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A Simple Mucus Test for Cancer Screening*

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Abstract. *Comparative and correlative studies of the pathology and pathogenesis of colon cancer in animal models and human disease have resulted in conceptualization of "field effect" theory and identification of markers that are expressed early during carcinogenesis. This assimilated body of knowledge has resulted in development of a simple rectal mucus test for colon cancer screening. The marker galactose-N acetylgalactosamine (Gal-GalNAC) is expressed in the rectal mucus of patients with colonic cancer or precancerous lesions and is detected by enzymatic oxidation (10 minutes) followed by color reaction (1 minute). The high sensitivity, specificity, positive predictive value and negative predictive value, as well as the cost-effectiveness of this test makes it a great tool in our strategies for early detection, hence control of colon cancer. Because of its high accuracy (as opposed to the fecal occult blood tests), it would reduce the number of unnecessary colonoscopies, thereby decreasing the total national health-care cost to the society. Similar expression of this marker in cancers of breast, lungs, prostate, pancreas, makes it a potentially useful general cancer screening test.*

Colorectal cancer is one of the commonest cancers in the industrialized world. It is ranked among the major causes of cancer death in the United States and other Western countries (1). Because of the magnitude of the health problem and associated cost in life and materials, control of this cancer is vital, as is any other dreadful disease. Prevention is one of the methods of cancer control, and detection of the cancer at the very early stage of the disease is fundamental to prevention. Early detection in its turn is dependent on screening the population for the disease (or those at risk). Without screening, a 50 year old person at average risk has approximately a 530-in-10,000 chance of developing invasive colorectal cancer during the rest of her/his life (2). A host of currently available diagnostic assays have been recommended and are in use for screening (3). The common ones are the fecal occult blood tests (FOBT), barium enema X-rays, and endoscopic visualization (3). The cost effectiveness of these vary tremendously; thereby, their use as screening assays are seriously in question, since to qualify as screening assays, they should be accurate, reliable, cost-effective with high acceptance by the population to be screened (2-7). On one hand, FOBTs are relatively cheap (~$10), compared to the cost of barium enema and colonoscopy, which could range from $250-800. While FOBTs are inexpensive, they are notoriously inaccurate and therefore not cost effective (2,3). On the other hand, the high accuracy of barium enema and endoscopies are marred by their high cost and subject discomfort (3). Notwithstanding the strong and persistent recommendation of radiologists and gastroenterologists respectively, these two diagnostic assays do not fit into the criteria of screening assays. Table I shows the difference in net saving in terms of cost per year of life gained from screening with assays that have different sensitivities.

The cost effectiveness of screening assays depend not only on their actual expense, but also on the sensitivity and speciffcity of the assays (sensitivity is the proportion of diseased subjects who have a positive test and specificity is the proportion of nondiseased subjects who yield a negative test result - ref 3). Perhaps because of the relative simplicity and low price, FOBTs have become part of the screening strategy for colorectal cancer despite their notoriously high inaccuracy. The sensitivity and specificity of the FOBTs are so poor that "Occult blood testing is, at best an imperfect approach to the screening of colorectal cancer" decries Ahlquist (8).

By merely increasing the sensitivity of FOBT from 25% to 40%, the U.S. Congressional Office of Technology Assessment estimates that the cost per year of life gained could be reduced by nearly 20%. Table I also shows that the

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cost as estimated, could easily be reduced by enhancing the sensitivity even when using FOBTs. The fundamental problem with the FOBTs are that they are based on the faulty premise of blood in stool being a marker of the cancer. The fact that our current strategies for colorectal cancer screening have failed (9) is a testament to the 2 faulty premises upon which they are based, viz: a) most cancers arise from pre-existing polyps and b) fecal blood is a marker of the presence of colorectal neoplasms. That blood is not a marker has been well acknowledged (9). The basis for the first premise is the study by Muto *et al* (10) who have shown merely the presence of cancer on polyps, but concluded without scientific evidence that most colorectal cancers arise from polyps. Using the same supposition, Winawer *et al* (11) report to have reduced the incidence of colorectal cancer by colonoscopic polypectomy. Now more than ever, critical analysis of these reports (as for any others) is warranted since we are in the midst of a debate on health care and its cost; reports like this (11) may have potential impact on our national health policy.

