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Light-scattering Technologies for Field Carcinogenesis Detection: A Modality for Endoscopic Pre-screening

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Abstract

Colonoscopy has revolutionized colorectal cancer (CRC) screening resulting in a decrease in both CRC mortality and incidence. Despite this, CRC still ranks as the second leading cause of cancer deaths among Americans underscoring the need to both increase availability and accuracy of colonoscopy. The latter considerations provide the impetus for much of the current research into adjunctive imaging technologies. Recent advances in improving detection of dysplasia that have translated into clinical practice include high-definition scopes, narrow-band imaging, and chromo-endoscopy. Another major direction of research into improving endoscopy is determining histology of lesions *in situ* (“optical biopsy”) with confocal endomicroscopy, fluorescence and elastic scattering spectroscopy. All these techniques are of great promise in improving delivery of endoscopy but, to date, have not addressed the potentially more important hurdle associated with logistic challenges of providing accurate CRC screening for the entire at-risk population.

The Need for Risk-stratification

The critical but heretofore relatively unexplored issue in endoscopic screening is the need for risk-stratification. According to existing guidelines, every patient over the age of 50 is a candidate for colonoscopy¹. However, the majority of the eligible population does not receive recommended screening colonoscopies. Providing colonoscopy for the entire average-risk population (>100 million Americans over age 50) is impractical because of financial concerns (estimates of cost plus the economic impact range from \$22 to 50 billion annually), resource constraints (insufficient number of endoscopists), complication rate (small but significant when applied to large populations), and patients’ non-compliance (due to fear of complications, discomfort of the colonic purge, etc.). This is juxtaposed with the fact that in the average-risk population the yield of screening-relevant neoplasia (advanced adenomas or early stage carcinomas) is remarkably low (~5–6%). Even in patients with a personal history of adenomas (indication for ~20% of colonoscopies), the yields are low, ~10%. Thus, over 90% of the procedures do not engender cancer preventive ramifications (removal of significant lesions)².

In order to maximize the benefit to the population from the ~18–20 million colonoscopies performed in the United States annually, it has been advocated to increase the intervals between colonoscopy since the most of the benefit from colonoscopic surveillance is derived from the first procedure^{3–4}. While justified from the societal point of view, from an individual’s perspective, this is balanced by concerns of missed lesions (~5% of patients diagnosed with CRCs have had “negative” colonoscopies within the previous 3–5 years)⁵.

Furthermore, emerging evidence suggests that colonoscopy may be less effective (and possibly ineffective) in preventing right-sided CRCs⁶. These may be the central reasons behind the general overuse of colonoscopy with the negative consequences for cost, complications and endoscopic capacity.

Thus, instead of a triage based simply on age or endoscopic findings, more accurate assessment of risk is urgently needed. Instead of performing colonoscopy on the entire population over 50, preselecting patients harboring significant lesions would allow the focusing of the finite endoscopic resource on subjects who would have a cancer preventive benefit (i.e. undergo concurrent polypectomy) (Supplement Fig. 1)². For a risk-stratification test to be practical, it has to be cost-effective, have good patient acceptability, performed by primary care physicians and have a high sensitivity for significant lesions.

Field Carcinogenesis

For CRC screening, the rectum represents a site that could both be minimally-intrusively interrogated and serve as a surrogate for neoplastic transformation elsewhere in the colon through the concept of field carcinogenesis (also known as field cancerization, field effect, and field defect). Field carcinogenesis is a common theme in a variety of malignancies (colon, lung, pancreas, esophagus, stomach, ovarian, cervical, head and neck, liver, breast, prostate, etc.)⁷. It is the notion that the genetic/environmental milieu that leads to a focal tumor exists not only at that particular location but affects the organ diffusely⁸. For instance, if a patient develops a cecal CRC, this occurred through interplay of both genetic and exogenous factors (e.g. fecal stream mutagens) leading to stochastic mutations. Thus, the diffuse field changes provide a fertile mutational environment and predispose to carcinogenesis while focal neoplastic lesions are determined by stochastic mutations. It follows that the uninvolved mucosa throughout the colon may serve as a surrogate site for assessing the risk of developing neoplasia with the rectum being the most readily accessible site.

