Metabolic Profiling Detects Field Effects in Nondysplastic Tissue from Esophageal Cancer Patients

Danny Yakoub1, Hector C. Keun1, Robert Goldin2, and George B. Hanna1

Abstract

The variable rate of missed cancer in endoscopic biopsies and lack of other biomarkers reduce the effectiveness of surveillance programs in esophageal cancer. Based on the “field cancerization” hypothesis that tumors arise within a transformed field with an altered biochemical phenotype, we sought to test if metabolic profiling could differentiate between histologically normal tissue from individuals with and without esophageal cancer. Thirty-five patients with esophageal adenocarcinoma and 52 age-matched controls participated in the study. Using 1H magic angle spinning–nuclear magnetic resonance spectroscopy of intact tissue, we generated metabolic profiles of tumor tissue, proximal histologically normal mucosa from cancer patients (PHINOM), and proximal histologically normal mucosa from a control group. Using multivariate regression and receiver-operator characteristic analysis, we identified a panel of metabolites discriminating malignant and histologically normal tissues from cancer patients and from that of controls. Whereas 26% and 12% of the spectral profile regions were uniquely discriminating tumor or control tissue, respectively, 5% of the profile exhibited a significant progressive change in signal intensity from controls to PHINOM to tumor. Regions identified were assigned to phosphocholine (PC), glutamate (Glu), myo-inositol, adenosine-containing compounds, uridine-containing compounds, and inosine. In particular, the PC/Glu ratio in histologically normal tissue signified the presence of esophageal cancer (n = 123; area under the curve, 0.84; P < 0.001). In conclusion, our findings support the hypothesis of the presence of metabonomic field effects in esophageal cancer, even in non-Barrett’s segments. This indicates that metabolic profiling of tissue can potentially play a role in the surveillance of cancer by reporting on the phenotypic consequences of field cancerization. Cancer Res; 70(22); 9129–36. ©2010 AACR.

Introduction

Esophageal cancer accounts for 6% of all cancer deaths worldwide (1). Early detection of esophageal cancer is the most effective strategy to improve its outcome (2). Conventional white light endoscopy remains the gold standard surveillance procedure for premalignant conditions of the upper gastrointestinal tract, although its cost-effectiveness is debated (3, 4). The surveillance endoscopy, as mandated by the American College of Gastroenterology guidelines, yields ~20 biopsies, most of which are normal on histologic assessment. Even with stepwise endoscopic biopsy in patients with confirmed high-grade dysplasia, variable rates as low as 7% and as high as 41% to 66% of resected specimens had missed cancer lesions (5, 6).

The concept of “field cancerization” was introduced by Slaughter in 1953 to describe the existence of generalized carcinogen-induced early genetic changes in the epithelium from which multiple independent lesions occur, leading to the development of multifocal tumors (7). This idea came from the belief that the lateral spread of tumors was due to progressive transformation of cells adjacent to a tumor rather than the spread and destruction of the adjacent epithelium by pre-existing cancer cells (8). Since this time, many studies have been conducted to test this hypothesis in various cancers (9–15) by investigating genetic and epigenetic mutations, transcriptional effects, and the proteome of tumors (16).

Metabonomics is defined as “the quantitative measurement of the time-related multiparametric metabolic response of living systems to pathophysiologic stimuli or genetic modification” and exploits analytic techniques such as nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry to gain a global perspective on metabolic events (17). Recently, the development of high-resolution 1H magic angle spinning (MAS)-NMR spectroscopy combined with multivariate data analysis and pattern recognition techniques has had a substantial effect on the ability to analyze intact tissue specimens in metabonomics studies (18). This...
approach has been applied to many cancerous and precancerous tissues, including those of the cervix (19), colon (20), brain (21, 22), breast (23), prostate (24, 25), stomach (26), and esophagus (27). Metabonomic profiling has the advantage of simple preparation of samples as well as relatively cheap cost. It has also the ability to interrogate complex metabolic profile data, with the potential to identify biomarkers or biomarker patterns reflective of early events in the carcinogenic process (28).

