

Original Article

Correlation of Tn antigen expression with mucins in Chinese patients with colorectal cancer

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Abstract: *Objective:* Tn antigen expression, indicative of aberrant O-glycosylation, is frequently observed in human colorectal cancer (CRC) and is proposed to play key roles in tumorigenesis and cancer progression. Tn antigen appears to produce global effects on O-glycosylation of proteins, particularly on mucins. However, the association between expression of Tn antigen and mucins in CRC remains unclear. Here, we investigated the expression profile of Tn antigen as well as MUC1, MUC2, and MUC4 in a series of human CRC tissues, with the aim of determining whether the Tn antigen has an influence on mucins in the development of CRC. *Methods:* Expression and localization of Tn antigen, MUC1, MUC2, and MUC4 were determined by multiplex immunohistochemical staining in formalin-fixed, paraffin-embedded colonic sections from Chinese patients with primary CRC. *Results:* The data show that 65 of 78 (83.3%) patients with CRC were found to express Tn antigen, which was most often stained in the apical cell membranes, mucin droplets, and cytoplasm of the cancer tissues. No Tn antigen was detected in normal colonic tissues. Correspondingly, there were altered patterns in the expression of mucins. Compared with normal colonic tissues that were absent of Tn staining, MUC1 and MUC4 showed an up-regulated and diffuse expression pattern in cancer tissues that expressed Tn antigen, whereas MUC2 expression was significantly decreased in Tn-positive cancer tissues. *Conclusions:* These results indicate that Tn antigen expression is closely associated with altered expression of mucins in human CRC. Tn antigen may promote development of CRC through affecting the associated mucins expression.

Keywords: Tn antigen, O-glycosylation, mucins, colorectal cancer

Introduction

Colorectal cancer (CRC) is the third most common human malignancy and the fourth leading cause of cancer-related death worldwide [1]. In China, the estimate of new cases diagnosed with CRC in 2015 was 376,300, with an estimated 191,000 deaths [2]. So far, understanding of the pathogenesis of CRC is limited. Alterations in glycosylation have recently received attention as a key component of neoplastic progression. Studies have demonstrated that aberrant glycosylation of proteins and lipids, resulting in exposure of the immature truncated O-glycans such as Tn antigen, is closely associated with tumorigenesis and malignant transformation of human CRC [3-5]. Tn antigen is the basic O-linked structure for all O-glycans. In normal cells, Tn antigen is capped, elongated, and branched by specific glycosyl-

transferases to form complete O-glycans that exert diverse biological effects. By contrast, cancer cells express only immature truncated O-glycans such as Tn antigen, possibly resulting from a number of different mechanisms, including abnormal expression of glycosyltransferases, somatic mutations [6] or hyper-methylation of COSMC [7, 8], relocation of polypeptide GalNAc-transferases from Golgi to endoplasmic reticulum [9], etc. Tn antigen appears to not only serve as a tumor carbohydrate marker but may also directly impacts cell growth and survival and even metastasis. It has been reported that Tn antigen expression may induce oncogenic features in many epithelial-derived cancers including CRC [10, 11]. The mechanisms, however, by which Tn antigen contributes to tumorigenesis are unclear. It is known that glycosylation ultimately results in the modification of many different protein products within a

