SUMMARY - The sialic acid-free disaccharide D-galactose-β-(1→3)-N-acetyl-D-galactosamine (Gal-GalNAc) is expressed in mucous secretions of various epithelial cancers including those of the colon, breast, lung, prostate, pancreas, etc. The expression of Gal-GalNAc in "normal" tissues and their secretions away from, but within the general field of cancers indicate the operation of a field-effect phenomenon (Shamsuddin et al., Cancer Res 1995;55:149-52). Gal-GalNAc in tissues or in mucous secretions can be easily detected by enzymatic oxidation (10 minutes) followed by color reaction by Schiff's reagent (1 minute). Using the rectal mucus as a sample, this test has yielded a very high sensitivity, specificity, positive predictive value and negative predictive value for colon cancer screening. Together with its cost-effectiveness it offers as a great tool in our strategies for early detection and, hence the 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 health-care cost to the society.

Similar expression of this marker in cancers of breast, lungs, prostate, and pancreas makes it a potentially useful general cancer screening test by using their secretions, eg, nipple secretions or aspirate, prostatic massaged secretions, bronchial secretions and sputum, etc. Quantitative assays could be of additional benefit; alternatively, the same principle could be used for other markers.

Key words: tumor marker, Gal-GalNAc, field-effect, colorectal cancer
INTRODUCTION

Cancer is one of the commonest causes of death in the industrialized countries; in the United States for example, it is the cause of an estimated >500,000 deaths, ranking second after heart disease (1). 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, for instance, a 50-year-old person at average risk has approximately a 0.5% 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). Screening tests must, however, meet certain criteria. They must have high sensitivity and specificity (Table 1) and, more importantly, acceptable predictive values (positive and negative). The positive and the negative predictive values (PPV and NPV) are dependent on the prevalence of the disease in the population. As an example, a screening test with a 99% sensitivity and specificity will have a PPV = 0.97 in a population with 25% prevalence of the disease, as opposed to only 0.09 if the disease has a prevalence of about 1 per 1,000 in another population. Besides, there are issues of reliability and biases to be considered, let alone the cost-effectiveness of the assays (3, 4).

<table>
<thead>
<tr>
<th>Test result</th>
<th>Disease present</th>
<th>Disease Absent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>a</td>
<td>b</td>
</tr>
<tr>
<td>Negative</td>
<td>c</td>
<td>d</td>
</tr>
</tbody>
</table>

a: true positive  b: false positive  c: false negative  d: true negative
Sensitivity: a/(a + c)  Specificity: d/(b + d)

Note that positive predictive value (a/(a + b)) and negative predictive value (d/(c + d)) are dependent on the prevalence of the disease in the population.

Colorectal cancer screening

Let’s use colorectal cancer as an example. Assays that are commonly used for screening are the fecal occult blood tests (FOBT), barium enema X-rays, and endoscopic visualization (3). Their cost-effectiveness vary tremendously so that their use as screening assays are seriously in question, since to qualify as screening assays they should be accurate, reliable, and cost-effective, with high acceptance by the population to be screened (2-12). On one hand, FOBTs are relatively cheap (~$10), compared to the cost of barium enema and colonoscopy, which could range from $150-1500 (12). While FOBTs are inexpensive, they are notoriously inaccurate and therefore not cost-effective.
Abulkalam M. Shamsuddin Carbohydrate Tumor Marker

(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 forceful and relentless advocacy of radiologists and gastroenterologists, these two diagnostic assays do not fit into the criteria of screening assays.

The fundamental problem with the FOBTs are that they are based on the faulty premise of blood in stool being a marker of cancer. The fact that our current strategies for colorectal cancer screening have failed (11) is a testament to the 2 faulty premises upon which they are based, viz: a) fecal blood is a marker of the presence of colorectal neoplasms, and b) most cancers arise from pre-existing polyps. That blood is not a marker has been well acknowledged (11). Insuff as screening for colorectal cancer is concerned, Ransohoff and Lang (11) outlined the worthlessness of FOBT and the prohibitive discomfort and high cost of screening colonoscopy is a common knowledge. Therefore we need better assays for colorectal cancer screening that would be scientifically based on expression of reliable tumor markers, yet simple and acceptable not just to the health-care providers, but to the population at large. The new strategies must take into consideration the correct histogenesis of the cancer and expression of markers of both cancer and precancerous lesions (3). If a marker detects only cancers, but not precancerous lesions or diseases, then it may not be effective [too late] in offering any realistic chances of prevention. The new assays must be based on expression of tumor markers (phenotypic or genetic), yet technically simple and easy to administer; 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.

Comparative and correlative studies of colon cancer across species and model systems have pointed towards alterations in mucin biochemistry of both the intracellular and the secreted mucus in the large intestine as being a consistent marker during the formation of cancer of the large intestine in humans (3, 12-22). Along with its emergence during cancer formation, its expression occurs 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 cancerous and precancerous cells, but is also found in the otherwise morphologically normal cells away from the 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 the explanation for this phenomenon as being the result of generalized field-effect of the carcinogenic stimuli and proposed that in light of these observed changes, it should be possible to devise alternate strategies for early detection of cancer (3, 13, 16, 18, 22).