Based on the concept of field cancerization, we examined the hypothesis that metabolic differences between histologically normal mucosa from esophageal cancer patients and from nondiseased controls would be detected by MAS-NMR analysis and aimed to identify putative biomarkers of potential value in clinical applications.

Materials and Methods

The study was granted the necessary approval by the local research ethics committee (PROJECT 05/Q0403/165). Informed consent was obtained from all patients prior to sample collection. Eighty-seven patients were recruited from five hospitals in the North West London region: 35 with esophageal adenocarcinoma and 52 age-matched, noncancer controls. Sixteen patients from the cancer group had chemotherapy during the course of the study, typically three to six courses of cisplatin + 5-fluorouracil or epirubicin + capecitabine. Sampling of cisplatin + 5-fluorouracil or epirubicin + capecitabine during the course of the study, typically three to six courses of cisplatin + 5-fluorouracil or epirubicin + capecitabine.

Sample collection

Samples ranged from 30 to 50 mg in weight and were taken from cancer patients either (a) endoscopically during staging laparoscopy under a general anesthetic, (b) during endoscopic ultrasound staging under sedation, or (c) surgical specimens immediately after retrieval in the course of esophagectomy. Samples were collected from both tumor tissue as well as normal mucosa 20 cm from incisors at endoscopy or at least 5 cm away from a visible tumor in surgical specimens. Control samples were taken from noncancer patients during endoscopy for indications other than suspicion of malignancy (initial and follow up endoscopy for heart burn, hiatus hernia, and history of gastritis/duodenal ulcer). All but 21 of these patients received local anesthetic during their endoscopy. In patients who received neoadjuvant chemotherapy, samples were collected 3 to 5 weeks after the last chemotherapy dose, as this is the recommended time for postchemotherapy surgical intervention. Samples for NMR analysis were snap frozen in liquid nitrogen within 12 minutes of extraction from the body and stored at –80°C. Another set of matched samples from all sampled areas were fixed in paraffin for pathologic examination. All samples were collected after an overnight fasting. Samples were annotated with full clinical details including demographic data, comorbidities, and drug intake. Patient and sample characteristics are summarized in Table 1.

Intact sample preparation and NMR analysis

Tissue samples were thawed at room temperature and washed with 200 μL of 95% D₂O for a maximum of 10 seconds to prevent leakage of solutes. Wet samples were loaded in a zirconium rotor (Bruker), weighed, and then spun in a 600 MHz MAS-NMR spectrometer (Bruker) at 5 kHz with probe temperature preserved at 283 K.

NMR data acquisition

NMR spectra were acquired on a Bruker DRX600 spectrometer (Bruker Biospin) operating at 600.13-MHz ¹H NMR frequency and 283 K. Shimming was done manually for each sample. ¹H NMR spectra of the samples were acquired using a one-dimensional Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence (RD-90°-τ-180°-τ-n-acquire). The CPMG sequence generates spectra edited by T₂ relaxation times, i.e., with reduced signals from high molecular weight species or systems in intermediate chemical exchange. In our experiments, τ = 300 and τ = 400 μs, for a total T₂ relaxation time of 240 ms. For all spectra, 256 free induction decays (FID) were collected into 32 K complex data points, using a spectral width of 12,019 Hz (20 ppm), with a 2-second pulse width.
relaxation delay between pulses. A water presaturation pulse was applied throughout the relaxation delay.

**NMR spectral data processing**

Data were zero-filled by a factor of 2, and the FIDs were multiplied by an exponential weighting function equivalent to a line broadening of 1 Hz prior to Fourier transformation using XWINNMR software (Bruker Biospin). The improved resolution gained by zero filling often benefits the upstream (visual) and downstream (integration) analysis surrounding pattern recognition. The acquired NMR spectra were calibrated to the lactate-CH₂ resonance at 1.33 ppm and manually corrected for phase and (linear) baseline distortions. All subsequent data processing and analysis, unless specifically stated otherwise, was conducted using in-house software written and compiled in MATLAB (Mathworks) by O. Cloarec, T.M.D. Ebbels, and H.C. Keun. Spectra were interpolated from 32 K to 42 K data points using a cubic spline function to regularize the abscissa and improve calibration accuracy (final resolution, 0.29 Hz/pt) prior to pattern recognition analysis.