Field carcinogenesis is well established in clinical practice. For instance, the distal adenoma found on flexible sigmoidoscopy portends a higher risk of synchronous proximal lesions and thus mandates colonoscopy⁹. An adenoma on colonoscopy represents a higher risk of future neoplasia (metachronous lesions) thus providing the biological underpinnings behind post-polypectomy surveillance colonoscopy¹⁰. A number of other biomarkers of field carcinogenesis in endoscopically-normal mucosa have been reported including rectal aberrant crypt foci (ACF)¹¹, proliferation¹², decreased apoptosis¹³, nuclear karyometry¹⁴, and genomic (microarray)¹⁵, proteomic¹⁶, methylation¹⁷, TGF- α ¹⁸, and crypt-restricted cytochrome C oxidase subunit I¹⁹ markers. However, the diagnostic performance of current biomarkers has been suboptimal. These molecular changes would be anticipated to give rise to morphological and functional alterations in colonic mucosa. Therefore, field carcinogenesis detection through evaluation of the endoscopically/microscopically normal rectal mucosa is biologically plausible. The central hurdle has been finding an accurate and practical biomarker, which is an emerging frontier for biophotonics.

Biophotonics Detects Multiple Facets of Field Carcinogenesis

The fact that the rectal epithelium in field carcinogenesis appears normal under light microscopy is related to the diffraction limit of resolution: it is not sensitive to structures under 200–500nm. Therefore, the intracellular structures that are dysregulated in early carcinogenesis (e.g. mitochondria, higher-order chromatin structure, cytoskeleton) are not detectable by conventionally light microscopy. Thus, there can still be profound functional, micro- and nano-architectural alterations in histologically-normal epithelial cells undergoing field carcinogenesis. In order to detect these changes we developed a suite of light-scattering

technologies. There are several salient features of this platform including ability to probe for structures at submicron scale, depth-selectivity given the heterogeneous nature of the epithelium (e.g. the earliest changes are believed to occur in proliferative/stem cells at the bottom third of the crypt), and ability to provide quantitative information. As opposed to imaging modalities that are able to visualize tissue structure but are qualitative, these approaches provide quantification of tissue/cell structure at submicron scales.

We focused on three facets of field carcinogenesis alterations, each representing a different level of tissue organization and a specialized technological solution for detection: 1. Physiological targets such as microvascular blood content (reflecting the hyperproliferative state of the premalignant epithelium).^{20–22} 2. Ultrastructural changes at the tissue level.²³ 3. Intracellular nanoarchitectural alterations^{24–26} (Fig. 1). All of these approaches provide the ability to accurately sense field carcinogenesis with slightly varying performance characteristics. There are, however, important differences in their clinical applications (*in vivo* measurements using fiber-optics probes versus analysis of cytological slides from rectal brushings).

Increased Microvascular Blood Supply as a Marker of Field Carcinogenesis

Cells in field carcinogenesis are hyperproliferative and thus would be expected to be hypermetabolic. Thus, the logical corollary is that there is a need for an increased blood supply to the colonic epithelium. Previously, this has been difficult to detect since the pericryptal capillary plexus supplying blood to the epithelium represents only a very small portion of the total colonic blood supply and even marked changes in this compartment can be obscured by the rest of the vasculature.