Field effect theory of carcinogenesis

In the colon, the normal appearing mucosa remote from the carcinoma sporadically harbors a wide variety of progressive changes. These multifocal

BIOCHEMIA MEDICA god. 6, br. 4, 1996. 267
changes are commonly observed in the entire colon not only from the experimental animals injected with carcinogens, but also from human cancer specimens resected at surgery. Based on the morphological and histochemical observations, I hypothesized that the alterations in the normal appearing mucosa are perhaps multifocal areas of initiated but not promoted foci and that these may be predictors of the cancer away from their site of sampling (3, 13, 16). In other words, as a result of the generalized effect of the carcinogen throughout the entire field of the target tissue (viz. the colorectal epithelium), it is likely that the mucosa away from an obvious cancer would be abnormal. This is the basis for testing rectal mucin, 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) only some of many initiated sites 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.

Investigations of other epithelial cancers, including the lungs, breast, pancreas, etc., show that the same field effect phenomenon may be operational in them as well (23). The altered mucopolysaccharide was expressed by normal-appearing epithelium (e.g. ducts) away from the carcinoma. Not only was it expressed by cells, but the secretions were also positive for the marker (23). Clearly this provides an easy means of sampling of the marker to identify the underlying neoplasm; for instance, bronchial mucus or sputum for lung cancer, nipple aspiration or secretions for breast cancer, etc.

Since mucin is secreted by the colorectal mucosa and can easily be sampled from the rectum, I first embarked on exploiting this fact in developing screening assays for colorectal cancer. Since the same marker alteration is observed in a host of other epithelial cancers by way of the field effect, it is quite plausible that they too could be candidates for screening by using their secretions.

The carbohydrate tumor marker

The disaccharide D-galactose[β(1→3)]N-acetyl-D-galactosamine (abbr. Gal-Gal-NAc), also known 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 Aβ2-acetyl-neuraminic acid) residues to T-Ag confers blood group M and N specificity. In normal human epithelial and other cells, the terminal sialic acid residue is attached to the penultimate galactose through α2→4 or α2→6 linkage. Derivatives of d-galactose having substituents on the hydroxyl group at C-4, or the 2-amino-2-deoxy-d-galactose having glycosyl substituents at C-3, are not oxidized by the enzyme d-galactose oxidase (24).
On the other hand, removal of the sialic acid by sialidase or neuraminidase results in unmasking of the Gal-GalNAc residue which can then be visualized. 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.

Rectal mucin test for colorectal cancer

Exploiting the mucous samples in rectum obtained at the occasion of digital examination, the marker Gal-GalNAc is detected by a very simple two-step procedure. Concerning the field effect theory, 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 cancer 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 increase the risk of the patient for cancer. Although several assays using rectal mucin have been developed (3), only the Galactose Oxidase Test will be described here. The test procedure is as follows:

1. Smear rectal mucus sample onto nitrocellulose membrane filter
2. React with galactose oxidase 100U/ml, 10 min, room temp.
3. Wash briefly with distilled water
4. React with Schiff’s reagent (1% basic fuchsin, 1 min)
5. Rinse in running tap water, dry, evaluate for color

It should be kept in mind that a false 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 (25-27), many others have evaluated the sensitivity and specificity of this test for detecting colorectal cancer (28-41). Please see reference 22 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. (39) first used this test to screen asymptomatic population and detected one case with focal cancer in adenoma, reporting 92.9% specificity.

That study, as well as the rest were done on a relatively small (hundreds) population size. Like Sakamoto et al. in Japan, Zhou and co-workers performed a similar second study on 6,480 asymptomatic subjects in China (40). 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 (41) showed a similarly high sensitivity (94.4%) and specificity (98.23%); the positive predictive value was 58.02% and the negative predictive value was 99.86%. Considering the fact that China is a country with a relatively low prevalence of colon cancer, the predictive values were remarkably good.

The issues of sensitivity and specificity of the assay compared to the so-called gold standard of colonoscopy have resulted in conduction of studies that were less than well-designed (37, 38). In any event, compared to the FOBTs, it offers quite a few advantages (Table 2) in screening for colorectal cancer.

### Table 2 — Comparison between FOBT and Mucus Test in colon cancer screening

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Fecal Occult Blood Test</th>
<th>Galactose Oxidase Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity*</td>
<td>4.5% - 50.0%</td>
<td>80.0% - 100.0%</td>
</tr>
<tr>
<td>Specificity*</td>
<td>4.3% - 50.0%</td>
<td>92.4% - 100%</td>
</tr>
<tr>
<td>Stability</td>
<td>5 days</td>
<td>8 years</td>
</tr>
<tr>
<td>Restriction</td>
<td>diet / drug</td>
<td>none</td>
</tr>
<tr>
<td>Discomfort</td>
<td>aesthetie</td>
<td>minimal</td>
</tr>
<tr>
<td>Required #</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>Total cost</td>
<td>$10.00</td>
<td>-$10.00</td>
</tr>
</tbody>
</table>

* for cancer and polyp, the best and worst figures from published reports are given; please refer to specific references (# 48-50) for detail, for galactose oxidase test please see references # 3 and 22.