**Pathologic examination**

Samples matched to those undergoing NMR spectroscopy were subjected to staining with H&E to ensure the benign or malignant nature of tissues. Normal esophagus slides from 34 cancer patients and 50 noncancer patients underwent additional immunohistochemical staining for the markers Ki-67 (which identifies cells in the proliferative cell cycle, i.e., the G₁, S, G₂, and M phases, during which it is expressed), p53 (missense mutations or posttransitional modifications of which lead to an abnormality stable but inactive p53 protein that effectively promotes cell division and is detectable by immunohistochemistry), terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL; a marker for apoptosis), and mucin, wherein percentage of areas stained were accurately calculated by computational image analysis techniques. Two consultant pathologists with a special interest in gastrointestinal pathology collectively.

**Table 2. Discriminatory spectral regions exhibiting intermediate metabolic change in nontumor tissue from cancer patients**

<table>
<thead>
<tr>
<th>Marker region</th>
<th>Upper, ppm</th>
<th>Lower, ppm</th>
<th>Metabolite</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2.18</td>
<td>2.1</td>
<td>Glu</td>
</tr>
<tr>
<td>B</td>
<td>2.51</td>
<td>2.4</td>
<td>Glu</td>
</tr>
<tr>
<td>C</td>
<td>3.16</td>
<td>3.09</td>
<td>unassigned</td>
</tr>
<tr>
<td>D</td>
<td>3.21</td>
<td>3.2</td>
<td>free choline</td>
</tr>
<tr>
<td>E</td>
<td>3.3</td>
<td>3.28</td>
<td>unassigned</td>
</tr>
<tr>
<td>F</td>
<td>3.53</td>
<td>3.5</td>
<td>myoinositol</td>
</tr>
<tr>
<td>G</td>
<td>3.57</td>
<td>3.55</td>
<td>glycine</td>
</tr>
<tr>
<td>H</td>
<td>4.08</td>
<td>4.04</td>
<td>myo-inositol</td>
</tr>
<tr>
<td>I</td>
<td>4.22</td>
<td>4.15</td>
<td>PC</td>
</tr>
<tr>
<td>J</td>
<td>6.02</td>
<td>5.95</td>
<td>uridine/UXP*</td>
</tr>
<tr>
<td>K</td>
<td>6.13</td>
<td>6.17</td>
<td>AXP*</td>
</tr>
<tr>
<td>L</td>
<td>7.51</td>
<td>7.55</td>
<td>uracil*</td>
</tr>
<tr>
<td>M</td>
<td>7.91</td>
<td>7.87</td>
<td>uridine/UXP*</td>
</tr>
<tr>
<td>N</td>
<td>8.2</td>
<td>8.17</td>
<td>hypoxanthine</td>
</tr>
<tr>
<td>O</td>
<td>8.22</td>
<td>8.2</td>
<td>hypoxanthine*</td>
</tr>
<tr>
<td>P</td>
<td>8.25</td>
<td>8.29</td>
<td>adenosine/AXP</td>
</tr>
<tr>
<td>Q</td>
<td>8.52</td>
<td>8.6</td>
<td>AXP</td>
</tr>
</tbody>
</table>

NOTE: XP indicates the mono-, di-, and triphosphates collectively. *Tentatively assigned.
examined the slides independently. In addition, these pathologists examined 10 slides of each of the normal tissue from cancer and noncancer groups blinded to the origin of the tissues.