Flaws in current strategy. Since the rationale used by Winawer *et al* (11) is that adenomatous polyps are the precursor of colorectal cancer and removing them will prevent the cancer is also based on the paper by Muto *et al* (10), let's examine it. Muto *et al* (10) state:

1. "...the majority of adenomas do not become cancerous during a normal adult life span".
2. "The slow evolution of the polyp cancer sequence is stressed".

Figure 9 of their paper (10) shows the life history of 10 villous adenomas [who have an even higher potential of becoming malignant than the adenomatous polyps], "selected because they illustrate how these tumors can remain benign over a long period of time, although 2 eventually became malignant....In Case 8 the polyp-cancer sequence took at least 28 years"; 8 of those 10 villous adenomas (~80%) DID NOT become malignant for at least 22.5 years!

A rarely quoted paper by Kozuka *et al* (12), though published at the same time as Muto *et al* (10), report that the usual time for those rare polyps to become malignant (if and when) is 18 years! It is difficult to conceive as to how, an average follow-up period of merely 5.9 years (11) could be sufficient when the time span is at least 3-4 times as long. Even more interesting and to the point are the 5 cancers (rather "malignant polyps") detected during follow-up, "none of whom had rectal bleeding" pointing to a 100% false negative rate for the FOBT (11)!

If on the other hand, some cancers do arise from the flat non-polypoid mucosa, without having to go through the polyp (13), then how do we detect them? Shimoda *et al* (14) have shown that a large number, indeed the majority (~80%) of cancers of the large intestine do arise directly from the flat non-polypoid mucosa! Thus it is not totally surprising that the screening for colorectal cancer by FOBT has failed (9).

Insofar as screening for colorectal cancer is concerned

Table I. *Effect of sensitivity on cost-effectiveness*[4].

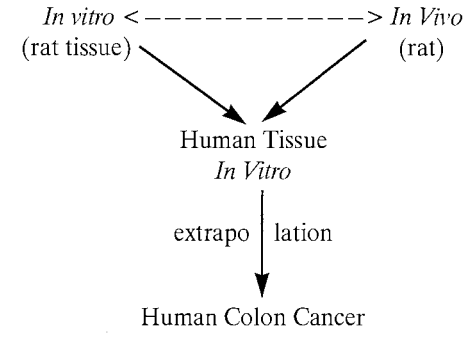
Sreening Regimen	Cost per year of life gained from screening FOBT		
	25% sensitivity	40% sensitivity	$ saving (%)
FOBT only[a]	$43,167	$35,054	$8,113 (19%)
FOBT + Sig[b]	$48,338	$42,509	$5,829 (12%)

[4]Adapted from Congressional Office of Technology Assessment-1990, [a]Once a year, [b]Sigmoidoscopy every 5 years.

Ransohoff and Lang (9) outlined the worthlessness of FOBT, and the prohibitive discomfort and high cost of screening colonoscopy is a common knowledge. Thus we need better assays for colorectal cancer screening that are based on expression of tumor markers, yet simple and acceptable not just to the health-care providers, but to the population at large; better assays for blood however is certainly not the answer. The new strategies must take into consideration the correct histogenesis of the cancer and markers of both cancer and precancers (3). The new assays must be based on expression of tumor markers (phenotypic or genetic), yet technically simple, and easy to administer; for turf, monetary and all other non-altruistic considerations aside, early detection strategy for this public health menace must be based on rational and established scientific facts taking into consideration the basic tenets of screening such as simplicity, cost-effectiveness, high sensitivity and specificity, compliance, acceptance by the public etc.

