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Invited Review

Gal-GalNAc: a biomarker of colon carcinogenesis

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Summary. The disaccharide tumor marker Gal-GalNAc visualized by galactose oxidase-Schiff sequence is commonly present in cancer cells and in rectal mucus of patients with colon cancer. The expression of this marker on tissue sections taken during experimental colon carcinogenesis shows excellent correlation with human precancerous lesions and cancers. A high proportions of human precancerous lesions and even higher percentage of colon cancers express this marker, whereas, no expression is seen in the normal human large intestine. Multifocal expression of the marker is seen throughout the entire colon of patients with precancer and cancer; these include dysplasia, dilated and distorted crypts, regenerative dysplasia and hyperplastic crypts, as well as the morphologically normal crypts remote from cancer. Nearly identical pattern of Gal-GalNAc expression throughout the entire colon also appear during rat colon carcinogenesis induced by azoxzymethane including non-expression by the normal and regenerative epithelia during wound healing following mechanical injury. Thus, Gal-GalNAc detected by the simple technique of galactose oxidase-Schiff sequence, is a biomarker that appears during the very early stages of progression of carcinogenesis. The expression pattern supports the field effect theory of carcinogenesis and also explains the basis for mass screening for cancer and precancerous conditions. Chemoprevention strategy using Gal-GalNAc as an intermediate marker detected by accurate and cost-effective rectal mucus test may have great potential.

Key words: Tumor marker, Gal-GalNAc, Field effect, Chemoprevention, Carcinogenesis

Introduction

In countries with Western lifestyle, large intestinal cancer is one of the most common cancers and a major cause of cancer death. There is little doubt that dietary habits play a very important role in the etiology of this cancer. High intake of fat, in particular animal fats, and a diet with a low fiber and inositol hexaphosphate ($InsP_6$ or IP-6) have been incriminated (Shamsuddin and Sakamoto, 1992; Willett, 1994). Prevention (both primary and secondary) is an important method for cancer control, which includes (a) detection of cancer at the very early stage of the disease (secondary prevention) to reduce cancer mortality and increase the survival rate of patients; and (b) etiology prevention or primary prevention attempts to reverse precancerous lesions or *in situ* carcinomas to normal or stop them from progressing to invasive malignancies in populations at high risk. Thus, early detection is fundamental to prevention, and the key is to find the marker which is differentially expressed in high risk tissues (cancer and precancer) but not in normal. Biomarker(s) meeting this criterion is/are logical choice for establishing accurate methods to detect cancers at infancy; it may also help in monitoring the efficacy of chemoprevention program by serving as intermediate endpoint marker (Kelloff et al., 1992; Shaw and Srivastava, 1993; Wattenberg, 1993).

The carbohydrate moiety D-galactose-ß(1-3)-N-acetyl-D-galactosamine (Gal-GalNAc, also called Thomsen-Friendenreich antigen or T-Ag, though it may be different) is such a biomarker of colonic cancer and other adenocarcinomas (Springer, 1984; Shamsuddin, 1991b; Shamsuddin et al., 1995). T-Ag is a precursor of the M and N blood group substances, which is recognized by the lectin peanut agglutinin (PNA) or detected by antibodies. 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 that react with basic fuchsin to give magenta/purple coloration. Thus, Gal-GalNAc can be visualized by a simple enzymatic reaction with galactose oxidase followed by Schiff's reagent (GO-Schiff sequence; Shamsuddin, 1991b; Shamsuddin and Sakamoto, 1994; Shamsuddin et al., 1995). Shamsuddin and co-worker have shown that the moiety recognized by GO-Schiff sequence may be different from the PNA-binding T-Ag and developed a simple test for early detection of colorectal cancer in the rectal mucus of patients with colorectal neoplasms (Shamsuddin and Elsayed, 1988;

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Shamsuddin, 1991b; Xu et al., 1992). Several studies of this test world-wide, have proven its high sensitivity and specificity in mass screening of colorectal cancer and precancerous lesions (Mackett et al., 1989; Sakamoto et al., 1990, 1993a; Zhou et al., 1993; please see Shamsuddin and Sakamoto, 1994 for a comprehensive review). Shamsuddin and co-workers propose that while D-galactose oxidase reacts with both D-galactose-ß(1-3)-N-acetyl-D-galactosamine and terminal monosaccharide galactose, D-galacto-hexoaldose converted from the latter may not be able to generate magenta coloration with basic fuchsin because of an atypical distance among the participating molecules (Avigad et al., 1962; Schlegel et al., 1968; Schulte and Spicer, 1983). In contrast, PNA or antibodies that bind to either Gal-GalNAc (or related structures), or terminal galactose may be equally visualized by PNA or first antibody (Shamsuddin and Sakamoto, 1994). This suggest that GO-Schiff sequence may be better for visualization of Gal-GalNAc. Furthermore, the rapidity and simplicity of GO-Schiff sequence makes it more practical.