Results

Using $^1$H MAS-NMR spectroscopy of intact tissue, we generated metabolic profiles of esophageal tumor tissue ("tumor") and of PHINOM together with proximal histologically normal mucosa from an age-matched control group ("control"; $n = 123$; Table 1; Fig. 1). For a detailed annotation of the NMR profile of esophageal tissue, please see Supplementary Data S1. Histopathologic examination after H&E staining and immunohistochemistry confirmed the presence of adenocarcinoma in all tumor tissue samples (Fig. 1B). Neither of the normal tissues from cancer nor noncancer patients showed evidence of malignancy or dysplasia, and the PHINOM group could not be separated from the proximal histologically normal mucosa of the control group on blinded examination. No significant differences were observed in proliferation (Ki67) or apoptosis (TUNEL).

To avoid confounding variation from chemotherapy, gender, and the route of sampling, a training set was established that contained data from samples obtained only from male individuals that were not receiving neoadjuvant chemotherapy and that were collected by endoscopy during routine diagnostic testing or staging laparoscopy ($n = 45$). From visual inspection of the profiles from the training set, several metabolites seemed to exhibit a progressive change in intensity from tumor tissue to PHINOM to tissue from controls, consistent with the hypothesis that tumors arise within a biochemically altered tissue field (Fig. 1C). Using PLS discriminant analysis (PLS-DA), we were able to generate a robust and significant model that could discriminate profiles based on the origin of the tissue, confirming that the metabolic profile of PHINOM is systematically different from that of both control and tumor tissue (Fig. 2; LV2; $Q^2_Y$, 0.46; $P < 0.01$ by permutation).

To test this hypothesis further, we derived two more discriminant analysis models trained to classify proximal histologically normal mucosa either from cancer patients (PHINOM) or from noncancer group (control). A discriminant analysis (PLS-DA) approach was used where the spectra

![Figure 1. Profiling by $^1$H-MAS NMR spectroscopy reveals systematic metabolic differences between malignant, PHINOM, and control esophageal tissue.](image-url)
data are regressed to a matrix, \( Y \), with each column representing one class of sample (tumor, PHINOM, or control), and the value “1” was used to indicate class membership or “0” if the sample is not of that class. The optimum number of factors (LVs) for the model and the overall goodness of fit was assessed by the \( Q^2 \) statistic, which is defined as the predicted explained variance in \( Y \) from 7-fold cross validation (29). The significance of \( Q^2 \) was assessed by comparison to the null distribution estimated from PLS-DA models regressed to random permutations (\( n = 100 \)) of the \( Y \) matrix. The variances in the regression coefficients were estimated by cross validation (7-fold), and spectral regions that were significantly correlated to the presence of cancer in each model were identified (by \( t \) test, \( P < 0.1 \)).