Measuring blood content is a very well studied application of biophotonics due to the pathognomonic light absorption spectrum of hemoglobin (Hb). Because the increased microvascular blood supply in early colon carcinogenesis is expected to reside in the pericryptal capillary plexus surrounding the bases of the crypts (a few hundred microns below tissue surface), it is critical to restrict the depth of blood supply detection. Among other approaches, this can be accomplished through polarization gating, in which a tissue is illuminated by linearly polarized light and the difference between co- and cross-polarized reflected signals is generated primarily by short-traveling photons²⁷. The depth of interrogation can be chosen from ~50 to a few hundred microns from below the colonocytes depending on the design of a fiber-optic probe that is used for both illumination and collection of light interacting with the tissue (Fig. 2(a)). Blood content is determined through the spectral analysis of the recorded signal. The measurable parameters include Hb concentration, oxygenation, and the average blood vessel diameter.

The phenomenon of increased microvascular blood content in early carcinogenesis (termed ‘early increase in blood supply’ or EIBS) was first observed in two animal models of CRC: the AOM-treated rat and the MIN-mouse with a germline APC mutation²⁸. EIBS was detectable in the normal-appearing mucosa preceding formation of aberrant crypt foci (ACF) and adenomas and progressed over time paralleling the course of carcinogenesis. The phenomenon was largely confined to the mucosa.

EIBS is a robust marker of field carcinogenesis in humans.^{20–22} This was confirmed in a study involving 222 unselected patients undergoing colonoscopy including 35 with non-advanced and 12 with advanced adenomas. EIBS readings (each taking 50 milliseconds) were acquired *in vivo* by a fiber-optic probe from the endoscopically normal mucosa (cecum, mid-transverse colon and rectum). The data demonstrated that, in patients with neoplasia, EIBS was present diffusely throughout the colon. In addition, the magnitude of EIBS progressively increased when the measurements were taken closer to an adenoma.

EIBS in the rectum was elevated regardless of the location of the advanced adenoma²². The effect was the most pronounced close to the bases of the crypts (within ~100µm below colonocytes). The area under the receiver-operator characteristic curve (AUC) for advanced adenomas based on a single marker, mucosal oxygenated-Hb concentration, was 0.88 with 83% sensitivity and 82% specificity. EIBS was not confounded by demographic factors or benign colonic disease.

The biological basis of EIBS appears to involve an induction of neo-angiogenesis that is most pronounced in the area adjacent to the bottom of the crypt (the proliferative compartment where teleologically one would expect greatest EIBS). While there are numerous potential molecular drivers, inducible nitric oxide synthase is at least one important factor²⁹.

Our data suggested a two component origin of EIBS: a diffuse component related to field carcinogenesis and a component related to factors elaborated by the tumor. This lends itself to a number of distinct applications: i) risk-stratification via detection of the field carcinogenesis component in the rectum and ii) guide-to-colonoscopy for adenoma detection via detection of the tumor-related gradient of EIBS. The latter has been facilitated by the development of real-time data analysis and a sensor that can automatically triggers readings upon a probe's contact with the mucosa. The EIBS gradient in the proximity to adenomas could serve as a "red flag" technology identifying the 10–30 cm of the colon that is likely to harbor neoplasia and thus require increased scrutiny (e.g. chromo-endoscopy).

Ultrastructural Markers of Field Carcinogenesis

The genetic and epigenetic alterations of field carcinogenesis can lead to significant ultrastructural consequences. For instance, many early events in colon carcinogenesis (e.g. APC, E-cadherin, Src)³⁰ interact with the cytoskeleton and would be expected to alter cellular ultrastructure. In turn, cellular ultrastructure is inherently linked to biochemical processes within the cell. Examples include the modulation of gene transcription by the higher-order chromatin structure, effects of macromolecular crowding on protein folding, and the regulation of gene expression by the extracellular matrix.

Since optical refractive index is linearly proportional to the local density of macromolecules (e.g. proteins, lipids, DNA), alterations in tissue/cell structure can be assessed by light-scattering. Importantly, light-scattering approaches are sensitive to sub-diffractive length scales: a scattering pattern becomes featureless only when the size of a structure falls below ~1/20th of the wavelength of light (~20 nm for the visible light)³¹. While visualization of such small objects with microscopy is impossible, light-scattering allows measuring their statistical properties.

