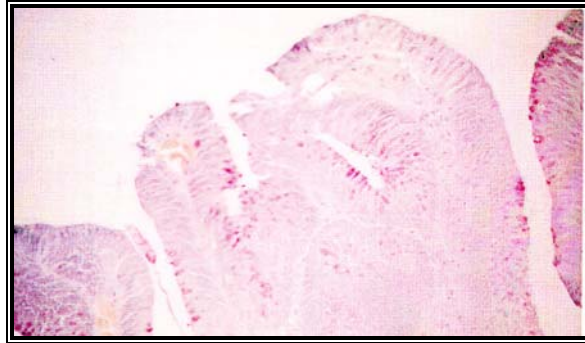


Figure 1. GOS reaction in adenoma specimens showed the magenta coloration in goblet cells ($\times 200$).

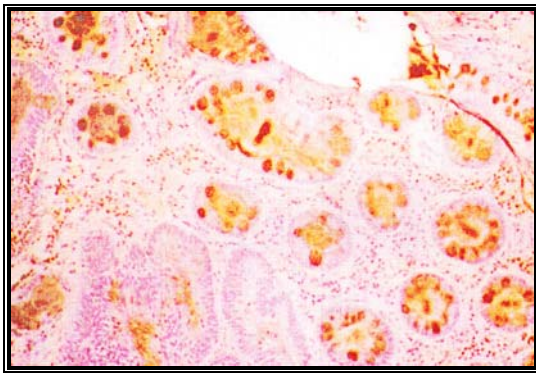


Figure 2. TF antigen immunoreactivity in adenoma samples using anti-TF antibody. The positive reaction is depicted in goblet cells ($\times 400$).

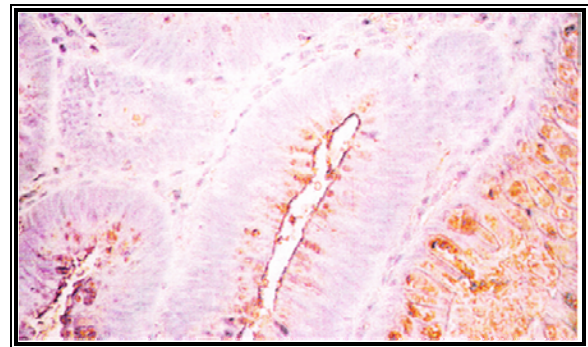


Figure 3. MUC2 immunostaining in adenoma showing positive reaction in goblet cells ($\times 400$).

The GOS reaction was positive in 21 of 25 (84%) cancer specimens. The well-differentiated and moderately differentiated adenocarcinomas (Fig. 4) showed more intense staining than poorly differentiated adenocarcinoma. The GOS reaction was strong in the five (100%) well differentiated carcinomas and six out of eight (75%) moderately differentiated carcinomas. Reactivity was observed in all six cases (100%) of mucinous carcinoma. A moderate to weak cytoplasmic staining was observed in four out of six (66%) poorly differentiated carcinomas. The expression of MUC2 was seen in 23 of the 25 (92%) cases of adenocarcinoma. Intense reactivity was observed in all cases of mucinous adenocarcinoma (Fig. 5) as well as all cases of well differentiated and moderately differentiated

adenocarcinomas. Two of the poorly differentiated adenocarcinomas did not show any degree of staining. Once again, the neoplastic glandular elements and to a lesser degree, the stromal cells showed positive reaction. The expression of TF antigen by anti-Tag antibody was documented in 15 out of 25 (60%) cases of adenocarcinoma. Intense staining was observed in only four cases, they were suffered from mucinous carcinoma. The well-differentiated and moderate differentiated adenocarcinoma showed a positive reaction in eight out of 13 (61%) cases, and in three of the six cases (50%) of poorly differentiated carcinoma (Table 1 & 2).

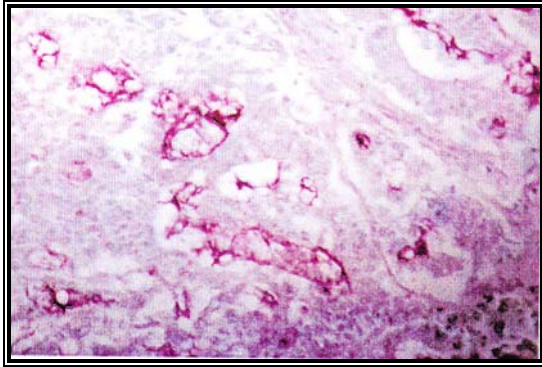


Figure 4. GOS reaction in adenocarcinoma showing the magenta colour in intraluminal-secreted mucous ($\times 400$).

