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

Danny Yakoub, Hector C. Keun, Robert Goldin, and George B. Hanna

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-seventeen 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.

Sampling

Samples 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—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.

Table 1. Patient and sample characteristics

<table>
<thead>
<tr>
<th>Patient group</th>
<th>Control</th>
<th>PHINOM</th>
<th>Tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>N (total patients)</td>
<td>52</td>
<td>38</td>
<td>32</td>
</tr>
<tr>
<td>Age (median years)</td>
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<tr>
<td>Gender</td>
<td>Male 18</td>
<td>30</td>
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</tr>
<tr>
<td></td>
<td>Female 34</td>
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<td>Grade</td>
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<tr>
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<td></td>
</tr>
<tr>
<td>2</td>
<td>4</td>
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<td>3</td>
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<tr>
<td>Sample Group</td>
<td>Control</td>
<td>PHINOM</td>
<td>Tumor</td>
</tr>
<tr>
<td>N (total samples)</td>
<td>53</td>
<td>38</td>
<td>32</td>
</tr>
<tr>
<td>Sampling</td>
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<td>15</td>
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<tr>
<td>Surgical</td>
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<td>21</td>
<td>17</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>Pre</td>
<td>24</td>
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</tr>
<tr>
<td>Post</td>
<td>14</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>N (training set)</td>
<td>19</td>
<td>14</td>
<td>12</td>
</tr>
</tbody>
</table>

NOTE: 80% of PHINOM and tumor samples were patient-matched.
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-CH₃ resonance at 1.33 ppm and manually corrected for phase and (linear) baseline distortions. All subsequent data processing and analysis, unless specified 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 absissa and improve calibration accuracy (final resolution, 0.29 Hz/pt) prior to pattern recognition analysis.

**Pattern recognition and statistical analysis**

For pattern recognition analysis, spectra were divided and signal integral was computed in 0.01 intervals across the chemical shift range δ0.24–9.96. The region δ4.7–5.2 was excluded to remove variation in the presaturation of the water resonance; also, regions δ < 0.8 and δ > 8.95 were excluded to avoid the effect of baseline noise. To remove the effect of signals from the local anesthetic, the following regions were further excluded, leaving 699 variables: δ0.075–1.255, 1.355–1.405, 2.185–2.245, 2.265–2.275, 3.335–3.395, 3.625–3.775, 4.295–4.325, and 7.185–7.305. Each integrated NMR spectral region was then divided by the sum of the total remaining intensity for each spectrum to normalize values. For pattern recognition, the reduced and normalized NMR spectral data were imported into SIMCA-P (version 11, Umetrics AB), and each variable was mean centered and Pareto scaled (divided by SD) prior to partial least squares (PLS) regression. PLS has an advantage over other algorithms for multivariate regression where there is high colinearity between predictors. The predicted explained variance in Y from 7-fold cross validation (29) was assessed by the Q² statistic, which is defined as the predicted explained variance in Y from 7-fold cross validation (29). The significance of Q² was assessed by comparison of the null distribution estimated from PLS-DA models regressed to random permutations (n = 100) of the Y matrix.

The t test was used for selection of variables showing a progressive metabolic change between tissue subtypes. Type I error was controlled for by taking only the intersect between variables showing pair-wise group differences and the expected false discovery estimated using the binomial distribution. Resonances corresponding to the selected intensity variables (spectral regions) were reintegrated to define the relative intensity of the entire resonance or the major resolved portion of the resonance in the normalized data (in house software written in MATLAB by H. Keun; see Table 2 for integrated spectral regions). Hierarchical clustering analysis was conducted on the resonance intensity data after conversion to z scores (scaled to unit variance) and using average linkage (within the R environment “ggplots” package: http://www.r-project.org).

**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 abnormally 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 percent of areas stained were accurately calculated by computational image analysis techniques. Two consultant pathologists with a special interest in gastrointestinal pathology.

<table>
<thead>
<tr>
<th>Marker</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>myoinositol</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>
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<td>hypoxanthine*</td>
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<tr>
<td>P</td>
<td>8.25</td>
<td>8.29</td>
<td>adenosine/AXP</td>
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<tr>
<td>Q</td>
<td>8.52</td>
<td>8.6</td>
<td>AXP</td>
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</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...
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 ($\delta 3.22$ 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

Figure 3. Identification of spectral features defining intermediate metabolic change in nontumor tissue from cancer patients. A, proportion of spectra area significantly differentiating between tissue groups based on two PLS-discriminant analysis models calculated as described in Materials and Methods, which classified tumor from PHINOM or control from PHINOM. B, heat map and hierarchical clustering analysis (HCA) of metabolite resonances occupying spectral regions exhibiting intermediate change in PHINOM in the training set. Data are presented as z scores (scaled to unit variance), and average linkage was used for the analysis. C, box plots of the PC/Glu ratio across the full sample set, clustered by tissue of origin and route of sampling. Outlying values of >2 SD (full circles) and >3 SD (asterisk) from the mean are indicated. ROC AUC values are indicated for the classification of each group relative to the sampling route match PHINOM group.
pheno...mal 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 metabonomic 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 metabonomic 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 translational 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

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