A comprehensive approaches to describe tissue ultrastructure is via a mass-density correlation function, which quantifies how spatial correlation between structures depends on distance³². The function can be measured by a light-scattering technique, low-coherence enhanced backscattering (LEBS).^{33–34} The depth of tissue interrogation can be controlled by varying the spatial coherence length of illumination from a tens to hundreds of microns³⁵. LEBS can determine the shape of the correlation function, the average amplitude and the length scale of mass density variations. These parameters serve as the ultrastructural markers of field carcinogenesis.

The initial evidence of ultrastructural alterations in field carcinogenesis came from the LEBS analysis of colonic mucosa of the AOM-treated rat and the MIN-mouse models with ultrastructural changes developing diffusely throughout the colon prior to formation of ACF³⁶.

The risk-stratification potential of rectal LEBS analysis has been tested in humans ($n=270$)²³ (Fig. 2(b)). The rectal ultrastructural alterations were sensitive to non-diminutive adenomas located elsewhere in the colon irrespective of adenoma location. The markers were progressively altered from patients with 5–9mm adenomas to those with advanced adenomas, thus paralleling the risk of progression to CRC. LEBS performance for advanced adenomas showed 0.90 AUC with 100% specificity and 80% specificity. There was no confounding by demographic, risk factors or benign colon pathology. The insensitivity to diminutive adenomas is probably of minimal clinical implications. Although this study was performed on rectal biopsies, LEBS enables a fiber-optics implementation. An LEBS fiber-optic probe can be delivered *in vivo* without bowel purging. To date, we have performed it with a 3mm probe through an anoscope in over 200 patients with diagnostic performance equivalent to that of the *ex vivo* analysis.

From a mechanistic perspective, there are three potential facets of the structural alterations: ultrastructure of colonocytes and extracellular matrix and crypt re-organization. Firstly, LEBS revealed that colonocytes' structure resembles a mass fractal³². This is consistent with other recent reports suggesting that key cellular components are fractal, including the chromatin.^{37–38} A myriad of molecular processes can be dramatically affected by a shift in a fractal dimension including gene co-localization, co-expression, and diffusion of transcription factors³⁹. LEBS showed that the fractal dimension is decreased in field carcinogenesis colonocytes – indicative of a more 'disordered' cell organization.

Secondly, a profound reorganization of the collagen matrix occurs with an increase in the fractal dimension of the matrix. There have been numerous studies showing altered gene expression and methylation in the matrix. Optically, these changes have been demonstrated using second harmonic generation (SHG) imaging in ovarian cancer⁴⁰. We have replicated these findings in the AOM-treated rat model (Fig. 2(c)). It is, however, not yet clear whether the matrix restructuring is initiated by pre-neoplastic colonocytes or whether it is a microenvironment phenomenon. Superimposed upon this are other facets including potential cryptal re-organization⁴¹. Taken together, these effects lead to an increase in the mass fractal dimension of the mucosa (Fig. 2(b)).

Other complimentary light-scattering technologies have also been used to detect microarchitectural alterations in the colon, including Fourier-domain low-coherence interferometry (Wax et al.) and elastic scattering spectroscopy (Bigio et al.) (see Supplement).

Nanocytology for Field Carcinogenesis Detection

Instead of using a fiber-optic probe (as in LEBS or EIBS), an alternate approach to the detection of ultrastructural, histologically-unapparent cellular alterations is by means of the analysis of cell nanoarchitecture in cytological samples from rectal brushings (hence the term nanocytology). Nanocytology has parallels to the conventional cytology except that it analyzes the nanoscale as opposed to the micron-scale morphology and is quantitative.

Recently, partial wave spectroscopic (PWS) microscopy was developed to enable nanocytology²⁴. By focusing on photons interacting with a cell substantially in one dimension, PWS is sensitive to essentially any length scale of density fluctuations (limited by the technical aspects of instrumentation). PWS measures a statistics of spatial mass density variations termed the disorder strength, which is related to the amplitude and the correlation length scale of the density variations.