Multiple foci of colonic mucosa show biochemical, histochemical or morphological alterations during the progression of carcinogenesis, and are recognized as precancerous lesions. This observation served as the basis for a) the field effect theory (Shamsuddin et al., 1981; Shamsuddin, 1991a) and b) the use of altered mucin as a biomarker. Since Gal-GalNAc is not expressed in the normal but in precancerous lesions and cancer; expression of Gal-GalNAc as detected by GO-Schiff sequence therefore justifies its inclusion as a biomarker for early detection and prevention of colon cancer. The modulation of its expression (viz. suppression) would be invaluable in monitoring patients with chemopreventive agents.

The simplicity of GO-Schiff sequence in mucus test as well as in tissue sections is another rationale for investigating human colon cancers, precancerous lesions, normal adult mucosa and fetal mucosa as well as tissues from rat colon carcinogenesis induced by the carcinogen azoxymethane (AOM) to investigate how and when the biomarker Gal-GalNAc is expressed during colon carcinogenesis; in particular is it expressed during the early stages and is it specific to carcinogenesis? Since AOM induces cancer of the colorectum in experimental animals, this well established model allows studies of the progression of the cancer (Shamsuddin and Trump, 1981).

Materials and methods

Sample data

Human tissues

Precancerous tissue specimens were from 14 cases of adenomatous polyps, and 13 cases of chronic ulcerative colitis. In addition, 7 cases of hyperplastic polyps with variable degrees of crypt atypia were also included since hyperplastic polyps have not been traditionally considered to be precancerous even though they express most tumor markers akin to premalignant and malignant lesions. Eighteen samples of fetal gastrointestinal tissues (6 each of large intestine, small intestine and stomach) from therapeutic abortions were examined. Eight cases of normal colonic mucosa from immediate autopsy and 20 cases of colorectal cancer with «transitional» and remote mucosa were also studied. The tissues were fixed either in 4% buffer formaldehyde-1% glutaraldehyde mixture or in 10% buffer formalin only and embedded in paraffin. All tissues were cut as 5 µm section.

Rat colon tissues

Eight samples of rat colon carcinomas were obtained from 8 rats induced by AOM (20 mg/kg one dose treatment). Two samples of rat colon injured mechanically by trocar under anesthesia and examined after 2 weeks to allow healing were also studied to see if Gal-GalNAc expression was non-specific as a result of injury and repair (wound healing).

Galactose oxidase-Schiff sequence

Paraffin tissue sections were dewaxed in xylene, rehydrated in graded alcohols according to the standard procedure and then transferred to distilled water for 10 min and in PBS (pH 7.0) for 10 min. Tissue sections were bathed overnight at room temperature with D-galactose oxidase (Sigma Chemical Company, St. Louis, Missouri) at 5 U/ml. The sections were then rinsed in distilled water for 10 min and stained with Schiff's reagent (basic fuchsin) for 5 min. The sections were rinsed in running tap water for 10 min and counterstained with hematoxylin for 5 sec and one drop of saturated lithium carbonate. After rinsing again in tap water, the sections were dehydrated, cleared and mounted.

Negative controls were done by replacing the galactose oxidase with PBS (pH 7.0). The positive control tissue specimen was a previously diagnosed mucinous adenocarcinoma of the colon that stained magenta with galactose-oxidase-Schiff sequence.

Staining evaluation

The entire area of the tissue were examined by low-power (x10) optical fields and the approximate percentage of positively stained neoplastic cells were estimated. The results were graded as follows: «-» for absent, «1+» for mild, «2+» for moderate, «3+» for intense magenta coloration by Schiff's reagent.

Results

Normal colon and fetal gastrointestinal tissues

No expression of Gal-GalNAc was seen in the 8

normal colon tissues and negative control slides. In the fetal gastrointestinal tract, the surface epithelia (mucin-producing cells) of stomach (6/6) and the goblet cells of colon (6/6) were reactive with GO-Schiff sequence (Fig. 1), but no expression was seen in the small intestine (0/6).