Thus assays which enjoy sensitivity rates higher than FOBT are most likely to reduce that cost even more and make screening cost-effective. These assays must be based on the markers that are expressed not only by the cancer, but also by the precancerous lesions so that the disease can be detected at a rather early stage.

Lessons from comparative pathology. Phenotypic alterations such as mucin histochemical changes associated with malignancy or premalignant lesions of the large intestine have been studied both in the human tissues as well as in the experimental models (15-19). An approach that compares and correlates the data obtained from *in vitro* and *in vivo* models using both experimental animals and human tissues and cells were utilized.



Based on such comparative and correlative studies across species and model systems, alterations in mucin biochemistry of both the intracellular and the secreted mucus in the large intestine has emerged as a consistent marker during the formation of cancer of the large intestine in the humans (16-19). Along with its emergence during cancer formation is its expression also in various conditions of the large intestine that are known to carry a high risk of subsequent progression to cancer (e.g. polyps, inflammatory bowel diseases etc.). The altered mucin is expressed not only in the cells that are cancerous and precancerous, but is also found in the otherwise morphologically normal appearing cells away from the cancer. Observation of mucin abnormalities in the normal appearing mucosa away from cancer was not new. Filipe and Branfoot (15) had earlier made such observation, its exact significance was however not understood perhaps in view of the rigid adherence to the "polyp-cancer only" theory of genesis of colon cancer. Based on parallel *in vivo, in vitro* studies in experimental models and extrapolation of the finding and comparison with human tissues bearing cancer or precancer, I forwarded an explanation for this phenomenon as being the result of generalized field-effect of the carcinogenic stimuli (16,19). I had also proposed that in light of these observed changes, it should be possible to devise alternate strategies for early detection of cancer(16,19).

Mucin markers. The mucin in the gastrointestinal tract consists of mucopolysaccharides, the glycoprotein. The characteristic feature of the mucopolysaccharide is that terminal moiety of the oligosaccharide side chain consists of *N*-acetylneuraminic acid (NANA, or sialic acid), and is negatively charged. The sialic acid residue is transferred to the terminal galactose or penultimate *N*-acetylgalactosamine (GalNAc) through the action of specific sialosyltransferase. Mucopolysaccharide is traditionally classified into either neutral or acidic.

Histochemical examination of a normal human large intestine indicates that the mucus in the crypts of ascending colon consists of a mixture of acidic and neutral mucin with a predominance of the latter. In contrast, the mucus in the rectal crypts is almost exclusively acidic in nature (20). These topological differences in the mucin histochemistry alter with malignant transformation, the phenomenon presumably attributed to the quantitative and/or qualitative composition of terminal sialic acid in the mucus glycoprotein. I had demonstrated in rats that, in contrast to the presence of sulfomucin in normal colon mucosa, abnormal sialomucin was detected both *in vivo* (16,17) and *in vitro* (16,18) shortly after treatment with the carcinogens. Similar to the experimental observations, a shift from normal sulfomucin to abnormal sialomucin was also demonstrated in the extensive comparative studies of human colon (16,19). The altered expression of the colonic mucin is observed not only in the carcinomas but also in the crypts of morphologically normal-appearing mucosa adjacent to and distant from the carcinomas both in humans (14) and mice with dimethylhydrazine (21) or rats treated with azoxymethane (17,22). As mentioned earlier, these observations were first reported by Filipe *et al* (15,22), but the reason and the exact significance of the abnormal mucin expression in the normal appearing mucosa were inexplicable. Based on my studies of comparative pathology, I have offered the field effect phenomenon (3,16,19).