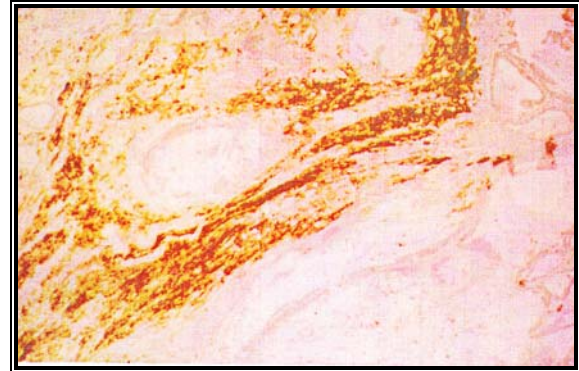


Figure 5. MUC2 antibody staining of adenocarcinoma specimens showing an intense positive reaction in mucus lakes and cytoplasm ($\times 400$).

Table 1. The expression of MUC2 and TF-antigen as detected immunohistochemically for both and by GOS for TF antigen in adenomas and adenocarcinomas.

Type	Total		TF Antigen (+)		GOS (+)		MUC2 (+)	
	No	%	No	%	No	%	No	%
Adenoma	25	100	15	60	20	80	24	96
Low grade	20	80	12	60	15	75	19	95
High grade	5	20	3	60	5	100	5	100
Adenocarcinoma	25	100	15	60	21	84	23	92
Poorly differentiated	6	24	3	50	4	66.7	4	66.7
Moderately differentiated	8	32	5	62.5	6	75	8	100
Well-differentiated	5	20	3	60	5	100	5	100
Mucinous carcinoma	6	24	4	66.7	6	100	6	100

Table 2. The sensitivity and specificity of MUC2 and TF-antigen as investigated immunohistochemically for both and by GOS for TF antigen to detect the adenomas and adenocarcinomas.

Type	TF Antigen		GOS		MUC2	
	Sens.	Spec.	Sens.	Spec.	Sens.	Spec.
Adenoma	60	100	80	100	96	80
Adenocarcinoma	60	100	84	100	92	80

4. Discussion

The process of colorectal carcinogenesis, which has been termed the polyp-carcinoma sequence, is believed to typically occur over 10 to 15 years and involves concurrent histologic and molecular changes. The subsequent effect of these genetic and epigenetic alterations on the cell and molecular biology of the cancer cells in which they occur is the acquisition of key biological characteristics that are central to the malignant phenotype. From the analysis of the molecular genetics of CRC, it has become clear that the formation of CRC involves a multistage process that is currently characterized based on genomic instability (i.e., the loss of the ability to maintain the wild-type DNA coding sequence and repair DNA mutations). In the background of genomic instability, genetic and epigenetic alterations accumulate and cooperate with each other to drive the initiation and progression of CRC (Kim et al., 1993).

Mucins are highly glycosylated, high molecular weight glycoproteins ($M_r > 200$ Kd) composed of oligomeric polypeptide backbones to which numerous linear or branched chains of one to 20 monosaccharides are attached. These polypeptide backbones also are called “

apomucins” and have been designated as MUC antigens (Senapati et al., 2010). The mucins play an important role in the protective lining of epithelial surfaces. Several studies have reported that during the course of carcinogenesis, the biosynthesis of MUC antigens has been altered with regard to the rate of synthesis and the extent of their glycosylation. Pattern of expression of membrane bound mucins including MUC2 have been illustrated in different tumours such as CRC, lung, oesophagus, gastric, pancreatic and hepatobiliary cancers (Andrianifahanana et al., 2006; Van der Sluis et al., 2006; Hadi et al., 2009; Jonckheere and Van Seuningen, 2010). In the current study, the pattern of MUC2 expression was altered in adenoma and adenocarcinoma patients in different grades and these data may reflect the central role of MUC2 in the CRC tumorigenesis.

