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Esophageal cancer phospholipids correlated with histopathologic findings: a ^{31}P NMR study

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ABSTRACT: We analyzed 36 esophageal tumor specimens for phospholipid content using phosphorus nuclear magnetic resonance spectroscopy (^{31}P NMR) and correlated the individual phospholipid profiles with specific clinical and histopathologic features. Among the 18 phospholipids identified in the esophageal tumor specimens, the mean mole percentage concentration of dimethylphosphatidylethanolamine, lysoalkylacylphosphatidylcholine, lysophosphatidic acid, lysophosphatidylcholine (deacylated at the glycerol-1 carbon), and lysoethanolamine plasmalogen correlated with pathologic *T* stage, nuclear grade, or the presence of lymphatic invasion. ^{31}P NMR produces well-dispersed phospholipid spectra and a precise determination of phospholipid relative mole percentages. These data provide a statistical correlation between histopathologic features and molecules known to play an important role in cellular activities and processes unique to malignant tissues. Copyright © 1999 John Wiley & Sons, Ltd.

KEYWORDS: NMR; ^{31}P NMR; esophagus; cancer; neoplasms; phospholipids

INTRODUCTION

Pathologic stage is the most important prognostic feature of esophageal cancer. Histopathologic features may be used to characterize an esophageal tumor and potentially provide additional prognostic information. Such information may be used to guide decisions regarding treatment. We previously demonstrated that tissue phospholipid extracts, analyzed using phosphorus nuclear magnetic resonance spectroscopy (^{31}P NMR), are capable of discriminating between esophageal cancer and adjacent normal tissues, including the non-involved esophagus and normal stomach.[1] ^{31}P NMR is an ideal technique for measuring and monitoring tumor biochemistry. For certain tumors it can be performed *in vivo*[2–4] or with extracts of surgical tissue specimens.[5–9] Studies performed under both conditions have demonstrated the usefulness of the technique to distinguish among malignant tumors, benign tumors, and normal tissues, discriminate tumor recurrence from treatment effect, and identify putative markers of malignant cells.[10–13]

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Abbreviations used: BVI, blood vessel invasion; LVI, lymphatic vessel invasion; ^{31}P NMR, phosphorous nuclear magnetic resonance spectroscopy.

It is unfortunate that the individual phospholipid components of cells cannot be readily measured *in vivo* using any available technology. However, specialized ^{31}P NMR spectroscopic techniques produce well-dispersed phospholipid spectral profiles that are quantitative to the fourth significant figure.[14] These important phosphorus-containing molecules are involved in many processes linked to malignant transformation, aggressive biological behavior, and cell destruction through apoptotic mechanisms.[15–21] Efforts are now underway to determine the pathological significance of aberrations in these profiles. This study examines the statistical correlation of phospholipid data with the histologic features of human surgical esophageal tissue specimens.

MATERIALS AND METHODS

Selection of tissues

A total of 36 clinically resectable malignant esophageal tumors were obtained for analysis from patients undergoing potentially curative surgery. No patients received preoperative radiation therapy or chemotherapy. All patients signed informed consent for surgery and the tissues were considered found specimens. Tissue specimens were submitted to the Department of Pathology in an unfixed state within 10 min after removal from the patient. A sample of the tumor weighing approximately

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0.25–0.50 g was removed from an area that macroscopically appeared to be the least necrotic. These representative samples of malignant tissues were frozen by immersion in liquid nitrogen. Tissue samples adjacent to those taken for NMR analysis were processed for routine pathologic evaluation.

Pathological evaluation

One pathologist (G.Y.L.) reviewed the routine hematoxylin and eosin stained histologic sections from the surgical specimens. Tumors were evaluated for histologic subtype (adenocarcinoma vs squamous cell carcinoma), predominant grade, pathological *T* staging according to the AJCC criteria,[22] blood vessel invasion (BVI), and lymphatic vessel invasion (LVI). BVI refers to vein invasion only; no cases of arterial invasion were noted. In addition, to heighten the identification of vascular invasion, special histochemical and immunohistochemical colorations were performed using standard techniques. EVG stain (elastic Van Gieson) highlighting the elastic fibers of the vessel wall and antibodies directed against factor VIII and UEA (*Ulex europeaus*), both antibodies decorating endothelial cells, were used on the same histologic sections.