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 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 (42). 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 confirming the operation of a field effect.
phenomenon (23). 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 are 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 have potential in detecting not only colorectal cancers, but also extra-colonic malignancies (22). Watanabe and co-workers (43) 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 have colorectal cancer? Obviously not, since the marker is not only for cancer, but also for precancerous lesions and conditions. Let’s take the example of an individual who has had repeatedly positive rectal mucin assay for Gal-GalNAc, but careful diagnostic examinations (such as barium enema, complete colonoscopy) reveal 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 high risk and give prophylactic chemopreventive agent, we could reduce or perhaps reverse the risk of cancer. In this instance the marker Gal-GalNAc is not expected to be expressed any more, indicating that the individual is no longer at high risk.

In vitro studies provide some support for such a strategy. The human colon cancer cell line HT-29 does express Gal-GalNAc. Inositol hexaphosphate (InsP6 or IP6), a naturally occurring carbohydrate is a potent anti-cancer agent with chemopreventive and chemotherapeutic properties (44). Treatment of HT-29 cells with IP6 results not only in the reduction of cell number, but also a near-total suppression of Gal-GalNAc expression (45, 46). Thus, Gal-GalNAc detected in rectal mucus has great potential as an intermediate marker for not only screening, but also in monitoring of people at high risk for cancer.

The U.S. National Cancer Institute outlined six criteria for intermediate endpoint biomarkers of use in chemoprevention (47). Here is how Gal-GalNAc lives up to these expectations:

1) Is the intermediate biomarker differentially expressed in normal and high risk tissue? The data (3, 19, 20-22, 42) 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: Data show that Gal-GalNAc is expressed very early during carcinogenesis, at least in the colon (19-21) and perhaps in the prostate (42).
3) Does the marker and its assay provide acceptable sensitivity, specificity and accuracy? Answer: Both the marker (3, 19-23, 42) and the assay (24-41, 43) enjoy 70%-100% sensitivity and specificity. The fact that it is not expressed by regenerating cells following wounding is an added evidence that Gal-GalNAc is carcinogenesis-specific (21).

4) How easily can the marker be measured? Answer: The detection of the marker for colon cancer screening is a non-invasive test done on mucus sample obtained during routine digital examination and the entire assay period is ≤15 min (3, 48). Plans to use other body secretions such as sputum or bronchial mucus, nipple aspirate or secretions, prostatic massage secretions, are all simple and non-invasive or minimally invasive.

5) Can the marker be modulated by chemopreventive agents? Answer: We have demonstrated that yes, indeed Gal-GalNAc expression can be suppressed by the chemopreventive/therapeutic agent IP6. Following IP6 treatment, HT-29 human colon carcinoma cells terminally differentiate and produce mucin, yet not Gal-GalNAc, akin to normal goblet cells (45, 46). Thus Gal-GalNAc and IP6 have great potential in our strategies for cancer prevention. Clinical studies are now needed to validate this and Criterion #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 testing for Gal-GalNAc by GO-Schiff reaction should be used in mass screening of cancer and preneosc and save health care cost. Gal-GalNAc may also serve as an intermediate biomarker to monitor the efficacy of chemoprevention.

Conclusion

This simple screening test is based on a tumor marker expressed during carcinogenesis. The marker i) is differentially expressed in cancer and precancerous lesions and conditions, but not in normal ones; 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 or an assistant, which is a routine procedure in 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 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, demonstrated to be high, is likely to reduce the number of colonoscopies that are now being performed unnecessarily, thereby reducing the health care cost of the society.

Extension of this assay in other body fluid secretions may prove to have similar potential in net savings of not just health-care cost, but also prolonged human health.

Sličan izražaj ovog biljega u karcinima dojke, pluća, prostate, i gušterače čini ga mogućom korisnom općom pretragom za pretraživanje karcinoma na temelju njihovih lučenja, upr. iscjetka dojke ili aspirata, sekreta prostate, bronhijalnih sekreta i ispljuvaka, itd. Kvantitativne analize bi mogle biti od dodatne koristi; u zamjenu, isto načelo bi se moglo upotrijebiti za druge biljage.

**Ključne riječi:** tumorski biljeg, D-galaktoza-P-[l->3]-N-acetil-D-galaktozamin, učinak polja, karcinom deblog crijeva.

**REFERENCES**


23. Shamsuddin AM, Tyner GT, Yong GV. Common expression of the tumor marker β-galactosidase (β-galactosidase): N-acetyl-D-galacto-


34. Veev A, Pongoljik B, Budi D, Cukelic I, Veger A, Prodl I, Kruce Z. The galactose...