Field effect theory of colon carcinogenesis. The normal appearing colonic mucosa that is far distant from the carcinoma site sporadically harbors a wide variety of progressive changes. These multifocal changes are commonly observed in the entire colon not only from the experimental animals treated with the carcinogens, but also from the human specimen resected at surgery (16,19). Based on the morphological and histochemical observations, I hypothesized a field effect carcinogenesis that the alterations in the normal appearing mucosa are perhaps multifocal areas of initiated (but not fully promoted) foci and that these may be predictors of the cancer away from their site of sampling (3,16,19). In other words, as a result of the generalized effect of the carcinogen throughout the entire field of the target tissue (viz. the colonic epithelium), it is most likely that the mucosa away from an obvious cancer would be abnormal. Thus the rationale for testing the mucin of the rectum, particularly since rectum is a convenient sampling site.

I rationalized that (a) the presence of cancer in the large intestine implies previous exposure of the host to carcinogens, (b) most carcinogens act by way of the "field effect" where the entire target tissue is subjected to the carcinogenic stimuli, (c) carcinogens induce multifocal changes throughout the entire target tissue viz. colorectal mucosa, (d) of the many initiated sites, only some of them may be promoted to a recognizable carcinoma. Thus the alterations in the normal appearing, initiated but not promoted mucosa may express some of the markers of cancer and precancer. Since mucin is secreted by the colorectal mucosa and can easily be sampled from the rectum, I thus embarked on exploiting this fact, in conjunction with the altered mucus of cancer in developing screening assays.

D-Galactose-ß(1→3)-N-acetyl-D-galactosamine. The disaccharide D-Galactose-ß(1→3)-*N*-Acetyl-D-galactosamine (abbr. Gal-GalNAc), also knows as T-Ag (for Thomsen-Friedenreich antigen) is a precursor substance of the M and N blood group antigen determinant. Transfer of sialic acid (NANA or *N*-acetyl-neuraminic acid) residues to T-Ag confers blood group M and N specificity. The T-Ag determinant Gal-GalNAc is recognized by the lectin peanut agglutinin (PNA) which is purified from *Arachis hypogaea (23). T* antigen is also detected by polyclonal or monoclonal anti-T antibodies.

T Ag is not expressed by the normal colonic mucosa, but extensively expressed by the fetal colon as well as by the colon

cancer cell, which is detected by PNA, anti-T Ag antibodies, or enzymatic oxidation (3,24-27). Not only is the T-Ag expressed by cancer, its expression by precancerous lesions as well as by the normal appearing mucosa remote from cancer has been observed (3,27). Since the enzymatic detection is simple and is the basis of the simple screening assay, the subject matter of this paper, here follows a description of the method.

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 (Text Figure 1).

Schulte and Spicer (28) first demonstrated the use of galactose oxidase - Schiff procedure (GO-Schiff) to study the T Ag in rat tracheal gland secretory glycoproteins. Shamsuddin *et al* then applied this technique to detect the marker Gal-GalNAc in the precancer and cancer of the colon (3,29-32). 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. In contrast, PNA that binds to either Gal-GalNAc (or related structures), or terminal galactose may be equally visualized by the second antibody (or conjugate) that is specific to PNA. This assumption is supported by the fact that not all tumors, tissues, or cells showing PNA reactivity may not be stained positive with GO-Schiff sequence, and *vice versa* (3,32). I had postulated that the abnormal mucin in the crypts or in the lumen of the carcinoma as well as normal appearing mucosa away from the carcinoma site could be exploited as one of the tumor markers (3,31).

Rectal mucin test. Rectal mucin test exploits the mucus samples in the rectum obtained at the occasion of finger examination, and detects the presence of the marker D-galactose-ß-[1→3]-*N*-acetyl-D-galactosamine (abbr. Gal-GalNAC) in the mucus. Thus, the presence of Gal-GalNAc in the rectal mucin would imply the existence of an abnormal mucosa somewhere in the colorectum. The abnormality may be either cancerous or precancerous lesions, or the clinical state of precancerous condition since the mucus samples from normal subject do not express the marker. The term 'precancerous lesion' indicates pathological lesions that carry a high risk of progressing to cancer, whereas 'precancerous conditions' are clinical diseases or conditions that increases the risk of the patient to cancer. Although several assay using the rectal mucin have been developed (3), only the Galactose Oxidase Test will be described here. The test procedure is as follows:

1. Examine the rectum with finger.
2. Smear mucus sample onto nitrocellulose membrane filter.
3. React with D-galactose oxidase (100U/ml, pH 7.2. 10 min, room temperature)

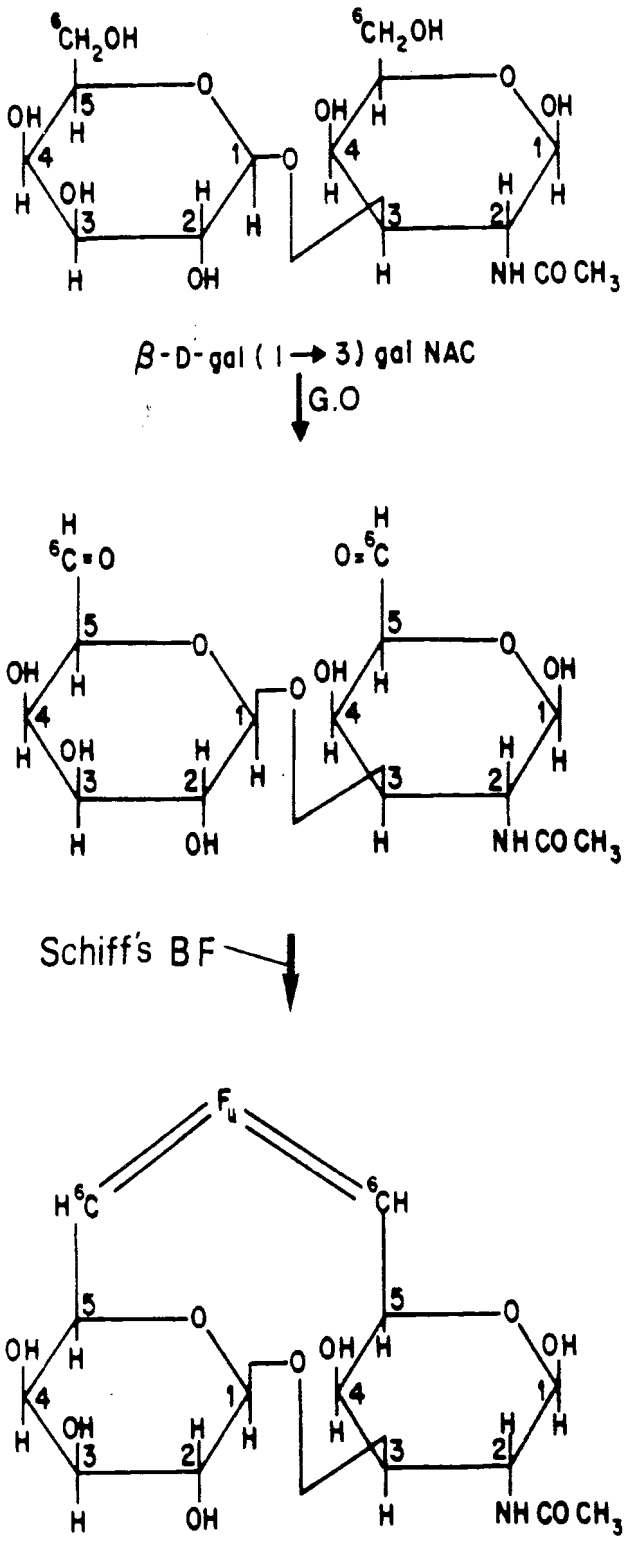


Figure 1. *Principle of the Galactose Oxidase-Schiff assay. The enzyme galactose oxidase specifically oxidizes C-6 hydroxyl groups of D-galactopyranose and N-acetyl-D-galactosamine residues of D-galactose-ß-[1→3]-N-acetyl-D-galactosamine. This generates two vicinal aldehyde groups that react with basic fuchsin (Schiff's reagent) to give magenta/purple coloration.*

4. Wash briefly with distilled water
5. React with Schiff's reagent (1 % basic fuchsin, 1 min)
6. Rinse in running tap water, dry, evaluate for color reaction.