Chemical extraction procedures and magnetic resonance spectroscopy

The 36 tissue specimens were extracted for phospholipids and prepared for analysis using methods previously described for ^{31}P NMR phospholipid analysis.[6,14,23–24] The tissue specimens were pulverized in liquid nitrogen and dissolved in 30 mL of 2:1 chloroform and methanol.[25] Each 30 ml chloroform–methanol extract was filtered into a separatory funnel (extracted tissues were discarded) and washed using 6 mL 0.2 M potassium (ethylenedinitrilo)-tetraacetic acid (EDTA) at pH 6.0.[26] After 24 h, the chloroform phase was recovered in a 125 mL round-bottom flask and evaporated to dryness at 37 °C using a rotary evaporator. This scrubbing procedure is essential to remove polyvalent metal cations from phospholipid samples that otherwise interfere with the ^{31}P NMR analysis. Dried phospholipid samples were dissolved in 3 mL of a chloroform–methanol–aqueous-Cs–EDTA NMR reagent specifically formulated for the analysis of phospholipids by ^{31}P NMR spectroscopy;[14,23,27] technical details and analytical precautions have been described previously.[14,24,27] A heteronuclear GE 500 NB, NMR spectrometer system (General Electric, Fremont, CA), operating at 202.4 MHz for ^{31}P was used to analyze the samples and determine the spectrum of phospholipids. The Spectrometer scan conditions used were: pulse sequence, one pulse; pulse width, 18 μs (45° spin-flip angle); sweep width 1000 Hz; acquisition delay, 500 μs; free-induction decay size, 4096 channels; interpulse delay, 500 μs; acquisition time, 2.05 s; and number of acquisitions, ca 1500. Additionally, a computer-generated exponential filter time-constant introducing 0.6 Hz line broadening was applied to reduce background noise. Data reductions, including peak-area and chemical-shift measurements and spectral curve analysis, when required,[28] were calculated using the spectrometer's software. Proton broad-band decoupling also was applied routinely (0.25 W). Resonance assignments were based upon accurate measurements of the chemical shift coupled with the addition of standards. Chemical-shift data are reported relative to 85% phosphoric acid, as is usual for ^{31}P NMR. The primary internal reference was the naturally occuring phospholipid phosphatidylcholine (PC, chemical shift −0.84 ppm).[14]

Spectral assignments were confirmed by the addition of standards to the samples. Phospholipid standards, including L-phosphatidyl(*N*-palmitoyl)-ethanolamine, dipalmitoyl and ammonium salt, were obtained from Sigma Chemical Co. (St Louis, MO). Phospholipid concentrations were determined through integration of the phospholipid resonance signals detected from each sample. (The veracity of ^{31}P NMR phospholipid quantification[23,26,29,30] and profile analysis has been validated previously.[31–33]) The relative mole fractions of each signal contributing to a given spectral profile were then calculated as a percentage of the total spectral integral.[34]

Data analysis

Phospholipid concentrations, in relative phosphorus mole percentages of the spectral profiles, were determined for all detected resonances using the curve resolution software of the spectrometer. Based upon the presence or absence of pathological features, groups were compared at the level of the individual phospholipids using univariate statistics (two-tailed *t*-test or one-way analysis of variance with a *post-hoc* Scheffé test).[35] The histologic findings were correlated with the phospholipid mole percentages using Pearson's[35] correlation procedure. The correlation coefficient is indicated as (r). Significance was determined at the $p < 0.05$ level. For the purposes of statistical analysis, missing values represented resonance signals lying below the level of detection and cases with indeterminant pathological features were excluded from the analysis.

RESULTS

Eighteen individual phospholipids (Table 1) were quantified[14] in the esophageal tumor spectral profiles. The following phospholipids were identified: alkylacyl-phosphatidylcholine (AAPC), 4,5-dihydrosphingomyelin

Table 1. Mean phospholipid mole percentages of esophageal tumor specimens ($n = 36$)

Phospholipid	Mean ± SD
Alkylacylphosphatidylcholine	3.90 ± 0.51
Dihydrosphingomyelin	1.87 ± 0.21
Dimethylphosphatidylethanolamine	0.06 ± 0.04
Diphosphatidylglycerol	2.63 ± 0.23
Ethanolamine plasmalogen	8.49 ± 0.81
Lysoalkylacylphosphatidylcholine	0.11 ± 0.08
Lyso ethanolamine plasmalogen	0.41 ± 0.20
Lysophosphatidic acid	0.69 ± 0.22
Lysophosphatidylcholine	1.56 ± 0.34
Lysophosphatidylcholine (LPC1)	0.56 ± 0.21
Lysophosphatidylethanolamine	1.41 ± 0.33
Phosphatidic acid	1.11 ± 0.35
Phosphatidylcholine	42.67 ± 1.64
Phosphatidylethanolamine	9.15 ± 0.58
Phosphatidylglycerol	0.25 ± 0.10
Phosphatidylinositol	8.42 ± 0.89
Phosphatidylserine	6.61 ± 0.62
Sphingomyelin	9.65 ± 0.63

[a] Phosphatidylcholine deacylated at the glycerol-1 carbon.