Whereas 26% and 12% of the spectral profile regions were uniquely discriminating tumor or control tissue, respectively, 5% of the profile exhibited a significant and progressive change in signal intensity from controls to PHINOM to tumor, significantly higher than expected by chance (\( \delta \approx 10^{-30} \) binomial distribution, expected value 0.005%; Fig. 3A). Regions identified as showing progressive change were interpreted as indicating the presence of putative biomarkers for a field effect, and resonances identified by these regions were integrated for further analysis. Several of these resonances could be assigned to specific metabolites, namely, PC, glutamate (Glu), myo-inositol, adenosine-containing compounds, uracil, and inosine by reference to literature data (Table 2). Hierarchical clustering of these metabolites indicated that they fell into two main groups, those increasing or decreasing with the presence of cancer, and clustering of the samples showed that this panel could distinguish most control tissue from PHINOM or tumor tissue in a progressive fashion (Fig. 3B). In principle, each metabolite cluster could provide complementary information for sample classification, leading to better discrimination. To test if this was the case, we examined the PC/Glu ratio, as these metabolites were members of different clusters and both are part of distinct metabolic pathways that are well documented as being altered in the presence of malignancy. The PC methylene resonance at \( \delta_{4.19} \) was selected by our algorithm and was used for calculation of the PC/Glu ratio in preference to the more commonly used \( N \)-methyl resonance (\( \delta_{3.22} \)) as it is better resolved from other choline-containing compounds such as glycerophosphocholine. This may make this resonance a better indicator of PC levels, particularly in nontumor tissue where PC levels are relatively low. Receiver-operator characteristic (ROC) analysis for the detection of cancer in histologically normal samples from the training set using PC/Glu gave an area under the curve (AUC) of 0.857 (\( P = 0.001 \), SEM = 0.069, under nonparametric assumption) compared with AUC values of 0.752 (\( P = 0.015 \)) and 0.637 (\( P = 0.184 \)) for PC and Glu, respectively. To validate our findings, we examined the distribution of the PC/Glu ratio in the full sample set, which included data from female patients and surgical excision specimens that were independent from the analysis thus far (\( n = 123 \); Fig. 3C). PC/Glu showed a clear progression to higher values from control to PHINOM to tumor tissue and that the difference between PHINOM and tumor tissue is retained in samples obtained from surgery, despite a systematic difference in the PC/Glu ratio between endoscopic and surgical PHINOM samples (Fig. 3C). ROC analysis produced AUC values of 0.84 for control versus PHINOM samples and 0.98 and 0.91, respectively, for endoscopic and surgical tumor versus PHINOM, showing both that discriminatory ability was retained in the full sample set and that the PC/Glu ratio is also a marker for tumorigenic progression. We examined the ability of further metabolite ratios to detect PHINOM in endoscopic samples based on identified resonances from each of the major clusters in Fig. 3B (Supplementary Data S2). The PC/Gly, myo-inositol/Gly, and choline/Gly ratios all exhibited AUC values of >0.8, indicating that a broad panel of potentially diagnostic features could be extracted from a single spectrum. The metabolic effect of surgical collection, presumably as a result of tissue ischemia, was the only apparent confounding factor of significance in our analysis.

**Discussion**

By comparing histologically normal mucosa proximal to a tumor (PHINOM) with proximal histologically normal mucosa from noncancer controls and tumor tissue, this study...
provides new evidence that field cancerization in esophageal cancer manifests a metabolic phenotype and that metabolic profiling via $^1$H MAS-NMR is sensitive to this phenomenon. Moreover, metabolites that show a progression in detectable levels from normal control to PHINOM to tumor tissue were identified. Some of these metabolites, such as PC, are well-established metabolic biomarkers for malignant potential. Alterations to choline metabolism and specifically the increase in PC have been shown to differentiate malignant from normal tissue in most tumors and also to discriminate Barrett’s esophagus (27). Also overexpression of choline kinase, which forms PC, is observed in several cancers (30). The immunohistochemical markers used [p53 mutations and cell proliferation (Ki-67)] are widely used by histopathologists in the diagnosis and assessment of dysplasia in Barrett’s esophagus (31) and squamous neoplasia (32). Normal epithelial cells transformed by oncoproteins or loss of tumor suppressors also show increases in PC, and conversely, reintroduction of p53 into p53 $^{-/-}$ (HCT116) cells has been shown to decrease intracellular PC levels (33). Given that loss of heterozygosity at loci for the tumor suppressors p16 (9p21) and p53 (17p13) has been used to map fields in the esophagus (34) and that PC levels are affected by p53 status, our observation that PC levels were elevated in PHINOM is consistent with the hypothesis that tumors arise within a widespread genetically predisposed field. Furthermore, it has recently been shown that loss of p53 function could directly contribute to other well-established aspects of the tumor metabolic...
phenotype, such as the Warburg effect (increased glucose uptake and metabolism to lactate), via regulation of mitochondrial metabolism and glycolysis (35). Although we did not see a progressive change in lactate or glucose levels, lactate levels were higher in tissue from cancer patients compared with controls (Fig. 2A). The observation that Glu was progressively decreased in PHINOM and tumor may also reflect alterations to mitochondrial function, because the increase in glutaminolysis in tumor cells is in part due to the consumption of Glu via a truncated TCA cycle (36).

