Use of novel autoantibody and cancer-related protein arrays for the detection of esophageal adenocarcinoma in serum

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Objective: The aim of this study was to evaluate the feasibility of using novel autoantibody and cancer-related protein arrays to identify potential biomarkers for the early detection of esophageal adenocarcinoma in serum.

Methods: Sera from 18 patients with esophageal adenocarcinoma and 14 with gastroesophageal reflux disease were added to microarrays designed to detect circulating autoantibodies to 51 tumor-associated antigens. Sera from the same patients were also added to a 53-plex assay for various cancer-related proteins. Cutoff values at 3 standard deviations above the mean expression of gastroesophageal reflux disease were used as a boundary for positivity.

Results: Nine proteins and 11 autoantibodies were able to individually segregate at least 1 esophageal adenocarcinoma sample from gastroesophageal reflux disease by means of cutoff values. The most discriminative marker was Fas ligand in the protein array, which was associated with 83.3% sensitivity and 100% specificity. The best performing autoantibody, NY-ESO-1, detected 3 esophageal adenocarcinoma samples. When both of these markers were combined, a sensitivity of 88.9% and specificity of 100% were attained.

Conclusions: Cancer-related protein and autoantibody arrays provide a technically simple and rapid method of identifying potential biomarkers for the detection of esophageal adenocarcinoma in serum. Furthermore, combining these platforms improves the diagnostic power of either platform alone. Integrating technologies that detect the expression of multiple proteins and autoantibodies in serum may provide a noninvasive and accurate method of detecting early esophageal adenocarcinoma.

The incidence of esophageal adenocarcinoma (EAC) in the United States has increased by 5-fold in the past 30 years, representing the most drastic rise of any cancer.¹ Epidemiologic data suggest that this rise may be due to similar increases in the incidences of gastroesophageal reflux disease (GERD) and associated Barrett esophagus, inasmuch as EAC is thought to follow a metaplasia–dysplasia–carcinoma sequence.² In addition, esophageal cancer has a dismal 5-year survival of 10% to 15% despite advances in medical and surgical therapy. This is most likely a result of more than 50% of patients having incurable disease on first examination, emphasizing the need for developing better methods of early detection.³

Current screening of patients with GERD who are at risk for EAC is limited to endoscopic surveillance, a method that requires sedation and is associated with high interobserver variability and a possibility of esophageal perforation. A noninvasive and inexpensive diagnostic tool that accurately detects EAC would allow for a broader screening program, increased probability of early detection, and ultimately an improvement in the dismal survivals of patients with EAC. These requirements may be fulfilled by technology that analyzes multiple biomarkers in serum.

The contents of tumor cells reach the blood to constitute part of the serum proteome either through active secretion or after cellular damage. The presence of elevated

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Abbreviations and Acronyms

EAC	= esophageal adenocarcinoma
FasL	= Fas ligand
GERD	= gastroesophageal reflux disease
TAA	= tumor-associated antigen

tumor-related proteins in serum, such as cytokines, epithelial cell markers, chemokines, growth factors, and angiogenic factors, have been demonstrated to be an important repository for experimental diagnostic tests in the detection of malignancy.⁴⁻⁷ The fact that there are very few individually sensitive cancer biomarkers underscores the need for multiple marker panels to achieve higher diagnostic performance.

The Luminex system (Luminex Corp, Austin, Tex) is based on laboratory multi-analyte profiling (LabMAP technology that uses up to 100 color-coded bead sets, each of which can be conjugated with a different reactant. This advanced LabMAP biomarker technology is capable of analyzing more than 100 different protein markers in an automated, internally controlled assay. Recent reports have demonstrated LabMAP technology's superior performance in identifying patients with early ovarian and breast cancer, stimulating the potential to establish a clinically reliable tool for the diagnosis of cancer.^{8,9}

In addition to secreted proteins, the host response to tumor-associated antigens can potentially be exploited for the early detection of EAC. Autoantibodies directed at tumor-associated antigens are thought to reflect aberrant expression and structural or functional changes in autologous intracellular proteins. A major advantage of analyzing these antibodies is that their production represents a dramatic amplification, allowing for the detection of circulating levels at earlier stages of tumor growth. CellCorrect microarrays (CeMines, Golden, Colo) take advantage of this biological phenomenon by containing selective cancer-specific antigen panels that interact with serum autoantibodies by reverse immunocapture. We hypothesized that these autoantibody arrays in combination with cancer-related protein profiling would provide a technically simple and rapid method of identifying potential serum biomarkers in EAC.