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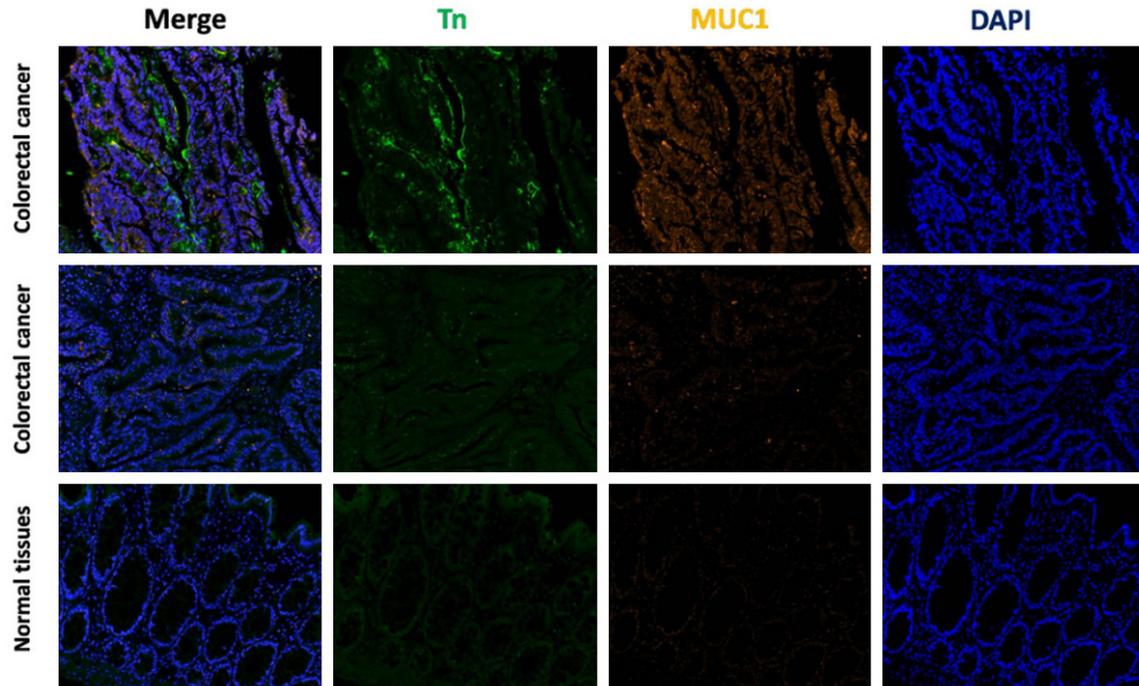


Figure 1. Expression profiles of Tn antigen and MUC1 in human colorectal cancer tissues. Staining shows that MUC1 is slightly expressed in normal tissues that were absent of Tn staining, but MUC1 expression and pattern is significantly altered in colorectal cancers that express Tn antigen.

given cell. Tn antigen may produce global effects on O-glycosylation of proteins, particularly mucins in the colon. Mucin MUC1, MUC2, and MUC4 are the predominant O-glycoproteins in colonic tissues and play essential roles in intestinal homeostasis [12-15]. Altered expression of these types of mucins has long been documented in patients with CRC and correlated with cancer metastasis and prognosis [16-18]. In this study, we investigated the expression profiles of Tn antigen as well as these types of mucins in a series of human CRC tissue samples, with the aim of analyzing whether Tn antigen mediated aberrant O-glycosylation has an influence on the expression of mucins. Our study will provide novel insight into mechanisms by which Tn antigen contributes to the development of CRC.

Materials and methods

Clinical specimens

Human tumor and adjacent normal tissues were obtained by endoscopic polypectomy or surgical resection from 78 patients with primary colorectal cancers. These patients were tr-

eated at Beijing Chao-yang Hospital, Capital Medical University, Beijing, China, from January 2015 to September 2017. The study was approved by the Ethics Committees of Beijing Chao-Yang Hospital, Capital Medical University, which followed the recommendations of the Declaration of Helsinki for biomedical research involving human subjects.

Multiplex IHC staining and evaluation

Formalin-fixed paraffin-embedded sections of human CRC tissues were stained with Tn antigen and mucins (MUC1, MUC2 and MUC4) simultaneously using Opal™ 4-Color Manual IHC Kit (PerkinElmer). Briefly, the sections were first incubated with a specific anti-Tn IgM mAb (1:300, kindly provided by Dr. Tongzhong Ju of Emory University School of Medicine in Atlanta, Georgia, USA) for 1 hour at room temperature and then incubated with goat anti-mouse IgM mu chain (HRP) (Abcam) and eventually labeled with Opal 520 fluorophore (PerkinElmer). For the other stains, MUC1 antibody (1:500, Santa Cruz, sc-7313), MUC2 antibody (1:500, Santa Cruz, sc-15334), and MUC4 antibody (1:500, Santa Cruz, sc-53945) were respectively ap-