It was found in mice that the loss of MUC2 in the intestine, and therefore breaches in the epithelial barrier, leads to an abnormal morphology marked by an increase in thickness of the gut mucosa, flattening and ulceration of epithelial cells, general

loss of architecture, a mild increase of inflammatory cells, and an increase in proliferation. Furthermore, It was showed that changes in the mucus composition, caused by MUC2 deficiency, lead to inflammation of the colon and that deficiency of MUC2 contributes to the onset and/or perpetuation of inflammatory bowel diseases (Hasnain et al., 2010). Moreover, in a more recent study, it was documented that the MUC2 gene deficiency in mice impairs host resistance to an enteric parasitic infection (Boland et al., 1982). Alterations or absence of MUC2 production can lead to colon carcinoma (Buisine et al., 199), ulcerative colitis (Crabtree et al., 1989) and celiac disease (Velcich et al., 2002). A role for Muc2 in the suppression of colorectal carcinoma has also been suggested because Muc2 knockout (KO) mice spontaneously develop colitis and adenomas that progress to invasive adenocarcinoma, suggesting an important function for this mucin in colonic protection (Hasnain et al., 2010). Furthermore, missense mutations in the MUC2 gene results in aberrant Muc2 oligomerization, leading to endoplasmic reticulum stress and subsequently increased susceptibility to colitis [Heazlewood et al., 2008).

The TF antigen (disaccharide Gal-GalNAc), also known as Tag, is a precursor of the M and N blood group substances. TF antigen normally contains a sialic acid that is attached to the terminal galactose. The removal of sialic acid allows the sugar moiety to be oxidized by the enzyme galactose oxidase (Gendler and Spicer, 1995; Wang and Fang, 2003). The enzyme D-galactose oxidase specifically oxidizes C-6 hydroxyl groups of D-galactopyranose and N-acetylgalactosamine residues of Gal-GalNAc, generating two vicinal aldehyde groups, which react with basic fuchsin to give magenta/purple coloration, called galactose-oxidase-Schiff or GOS reaction (Shamsuddin, 1996). It is believed that sialic acid free Gal-GalNAc is present in mucus of colorectal cancer of both human and experimental animals, but not in normal mucin [(Carter et al. 1997; Shamsuddin et al., 19981). The mucin composition is altered throughout the entire colon in patients with colonic cancer and in precancerous lesions by the way of the generalized field effect of carcinogens (Shamsuddin et al., 1994). One abnormality may be the loss of the terminal sialic acid residues, leaving Gal-GalNAc exposed. This, alteration may be due to reduction of glycosyltransferases, leading to aberrant or incomplete glycoprotein synthesis (Shamsuddin et

al., 1994). Based on this hypothesis, Shamsuddin developed a simple test, using the GOS technique, to detect the marker Gal-GalNAc in the rectal mucus of patients with precancerous and cancerous lesions of the colon (Croce et al., 2007). TF antigen can also be detected by using monoclonal or polyclonal antibodies [7]. In the current study, the expression of TF antigen (Tag) being none in the normal colonic mucosa, and is elevated severely in most of patients with adenoma and adenocarcinoma with different grades as detected by GOS and immunohistochemically and these results may reveal its importance during CRC carcinogenesis. In agreement with our results of TF immunostaining, Croce et al. 2007 found the TF positivity in none of colorectal normal tissues.

Over the past few years, increasing evidence suggests that the increased TF occurrence in cancer cells may be functionally important in cancer progression by allowing increased interaction/communication of the cells with endogenous carbohydrate-binding proteins (lectins), particularly the members of the galactoside-binding galectin family [Yu, 2007]. It was reported that the TF antigen is strongly expressed in the colorectal adenoma-carcinoma sequence, in well- and moderately differentiated carcinomas and in mucinous carcinomas Baldus et al., 1998).

Thus, it is concluded that the expression of MUC2 and TF antigens are altered during CRC carcinogenesis. Furthermore, MUC2 and TF antigens may have a diagnostic and or prognostic potential in CRC.

Correspondence to:

Dr. Ibrahim El-Sayed

Molecular Biology Department, Genetic Engineering and Biotechnology Research Institute, Minufiya University, Sadat City, P.O 22857-79, Minufiya, Egypt. E-mail: ibrahimelsayed@yahoo.com

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