The presence of field cancerization in upper gastrointestinal cancer is widely accepted, with well-documented clonal expansion in Barrett’s dysplasia, the major premalignant lesion (37). The results present in this report are of particular significance, because the tissue used to define the metabolic field effect was neither dysplastic nor Barrett’s segments. This implies that metabolic analysis is sensitive to molecular changes that are relevant to esophageal cancer in general and may relate to field-related phenomena such as the well-documented association between esophageal and head and neck cancers and satellite/skip metastasis (38,39). In addition to being an alternative route to genetic measurements to assess the state of the cancer field, metabolic profiling has several advantageous features. MAS-NMR profiles can be measured within 30 minutes of obtaining tissue from surgery or endoscopy so it has the potential to inform early decisions. Although histologic assessment of frozen section can be carried out within the same time scale, MAS-NMR has the advantage of being able to detect changes in histologically normal tissue. Metabolic biomarkers in general have other advantages: as a phenotypic end point, these can reflect a number of genetic lesions that produce the same loss of function; also, a metabolite has a defined chemical structure and hence analytic protocol for analysis can translate across species and sample type. More generally, the ability to characterize the state of the field might improve accuracy of diagnosis and staging. It is of note that two of the samples, which were collected endoscopically from a visible tumor mass, were unexpectedly deemed “normal epithelium” by pathologic examination. In our model, they were classified as metabolically malignant, and analysis of samples obtained from the patient after surgical resection of those lesions confirmed its malignant nature. In a MAS-NMR profiling study of postoperative prostate tissue, metabolic profiles of histologically normal tissue were shown to systematically vary with stage (25). The findings of our study have significant clinical implications as metabolic profiling of histologically normal tissue of cancer patients has the potential to predict the presence of malignancy within the sampled field. This would reduce the incidence of missed cancer because of sampling errors with significant effect on increasing the pick-up rate in diagnostic endoscopy. Also, the negative predictive power of the test will ensure the “no cancer miss” value of diagnostic endoscopy with specificity of 84% but in a relatively uncommon disease. The diagnostic power of the metabolic profiling of proximal histologically normal esophageal mucosa and its clinical value in the reduction of missed cancer should be investigated in a prospective clinical blind study. This will determine the best placement of the test in the diagnostic and surveillance pathway and the implication of its use in clinical services. Nevertheless, technical challenges do remain for the use of metabolomic approaches in clinical practice, for example, we observed systematic differences in the metabolic profile discriminating postsurgical tissue from that obtained by endoscopy implying that sample collection will have to be optimized and well controlled. However, several laboratories including our own are currently establishing more automated MAS-NMR facilities within rapid reach of surgical theaters, providing the ideal opportunity for translation and evaluation of our findings (40). The progress in magnetic resonance technologies such as hyperpolarized 13C imaging (41) may also provide new opportunities for the clinical use of metabolic biomarkers. In this way, biomarkers of field effects in esophageal and other more prevalent gastrointestinal malignancies, such as colorectal cancer, are important for improving strategies for surveillance and disease prevention and could have enormous benefits to health and health care expenditure. Further studies are required to elucidate the molecular mechanisms that produce the phenomenon.

Disclosure of Potential Conflicts of Interest

No conflict of interest whatsoever is involved with any authors or scientific or industrial parties.

Acknowledgments

We thank Dr Jo Lloyd (Department of Histopathology, St Mary’s Hospital, Imperial College Healthcare NHS Trust) for the independent review of histologic slides involved in the study.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 05/07/2010; revised 08/17/2010; accepted 09/18/2010; published OnlineFirst 09/30/2010.

References


www.aacrjournals.org Cancer Res; 70(22) November 15, 2010 9135


Metabolic Profiling Detects Field Effects in Nondysplastic Tissue from Esophageal Cancer Patients


Cancer Res 2010;70:9129-9136. Published OnlineFirst September 30, 2010.

Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-10-1566

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2010/10/01/0008-5472.CAN-10-1566.DC1

Cited articles
This article cites 40 articles, 11 of which you can access for free at:
http://cancerres.aacrjournals.org/content/70/22/9129.full.html#ref-list-1

Citing articles
This article has been cited by 6 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/70/22/9129.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.