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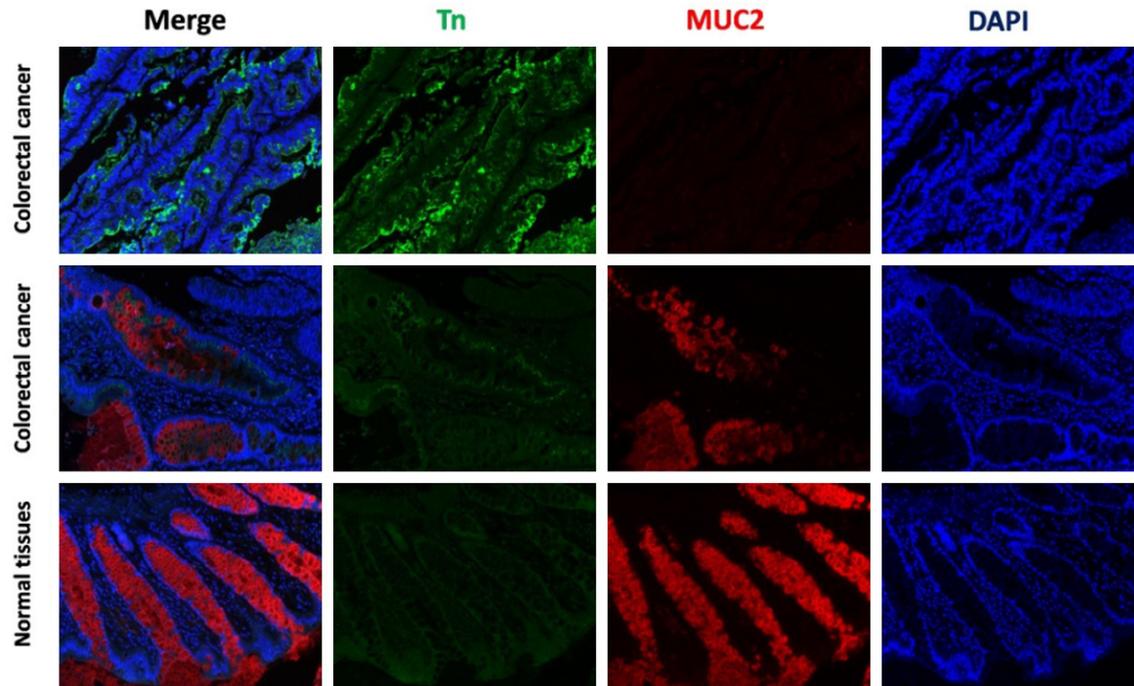


Figure 2. Expression profiles of Tn antigen and MUC2 in human colorectal cancer tissues. Staining shows an inverse association between Tn antigen and MUC2 expression. MUC2 expression decreased significantly in colorectal cancer tissues that were stained by Tn antigen.

plied and Opal polymer HRP Ms+Rb (PerkinElmer) was subsequently added to the sections followed by incubation with Opal fluorophore (PerkinElmer). Finally, all sections were counterstained with DAPI and mounted with anti-fade mountants. The inForm software (PerkinElmer) was used for analyzing the multi-spectral images.

The immunostaining was assessed according to staining intensity and distribution. Staining intensity (I) was graded as 0 (no staining), 1 (weak staining), 2 (moderate staining), and 3 (strong staining). The percentage (0-100%) of the extent of reactivity (R) scored as follows: 0 (no positive cells); 1 (positive cells rates <5%); 2 (positive cells rates \geq 5%); and 3 (positive cells rates \geq 50%). Histochemistry score = $I \times R$.

Statistical analysis

Correlations between the expression of Tn antigen and mucins and clinicopathological parameters of CRC tissues were analyzed using Spearman's correlation analysis. Results were considered statistically significant when $P < 0.05$. All statistical analyses were performed

using GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA).