The first demonstration of nanocytology was in CRC cell lines and animal models (the AOM-treated rat and the MIN-mouse): the disorder strength paralleled cell tumorigenicity in

otherwise histologically indistinguishable cells²⁴. In human studies (n=35), PWS-enabled nanocytology performed on rectal brushings from the endoscopically normal mucosa prior to colonoscopy enabled accurate detection of colon field carcinogenesis (Fig. 2(e,f))²⁶. Rectal colonocytes' disorder strength was progressively increased with the magnitude of neoplasia that the patients harbored, from non-advanced to advanced adenomas.

Multiple functional and genomic consequences would be expected to stem from this alteration. The disorder strength is increased in the entire cell, although the main effect appears to be in the nucleus. Experiments with pharmacological cytoskeleton disruption showed that of the cytoplasmic disorder increase is related to cytoskeletal changes⁴². The nuclear disorder increase appears to reflect chromatin compaction and decrease in its fractal dimension partially mediated by HDAC activity. Chromatin compaction, in turn, is expected to affect locally multiple facets of genome regulation including the work for separation of a DNA double helix during pre-initiation of transcription, accessibility of DNA to transcription factors, DNA-histone interactions, and the diffusion of transcription factors and mRNA within the nucleus. The potential consequences of decreased chromatin fractality include gene de-localization and altered co-expression.

Just as field carcinogenesis is a ubiquitous cancer phenomenon, PWS nanocytology-detectable increased nanoscale disorder is not restricted to colon carcinogenesis and has been demonstrated in a number of other malignancies (pancreatic, esophageal, lung, and ovarian, see Supplement)²⁶.

Optical Detection of Field Carcinogenesis for Surveillance of Post-polypectomy, Family History and Chemoprevention

Applications of biophotonics detection of field carcinogenesis may go beyond risk-stratification (see on-line Supplement for detail). Approximately 20% of colonoscopies are performed for post-polypectomy surveillance⁴³. Colonoscopic surveillance is recommended because patients with an adenoma detected on an initial colonoscopy are at a higher risk of recurrent neoplasia (metachronous lesions). This is highly inefficient with ~90% of post-polypectomy colonoscopies being negative and, still, 0.3–0.9% of patients undergoing polypectomy developing cancer within 3 years⁴⁴. Our retrospective analysis showed that the rectal ultrastructural markers are indicative of the risk of adenoma recurrence⁴⁵ (Fig. 2(d)). The ultrastructural and microvascular markers may help endoscopists determine patient-specific screening intervals, particularly in patients with negative colonoscopies: a positive ultrastructural/microvascular marker assessed via an endoscopically-compatible LEBS/EIBS probe during a negative colonoscopy may trigger a shorter interval.

Family history of CRC is another common indication for colonoscopy. Since frequently the genes involved and their penetrance are unknown, it is impossible to ascertain whether a family member has acquired the predisposition and rationally tailor a screening strategy. Our data showed the ability of the microvascular/ultrastructural markers to detect future neoplastic risk in the MIN-mouse (murine model of FAP) at a pre-adenoma time point (EIBS/LEBS)⁴⁶ and in neoplasia-free Lynch syndrome patients with germline mutations in hMLH1 or hMSH2 (~60–80% lifetime CRC risk) (PWS nanocytology) suggesting that the nanoscale disorder increase was proportional to the long term risk of CRC.

Finding a reliable intermediate biomarker of the efficacy of chemoprevention is critical for more rapid clinical trials and to personalize therapy. Currently, it is difficult to gauge effectiveness without many years of therapy⁴⁷. Since field carcinogenesis is an early event and exquisitely reflects risk, this has potential for rapid assessment of chemopreventive response. Our data in the AOM-treated rat model showed that short-term sulindac

administration resulted in a dose-dependent normalization of the ultrastructural markers (e.g. mass fractal dimension) providing the impetus for an ongoing Phase 2 trial.