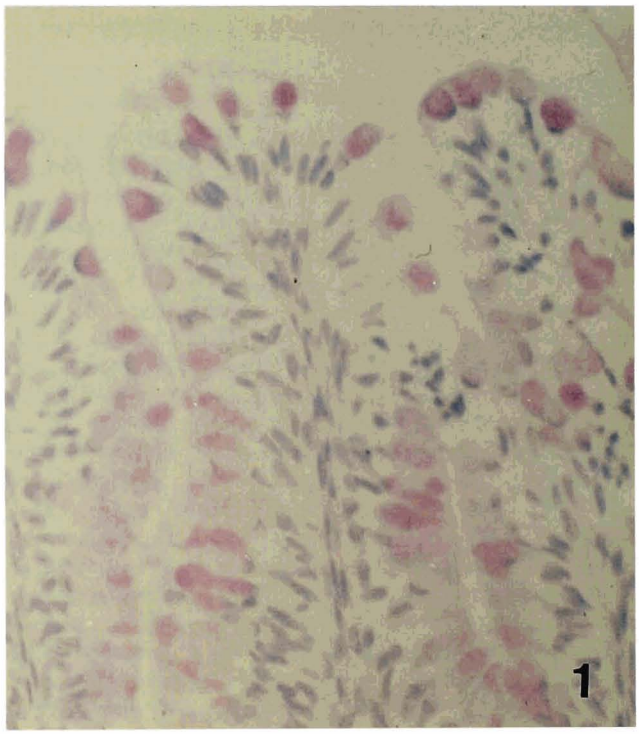

Fig. 1. Human fetal large intestine showing the expression of Gal-GalNAc tumor mucin marker (magenta color) in goblet cells. GO-Schiff sequence. x 500

Precancer of colon

The 34 tissue samples of colon from chronic ulcerative colitis and adenomatous polyps were stained by GO-Schiff sequence and the results are summarized in Table 1.

For chronic ulcerative colitis, the strongest expression of Gal-GalNAc was found in dilated and distorted crypts (also known as aberrant crypts) and in foci of dysplasia. The regenerative epithelia in ulcerative colitis, considered to be associated with the progression of carcinogenesis (hence called: regenerative dysplasia) also showed intense expression of Gal-GalNAc. Figure 2 shows a markedly dilated crypt from ulcerative colitis wherein the epithelial stains positive by GO-Schiff sequence. In polyps showing dysplastic crypts Gal-GalNAc expression can be seen in both the adenomatous components as well as in hyperplastic elements. Figure 3 shows expression of Gal-GalNAc by dysplastic (or early carcinomatous) crypts in a patient with ulcerative colitis.

Colon cancer and adjacent mucosa

Of the 20 cases of colorectal cancer, 15 (75%) expressed Gal-GalNAc. The pattern of expression was similar to the work published from this laboratory (Xu et al., 1992). Fifty percent of the «transitional» epithelia

Table 1. Gal-GalNAc expression in colonic polyps and ulcerative colitis.

	POSITIVE CASES/ TOTAL CASES	POSITIVE RATE
Chronic ulcerative colitis	6/13	46.2%
Adenomatous polyps	12/14	85.7%