Results

There was no Tn antigen detected in normal colorectal tissues. In contrast, a high percentage (65 of 78, 83.3%) of Chinese patients with CRC was found to express Tn antigen. Tn antigen showed a distinctive expression pattern, which was stained in the basolateral membrane, mucin droplets, and the cytoplasm of the cancer cells. Moreover, the frequencies and intensities of Tn antigen expression increased gradually from mild to moderate to severe dysplasia in cancer tissues. We next analyzed whether there was an association between Tn antigen expression and the mucins (MUC1, MUC2, and MUC4), which are essential for colonic tissues. Multiplex IHC staining enabled an observation of co-localization of Tn antigen and the associated mucins. The staining revealed that MUC1, a transmembrane O-glycoprotein, was expressed slightly in normal colorectal tissues, primarily detected on the apical surface of epithelium. By contrast, the intensity and pattern of MUC1 expression were

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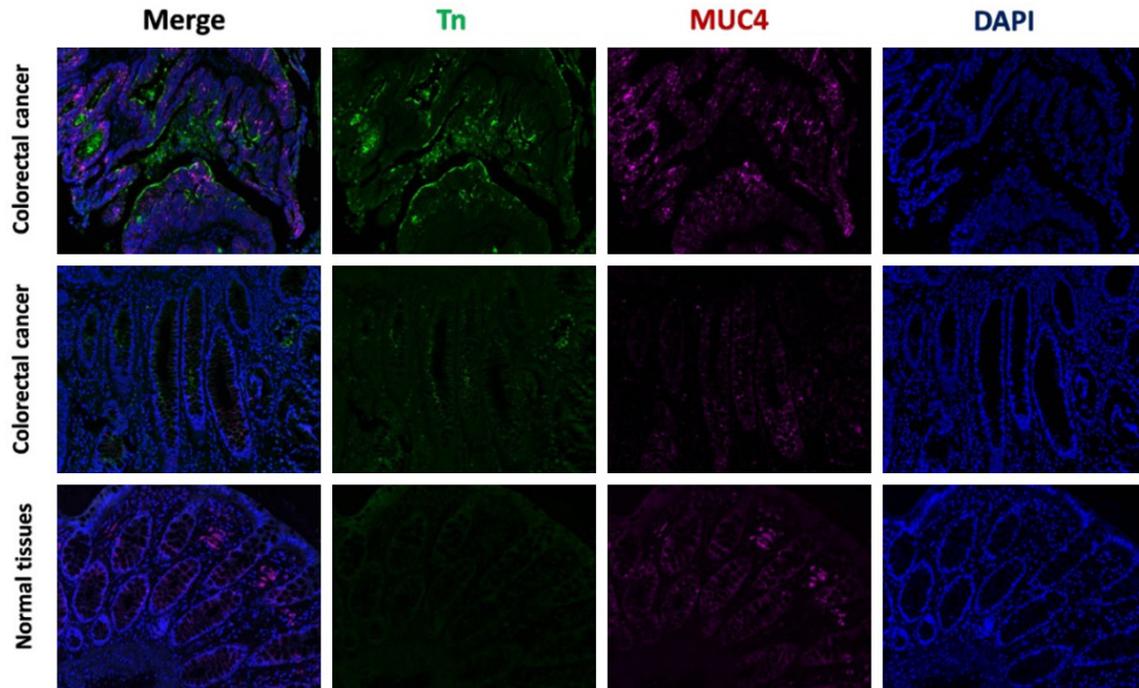


Figure 3. Expression profiles of Tn antigen and MUC4 in human colorectal cancer tissues. MUC4 is detected in normal colonic tissues. In a few cases of colorectal cancers, the expression and patterns of MUC4 were altered, similar to MUC1.

altered in cancer tissues (**Figure 1**). There was enhanced expression of MUC1 in cancer tissues that expressed Tn antigen but it presented in a diffuse expression pattern instead of apical localization (**Figure 1**). These observations support that Tn antigen may affect expression and localization of MUC1. As for MUC2, it is a secretory glycoprotein and is indeed the most predominant mucin in colonic tissues. We observed there was an inverse correlation between Tn antigen and MUC2 expression. MUC2 was expressed strongly in normal colorectal tissues that were absent of Tn antigen staining (**Figure 2**). But MUC2 expression decreased significantly in cancer tissues that had obvious Tn antigen staining, thereby suggesting that Tn antigen expression may impair MUC2 expression. MUC4 is also a transmembrane glycoprotein and is detected in normal colonic tissues, implying that it may participate in maintaining colonic functions. In a few severe cases, MUC4 showed an enhanced expression trend similar to MUC1 in cancer tissues (**Figure 3**). More CRC tissue samples, however, are required to confirm the correlation between Tn antigen and MUC4 expression. Together, our results indicated that Tn antigen expression

affects the expression of the colonic mucins, primarily MUC1 and MUC2.