(DHSM), dimethylphosphatidylethanolamine (DiMePE), diphosphatidylglycerol (DPG), ethanolamine plasmalogen (EPLAS), lysoalkylacylphosphatidylcholine (LAAPC), lysophosphatidic acid (LPA), lysophosphatidylcholine (LPC), lysophosphatidylcholine (deacylated at the glycerol-1 carbon) (LPC1), lysoethanolamine plasmalogen (LEPLAS), lysophosphatidylethanolamine (LPE), phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylserine (PS) and sphingomyelin (SM). The relative concentrations of the detected phospholipids for all cases are presented in Table 1. The esophageal profiles contain a large number of unusual phospholipids in relatively high concentrations: DiMePE, LAAPC, LPA, LPCl and LEPLAS. These phospholipids for the most part represent lysosomal activity.

By univariate analysis, the following phospholipids exhibited significant correlations with clinical and histopathologic parameters: DHSM, LAAPC, LPCl and PG. Statistically significant differences were noted in the levels of DHSM based upon the pathologic T stage of the tumor. The mean concentration of DHSM was 0.71 ± 0.31 in T_1 tumors and 2.14 ± 0.26 in T_3 tumors ($p < 0.05$). Similarly, the levels of LAAPC were significantly different when comparing T_1, T_2 and T_3 tumors. The mean concentration of LAAPC was 0.72 ± 0.51 for T_1 tumors compared to 0.02 ± 0.02 for T_2 tumors ($p < 0.05$). LAAPC was not detectable in T_3 tumors. There was a clear trend of decreasing DHSM and LAAPC with increasing T stage. These correlations were statistically significant at the $p < 0.02$ level for DHSM ($r = 0.40$) and the $p < 0.004$ level for LAAPC ($r = 0.46$).

The levels of PG were significantly different among the three tumor grades. The levels of PG in grade 1 tumors measured 1.03 ± 0.52 as compared to 0.22 ± 0.13 for grade 2 tumors ($p < 0.05$) and 0.04 ± 0.04 for grade 3 tumors ($p < 0.05$). There was also a clear trend of

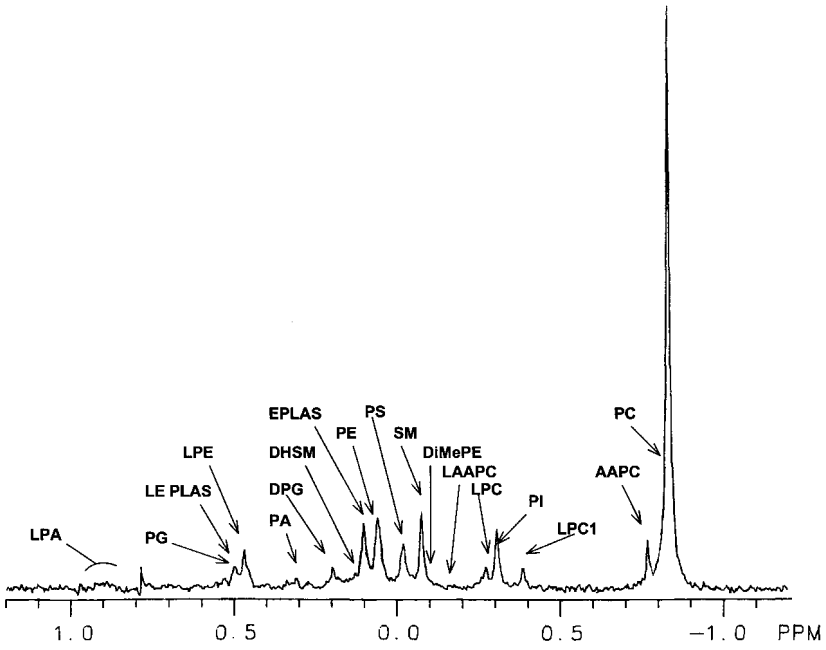

Figure 1. ^{31}P NMR phospholipid spectrum of a malignant esophageal tumor. **Legend**: lysophosphatidic acid (LPA), phosphatidylglycerol (PG), lysoethanolamine plasmalogen (LEPLAS), lysophosphatidylethanolamine (LPE), phosphatidic acid (PA), diphosphatidylglycerol (DPG), 4,5-dihydrosphingomyelin (DHSM), ethanolamine plasmalogen (EPLAS), phosphatidylethanolamine (PE), phosphatidylserine (PS), sphingomyelin (SM), dimethylphosphatidylethanolamine (DiMePE), lysoalkylacylphosphatidylcholine (LAAPC), lysophosphatidylcholine (LPC), phosphatidylinositol (PI), lysophosphatidylcholine (deacylated at the glycerol-1 carbon) (LPC1), alkylacylphosphatidylcholine (AAPC), phosphatidylcholine (PC)