It should be kept in mind that a false negative result could be due to sampling error. An additional step of reaction with periodic acid-Schiff sequence will ensure against that possibility. For further detail on this assay, please consult reference 3.

Performance of the galactose oxidase test. Since the publication of the pilot study by Shamsuddin and Elsayed (29,30), various investigators throughout the world have evaluated the sensitivity and specificity of this test for detecting colorectal cancer (33-43). Please see ref 33 for a summary of the results by various investigators. Most of these studies varied markedly in their design thus accounting for the variation in specificity; the sensitivity of the assay is rather consistently high. Sakamoto *et al* (44) first used this test to screen asymptomatic population and detected one case with focal cancer in adenoma. Although their evaluation of the subjects with colonoscopy and/or barium enema fluoroscopy was inadequate, they reported 92.2% specificity.

That study, as well as the rest were done on a relatively small (hundreds) sample size. Like Sakamoto *et al* in Japan, Zhou and co-workers performed a similar second study on 6,480 asymptomatic subjects in China (45). The specificity of the assay was evaluated in a subset of 2,660 asymptomatic individuals undergoing sigmoidoscopy. Only 228 individuals elicited a positive test result, of which 17 had adenomas and 2 carcinomas, giving a specificity rate of 97.61%. The assay done on an additional 924 individuals, reported in the Proceeding of Chinese Pathology Research Group for Colorectal Cancer, GO-S Team (46) showed a similarly high sensitivity (94.4%) and specificity (98.23%); the positive predictive value was 58.92% and the negative predictive value was 99.88%!

The importance of choosing the proper study population can not be over emphasized. The dilemma of the sensitivity and the specificity of the assay compared to the socalled gold standard of colonoscopy have resulted in, and will continue to conduction of studies that are less then well-designed. In a study of 670 persons undergoing colonoscopy, Kristal and co-workers (43) reported a sensitivity of 33% and 39% for colon cancer and 'cancer or polyp', respectively if the rectal mucus sample is collected after the colonoscopic preparation. It is however not the intention of this screening test, for that matter any screening test for colon cancer and precancer to be done after the patient is prepared for colonoscopy, it defeats the purpose of screening (47)! Nevertheless, being cognizant of this, Kristal *et al* subsequently tested the sensitivity and specificity of the assay by collecting the rectal mucus sample prior to colonoscopic preparation, a research method not too distant from the practical intended application; this simple modification of sampling time resulted in a rather dramatic increase of sensitivity for 'cancer or polyp' to 89%, and for cancer to 100% (43)! Curiously, the reader would note that this 100% sensitivity reported by Kristal *et al* (43) using 670 patients, and > 92% specificity reported by Sakamoto *et al* (39,44) on 330 asymptomatic Japanese and > 97% specificity by Zhou *et al* (45) on 6,480 Chinese are not different from the figures I had published from the pilot study done on only 73 people (30). Table II compares the accuracy and costs between the currently popular fecal occult blood tests (FOBT) and the galactose oxidase test if used for screening large intestinal cancer.

Table II. *Comparison between FOBT and Mucus Test in colon cancer screening.*

Parameters	Fecal Occult Blood Test	Galactose Oxidase test
Sensitivity[a]	4.5% - 50.0% [54,55]	80.0 - 100.0%
Specificity[a]	4.3% - 50.0%[56]	92.4 - 100%
Stability	<5 days	>8 years
Restriction	diet/drug	none
Discomfort	aesthetic	minimal
Required #	6	1
Total cost	$10. 00[57]	$10. 00

[a]for cancer and polyp. the best and worst figures from published reports are given: please refer to specific references for detail.