Discussion

Many secreted and membrane-bound glycoproteins are modified by mucin-type O-glycosylation. This process starts with addition of N-acetylgalactosamine (GalNAc) to either serine or threonine to form Tn antigen, which is the basic O-linked structure and is a biosynthetic intermediate to all O-glycans [19]. Expression of Tn antigen, representative of incomplete O-glycosylation, has been frequently observed in many epithelial-derived cancers including colorectal cancer, breast cancer, cervical cancer [20-23]. Studies have reported that Tn antigen is detected in around 70-90% of colorectal cancers while it is rarely detected in normal tissues [3]. Tn antigen is associated with a diverse range of important biological properties of cancer cells, such as immunological functions, tumor progression, and metastasis, and correlated with poor prognosis and eventual low survival [24]. However, it is yet unclear how Tn antigen plays a role in the development and progression of CRC.

Tn antigen and mucins in colorectal cancer

Tn antigen is carried by numerous glycoproteins such as mucins and its expression generally represents incomplete glycosylation and may result in the alteration of many different glycoproteins. Among these glycoproteins, mucins including MUC1, MUC2, and MUC4 are heavily modified by O-glycans and play essential roles in colonic tissues. Altered expression of MUC1, MUC2, and MUC4 has been associated with colorectal lesions including CRC, which may interfere with normal cellular signaling, and may be an early step in neoplastic transformation of a normal epithelial cell [25-27]. Therefore, we hypothesize that enhanced levels of Tn expression in colonic cancer tissues may affect the expression or stability of mucins. In this study, we sought to investigate whether there was a correlation between Tn antigen expression and the alterations of most associated mucins.

Here we observed that in contrast to the absence of Tn expression in normal colon, there was a significantly predominant expression of Tn antigen in Chinese patients with CRC, which was in accordant with previous other reports [3, 22]. We also found that Tn antigen expression was related with dysplasia in cancer tissues. As the relationship between Tn antigen and some clinical characteristics such as tumor stage, metastasis, and survival in CRC have been widely documented, it was thereby not our focus in this study [28-30]. We focused our effort on investigating the correlation of Tn antigen with most associated mucins in CRC. As a result, we observed a diffuse expression pattern of Tn antigen at high levels throughout the cancer tissues. Interestingly, the expression pattern of mucins was altered accordingly. For example, the expression of MUC1 was confined to the apical surface at relatively low levels in normal colonic tissues but presented in a diffuse cytoplasm and basolateral membrane pattern at very high levels in cancer tissues. We thereby conclude that Tn antigen may alter MUC1 expression pattern in tumor development. In addition, the secretory mucin MUC2 is stored in bulky apical granules of the goblet cells. MUC2 is heavily modified by O-glycosylation and is generally considered to be essential for epithelial protection [31, 32]. MUC2-lacking mice spontaneously developed colitis and colorectal cancer [26]. We show that there is an inverse correlation between Tn antigen expression and MUC2 expression in

human colonic cancer tissues, suggesting that Tn antigen may also affect MUC2 expression and/or stability. MUC4 is also a transmembrane O-glycoprotein that is expressed in normal colonic tissues [33]. In a few cases, we observed, similar to MUC1, that the expression pattern of MUC4 was altered in cancer tissues expressing Tn antigen but the correlation requires more samples to validate. We speculate that MUC4 may play a relatively minor role in cancer development in contrast to MUC1 and MUC2.

Together, our data indicated that the presence of elevated Tn expression was associated with altered expression of mucins, particular MUC1 and MUC2. These observations provide insight into the mechanisms by which aberrant O-glycosylation affects tumor development and progression in CRC.

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Disclosure of conflict of interest

None.

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