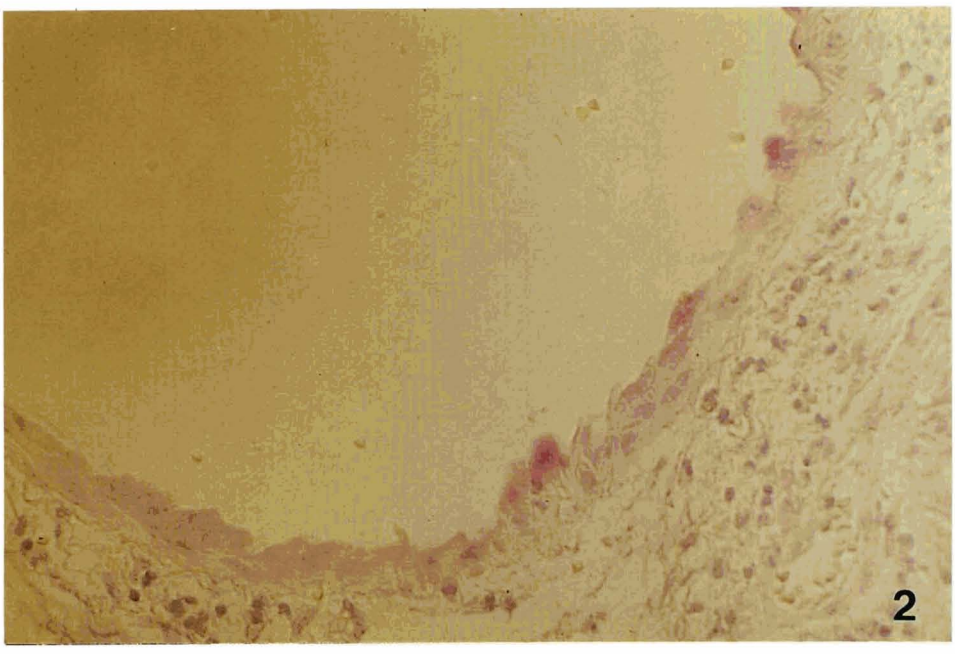

Fig. 2. Chronic ulcerative colitis, a markedly dilated crypt showing cytoplastic staining in epithelial cells. x 312.5

(adjacent to cancer), showed a positive reaction. The remote mucosa (far from cancer) though morphologically normal showed foci of Gal-GalNAc expression in 20% of samples as was reported by Xu et al. (1992).

Rat colon carcinogenesis

No expression of Gal-GalNAc was found in the normal and regenerative epithelia during wound healing in the 2 rats (Fig. 4).

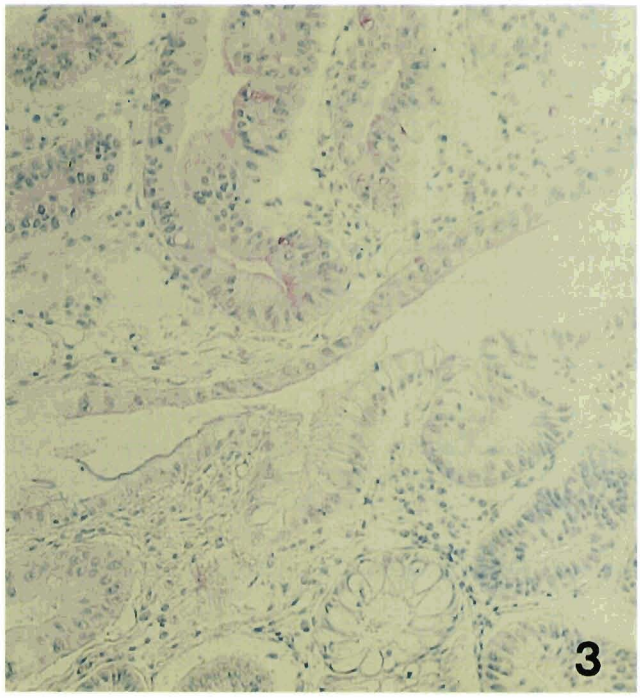

Fig. 3. Dysplastic crypts (or early carcinoma) of human chronic ulcerative colitis show Gal-GalNAc expression. x 312.5

In 8 cases of rodent colonic mucosa from carcinogenesis studies, Gal-GalNAc was not only expressed in cancer, but also in foci of dysplasia, in dilated and distorted crypts (so-called aberrant crypts) and in the morphologically «normal» glands away from cancer (Fig. 5), identical to the human remote mucosa.

Discussion

The disaccharide Gal-GalNAc, also known as T-Ag is a precursor of the M and N blood group substances, which is recognized by the lectin PNA or detected by antibodies. 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 that react with basic fuchsin to give magenta/purple coloration. While D-galactose oxidase reacts with both Gal-GalNAc and terminal monosaccharide galactose, D-galacto-hexoaldose converted from the latter may not be able to generate magenta coloration with basic fuchsin because of an atypical distance among the participating molecules (Avigad et al., 1962; Schlegel et al., 1968; Schulte and Spicer, 1983; Shamsuddin and Sakamoto, 1994). In contrast, PNA or antibodies that bind to either Gal-GalNAc (or related structures), or terminal galactose may be equally visualized by PNA or anti-T antibody (Shamsuddin and Sakamoto, 1994). This suggest the GO-Schiff sequence may be better for visualization of Gal-GalNAc. Furthermore, the rapidity and simplicity of GO-Schiff sequence makes it more practical.