decreasing PG with increasing grade. This trend was statistically significant at the $p < 0.01$ level ($r = 0.40$).

The results of LPC1 were parallel to those of PG with regard to tumor grade. The levels of LPC1 measured 2.15 ± 1.48 in grade 1 tumors as compared to 0.49 ± 0.20 in grade 2 tumors ($p < 0.05$) and 0.13 ± 0.13 for grade 3 tumors ($p < 0.05$). Likewise, there was a clear trend of decreasing LPC1 with increasing tumor grade that was significant at the $p < 0.01$ level ($r = 0.40$).

With regard to lymphatic vessel invasion, the level of PG was diminished in tumors with lymphatic vessel invasion (0.04 ± 0.04) compared to those without lymphatic vessel invasion (0.36 ± 0.15) at the 0.04 level.

DISCUSSION

Our data reveal a correlation between decreasing levels of four of the phospholipids (DHSM, LAAPC, LPC1 and PG) and increasing tumor aggressiveness as indicated by *T* stage and tumor grade. In addition, for the phospholipid PG, the concentration is lower in the presence of lymphatic vessel invasion, another indicator of tumor aggressiveness.

The importance of these lipids to the normal function of the esophagus is unknown. There is increasing evidence that phospholipids act as a surfactant in the esophagus to protect normal esophageal epithelium from exposure to noxious agents, specifically acids, produced during digestive and stress-related processes.[36,37] The outstanding feature of the esophagus, based on the findings of this study, is the presence of PG in substantial amounts relative to profiles of other tissues of the human body. The only other tissues known to contain substantial concentrations of PG in the human are the adult lung and fetal amniotic fluid.[14] For all of these tissues, the outstanding feature of the interface between the surface and air or other tissues is the presence of a lubricating mucin layer that in all cases appears to exert some degree of protection against chemical toxins or immune response cells.

In the lung, surfactants play several roles, including maintaining epithelial hydration and reducing surface tension at the air–fluid interface, and they also appear to have an inhibitory effect on immune response cells including lymphocytes.[38] Thus, surfactants appear to protect the lung from immune reactions in the setting of constant exposure to antigens. The purpose of the polyhydroxylated surfactants, for example PG,[39–41] is to organize a thickened water layer between the cell surface and air. This is accomplished by the hydrogen bond interaction of such polyhydroxylated polar headgroups with the liquid water structure. This action organizes liquid water into a relatively stationary gel,[42] thus creating a barrier between the epithelial cell surface and antigens or toxins impinging from the outside.

From this study it is not possible to determine whether the decreasing concentration of these four phospholipids is linked to the malignant transformation of the cells. It is possible to conclude that the decreasing phospholipid concentration correlates to the transformation of the cells from a less aggressive to a more aggressive phenotype. It is known that these phospholipids belong to a family known to promote transitions in membranes from lamellar to hexagonal II phases.[43,44] In doing so, this family of phospholipids imparts fluidity to membranes.[44]

In a separate report, we showed in a series of esophageal tumor specimens and normal tissues obtained using the same techniques, that the ethanolamine plasmalogen and phosphatidylserine are significantly diminished in esophageal tumors when compared to normal esophageal tissues obtained from the same patients. The two phospholipids are prominent components of the inner leaflet of the cell membrane. They also are known to be responsible, particularly the ethanolamine plasmalogen, for bilayer transitions from the lamellar to hexagonal II phase. ^{31}P NMR phospholipid studies characterize esophageal cancer. Other pathologic features have been examined and correlated with survival. For example, Sarbia et al.[45] proposed a prognostic score based on the pattern of tumor invasion and inflammatory response. By multivariate analysis these were found to be independent prognostic predictors for survival. Ongoing studies will determine whether our NMR findings correlate with clinical outcome. The ultimate goal of NMR analysis is to provide a method to predict the biologic behavior of these tumors. This may help select patients who would benefit from specific therapies such as neoadjuvant and post-operative therapies.

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