Conclusions

Optically detectable biomarkers of field carcinogenesis represent a number of levels of tissue organization and function including microvascular, mucosal ultrastructural and intracellular nanoscale alterations. Optical detection of field carcinogenesis has the potential to allow individualization of screening regimens. We envision the use of the no-bowel-preparation-required rectal fiber-optic test (e.g. LEBS) or nanocytology of cells brushed from the rectal mucosa in the primary care setting to determine the need for colonoscopy. The strength of these markers is their accuracy and ease of implementation. This would avoid unnecessary procedures and open up the limited endoscopy capacity. This pre-screen strategy is analogous to the Pap smear—colposcopy paradigm, which has relegated cervical cancer from the number 1 to the 14th cause of cancer deaths in women. Given that field carcinogenesis is a common theme in a variety of cancers, increased nanoscale disorder detectable by nanocytology may find broad applications as an initial screening tool for a wide range of malignancies (see Supplement).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Field carcinogenesis has manifestations at the number of levels of tissue physiology and morphology including alterations in mucosal microvasculature (detectable with a fiber-optic polarization-gated spectroscopy probe) and ultrastructure (detectable with low-coherence enhanced backscattering (LEBS) and partial wave spectroscopic (PWS) microscopy). The ultrastructural changes include alterations in the structure of collagen matrix and cryptal architecture as well as the nanoarchitecture of colonocytes (e.g. increase in the nuclear nanoscale disorder associated with alterations in the fractal organization of chromatin and chromatin compaction).

A **B** **C** **D**

Figure 2.

(a): A photograph of a fiber-optic probe for quantitative measurement of mucosal microvasculature (e.g. early increase in microvascular blood supply, EIBS). The probe is about 2 mm in diameter and can be used either as an endoscopically compatible device or a stand-alone device for detection of EIBS in rectal mucosa.

(b): Fractal dimension of rectal mucosal microarchitecture is altered in patients harboring significant neoplasia (advanced adenomas) elsewhere in the colon. The measurements were obtained using low-coherence enhanced backscattering (LEBS) from the rectal histologically and endoscopically normal appearing mucosa.

(c): Microscale organization of collagen matrix in the lamina propria and the upper submucosa is a marker of field carcinogenesis. The images were obtained by use of second harmonic generation microscopy (SHG) performed on histologically normal appearing colonic mucosa in the AOM-treated rat model of colon carcinogenesis. The top two panels (i and ii) show representative SHG images of collagen matrix structure from saline-treated control rats while the lower panels (iii and iv) show the matrix structure in the AOM-treated rats. The two left panels (i and iii) show three-dimensional reconstructions while the panels on the right (ii and iv) show the images integrated over all depths.

(d): Effect of past history of adenomas on rectal mucosal ultrastructural marker of field carcinogenesis. The ultrastructural marker was created as a linear combination of ultrastructural alterations measured with LEBS from histologically normal rectal mucosa. Patients with adenomas removed on a prior colonoscopy but with no concurrent adenomas ($n=14$) had statistically insignificantly elevated LEBS marker ($p=0.12$) compared to patients with no prior history and no concurrent adenomas ($n=121$), whereas patients with both prior history and concurrent adenomas ($n=14$) had significantly elevated LEBS marker ($p=0.001$).

(e,f): Nanocytology enables detection of field carcinogenesis and colon cancer screening. Nanocytological analysis of histologically normal colonocytes demonstrated that the disorder of their nanoscale architecture was a marker of field carcinogenesis. Nanocytology was performed by use of partial wave spectroscopic (PWS) microscopy (e). In the nucleus, the increased disorder is associated with altered higher-order chromatin structure. (f)

Increased nanoscale disorder of rectal histologically normal colonocytes is a marker of adenomas located elsewhere in the colon.