With regard to the study of the tumor development in the colon, especially based on the result of the field effect of the carcinogen, the administered carcinogen

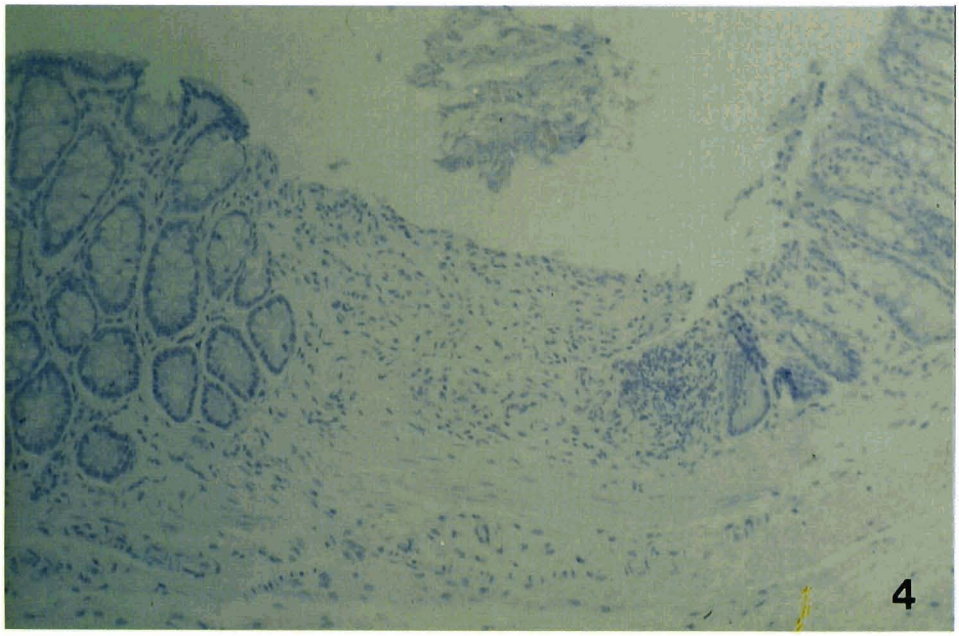

Fig. 4. Following wound healing and repair due to mechanical injury of colonic mucosa from rat show no Gal-GalNAc expression. x 312.5

acts on the entire epithelium causing multifocal changes throughout the field of action (Shamsuddin et al., 1981; Shamsuddin, 1991a), as evidenced by the presence of focal areas of atypia involving single or multiple crypts, dilated and atypical crypts in the large bowel mucosa found in the animal models treated with a chemical carcinogen and in the human samples (Deschner, 1974; Shamsuddin et al., 1981). These foci of dilated and distorted crypts as visualized by methylene blue are now called aberrant crypt foci (Bird, 1987; Pretlow et al., 1992) and have been observed in both experimental animals and in humans, which further support the hypothesis of field effect carcinogenesis (Shamsuddin and Sakamoto, 1994). In this study, we used the GO-Schiff technique to assess how and when Gal-GalNAc is expressed in the colonic mucosa during the progression of carcinogenesis. In other words, what kind of lesions or crypts express Gal-GalNAc marker during the early stage of carcinogenesis? In the human, while none of the normal colon tissues show expression of Gal-GalNAc, a high percentage of colon cancer and lesser proportion of precancerous lesions express the marker Gal-GalNAc. In the precancerous lesions and conditions, multifocal expression of the tumor marker Gal-GalNAc is seen in atypical or dysplastic crypts, dilated and distorted crypts and crypts showing regenerative dysplasia; some of the morphologically normal crypts remote from cancer also show Gal-GalNAc expression. The expression of Gal-GalNAc may be one of the earliest phenotypic abnormality that precede morphological changes of neoplasia (McKenzie et al., 1987; Xu et al., 1992) since identical results are seen in rat colon tissues undergoing carcinogenesis as shown in Figs. 4, 5. The expression is not non-specific since Gal-GalNAc was not expressed in the regenerative epithelia during wound healing following mechanical injury (Fig. 4). This strongly supports that a) Gal-GalNAc is a tumor marker and an intermediate end-point marker and b) multifocal premalignant and/or malignant changes may be seen in colon through field-effect action of the carcinogen(s).