Detection of extra-colonic malignancies. Following metabolic activation of the environmental carcinogenic agents, the active metabolite may be excreted *via* the bile, lungs, kidneys, large intestine, skin etc. Thus the hallmark of carcinogenic exposure, the phenotypic alterations may be observed in these organs as well. Studies in the prostate gland have shown that the same marker Gal-GalNAc is expressed by premalignant and malignant lesions but not the normal or hyperplastic glands (48). An ongoing trial shows the feasibility of the assay in detecting the marker in prostatic massage secretion in screening for prostate cancer. Evidence for the extension of this assay in the secretion of other organs such as the breast, lungs, pancreas etc is also provided by the fact that the same marker is expressed not only by these cancers, but also the remote non-cancerous areas as well as their secretions, thus operation of a field effect phenomenon (49). It is quite possible that the marker could likewise be detected in the nipple secretion or bronchial mucus specimen and thus aid in screening for malignancies in those organs.

Since changes in rectal mucus is indicative of the field effect of the carcinogen in the large intestine, could it also reflect exposure and malignancy in extra-colonic sites?.

According to pilot studies in Japan, the mucin test appears to be have potential in detecting not only colorectal cancers, but also extra-colonic malignancies (31,33). Watanabe and co-workers (50) detected not only colorectal cancers and polyps, but also gastric cancers and polyps by using rectum as the sampling site for mucus. Thus owing to the general field effect of the carcinogen(s), cancers from different organ sites may be detected by assaying for the marker(s) in rectal mucus.

Intermediate marker modulation in cancer prevention. Does every person with a positive test for Gal-GalNAc has colorectal cancer? Obviously not since the marker is not only for cancer, but also for precancerous lesions and conditions. Let's take the case of an individual who has repeatedly positive rectal mucin assay for Gal-GalNAc, but careful diagnostic examinations [such as barium enema, complete colonoscopy] reveals no obvious mass lesion. The presence of the marker indicates that the cells are abnormal, but not necessarily cancerous. If we consider this individual to be at a high risk and give prophylactic chemopreventive agent, we could reduce or perhaps reverse the risk of cancer. In this instance the marker Gal-GalNAc would not be expressed any more indicating that the individual is no longer at a high risk.

In vitro studies provide some support for such an optimistic scenario. The human colon cancer cell line HT-29 does express Gal-GalNAc. Inositol hexaphosphate ($Ins P_6$ or IP6), a naturally occurring carbohydrate is a potent anti-cancer agent with chemopreventive and chemotherapeutic properties (51). Treatment of HT-29 cells with IP_6 results not only in the reduction of cell number, but also a near-total suppression of Gal-GalNAc expression (52,53). Thus, Gal-GalNAc detected in the rectal mucus has great potential as an intermediate marker for not only screening, but also monitoring of people at high risk for cancer.

Conclusion

This simple screening test is based on a tumor marker expressed during carcinogenesis. The marker is i) differentially expressed in cancer and precancerous lesions and conditions, but not in normal; ii) appears early during carcinogenesis; iii) the marker and the assay enjoy a high sensitivity and specificity; iv) is easily identifiable and measured; and v) can be modulated by chemopreventive agents. Identification of this marker in mucus is a non-invasive process; the mucus sample can be obtained during rectal examination by a physician which is a routine procedure in the clinical practice. Besides the diseases of the rectum, that of the prostate in males and diseases of the uterus and adnexa in the females can be detected by this routine and simple examination. Expression of the marker in breast, prostatic, bronchial and pancreatic secretion makes it potentially useful for cancer screening in general. In the colon, the accuracy of this test is highly likely to reduce the number of colonoscopies that are now being performed unnecessarily, thereby reducing the health care cost of the society. Quantitative assays measuring the concentrations of Gal-GalNAc in mucin might be of use in predicting the malignant potential (prognosis) of precancerous lesions.

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