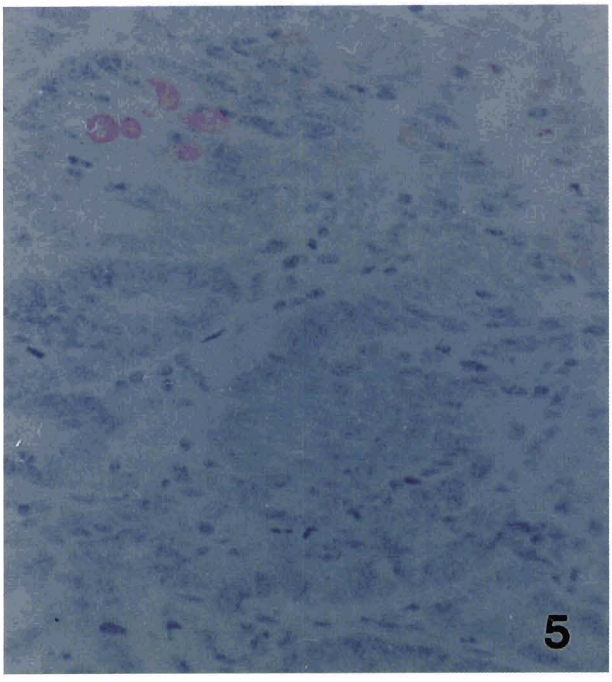

Fig. 5. Foci of neoplastic (or dysplastic or adenocarcinoma) gland from carcinogen treated rat show expression of Gal-GalNAc at the upper left. x 312.5

By using the 20-year estimate, simple calculation indicate that currently, there are approximately 10 million Americans in some phase of the development of the carcinogenic process that will result in their death (Parker et al., 1996). We need to identify these high risk or at risk individuals and to that end biomarkers are vital for screening strategy. Identification of such individuals as target population for chemoprevention is vital prior to beginning a trial. Our findings indicate that Gal-GalNAc is the biomarker of very early stage of colonic carcinogenesis, which is not only expressed in cancer and early cancer, but also present in various pre-cancerous lesions.

Kelloff et al. (1992) outlined six criteria for intermediate endpoint biomarkers of use in chemoprevention, and here is how Gal-GalNAc lives up to those expectations:

1) Is the intermediate biomarker differentially expressed in normal and high risk tissue? The data presented in this paper and elsewhere (Shamsuddin, 1991a) answer: Yes.

2) At what stage of carcinogenesis does the marker appear? The earlier a reliable marker appears in the carcinogenic process, the greater is the chance for successful intervention. Answer: Our data shown here demonstrate that Gal-GalNAc is expressed very early during carcinogenesis.

3) Does the marker and its assay provide acceptable sensitivity, specificity and accuracy? Answer: Both the marker (this report and Xu et al., 1992) and the assay (Sakamoto et al., 1993a; Zhou et al., 1993; Shamsuddin and Sakamoto, 1994) enjoy 70-100% sensitivity and specificity. That it is not expressed by regenerating cells following wounding is an added evidence that Gal-GalNAc is carcinogenesis specific.

4) How easily can the marker be measured? Answer: The rectal mucin test for detection of the marker is a non-invasive test done on mucus sample obtained during routine digital examination and the entire assay period is ≤ 15 min (Shamsuddin, 1991b).

5) Can the marker be modulated by chemo-preventive agents? Answer: We (Sakamoto et al., 1993b; Yang and Shamsuddin, 1995) have demonstrated that yes, indeed Gal-GalNAc expression can be suppressed by the chemopreventive/therapeutic agent IP-6. Following IP-6 treatment, HT-29 human colon carcinoma cells terminally differentiate and produce mucin, yet not Gal-GalNAc, akin to normal goblet cells (Sakamoto et al., 1993; Yang and Shamsuddin, 1995). Thus the Gal-GalNAc and IP-6 have great potential in our strategies for cancer prevention. Clinical studies are

now needed to validate this and Criteria#6 (Does modulation of the intermediate biomarker correlate with a decrease in cancer rate?); obviously the latter would require longer time and additional resources.

Thus the rectal mucus test for Gal-GalNAc by GO-Schiff reaction can be performed for mass screening of cancer and precancer and save health care cost. Gal-GalNAc may also serve as an intermediate biomarker to monitor the efficacy of chemoprevention.

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