Methods**Patient Population**

Sera from 18 patients with EAC (stage II, $n = 4$; stage III, $n = 9$; stage IV, $n = 5$) and 14 patients with GERD were obtained through protocols approved by the Institutional Review Board at the University of Pittsburgh Medical Center. Age (mean 58.9 vs 58.2 years), sex (all men), and smoking status (27.8% vs 28.6% nonsmokers) were similar between the EAC and GERD groups, respectively. Each sample was taken before anesthesia on the day of surgery (esophagectomy for EAC, Nissen fundoplication for GERD) and processed in an identical manner. Standard venipuncture technique

was used to draw peripheral blood into 10-mL glass red top tubes, and samples were left to stand for 30 minutes. Sera were then separated by centrifugation, an aliquot was taken, and frozen at -80°C . Freeze-thaw cycles were avoided.

Luminex Cancer-related Protein Profiling

Luminex LabMAP protein arrays were constructed with polystyrene microspheres dyed with variable ratios of two spectrally different fluorophores. These were used to create a family of 53 differentially addressed bead sets, with each set conjugated to a capture antibody specific for a unique soluble analyte. The 53 proteins that were analyzed in this particular array included cytokines, chemokines, angiogenic factors, apoptotic proteins, proteases, cancer antigens, and growth factors that have been demonstrated to be potential markers in a variety of other cancers (Table 1).

Assay buffer (25 μL) was added to background and sample wells in a 96-well microplate. Sera, 25 μL , from each EAC or GERD sample were added to appropriate wells in duplicate, followed by 25 μL of the bead sets, and the mixture was shaken for 1 hour in the dark with an orbital shaker. Wells were washed twice with 200 μL of wash buffer and then incubated with 25 μL of detection antibody for 30 minutes. After the addition of streptavidin–phycoerythrin solution, washing, and resuspension, results were read on the Luminex 100 machine. In internal validation studies, our Luminex facility has demonstrated low intra-assay (6.0%–8.2%) and interassay (5.7%–8.4%) coefficients of variation for multiplexed LabMAP assays using a variety of these biomarkers.

Dot plots were created to graphically express serum concentrations (average intensity of duplicate runs) of each of the 53 analytes. Cutoff values were established at 3 standard deviations above the mean GERD concentration. The most discriminative marker was chosen by assessing the associated sensitivities and specificities of each individual protein using these cutoff boundaries.

Autoantibody Arrays

CellCorrect microarrays were created with 51 preprinted synthetic, potentially immunogenic peptides that were selected on the basis of cancer gene expression and immunome databases (Table 2). These arrays were provided by CeMines free of charge for this analysis. Two microliters of each serum sample were diluted in binding buffer (Tris-buffered saline containing blocking solution and 0.5 Tween-20) and incubated overnight with the peptide arrays at 4°C . A secondary antibody (alkaline phosphatase conjugated anti-human immunoglobulins A, M, and G; Chemicon AP120A, Millipore, Billerica, Mass) was used to detect the immobilized serum autoantibodies. Slides were imaged with a flatbed scanner and the intensity of each spot was densitometrically assessed with software from the manufacturer. Each sample was run twice on two separate sets of arrays.

Similar to the Luminex analysis, dot plots were created to visualize concentrations (average intensity of two independent runs) in each EAC and GERD sample, and cutoff values were generated at 3 standard deviations above the mean GERD value to establish a strict boundary between a positive and negative test result. The most discriminative autoantibody was determined by identifying the marker associated with the highest sensitivity and specificity. The best performing autoantibody and cancer-related protein were also combined to assess whether their respective diagnostic powers were additive.

TABLE 1. Fifty-three–plex assay for cancer-related proteins

Biological groups	Proteins
Cytokines	IL-6, IL-8, IL-10, TNF α , TNFR I, TNFR II, IL-2R, sIL-6R, G-CSF
Chemokines	MIP-1a, MIP-1b, MCP-1, Eotaxin, MIF
Growth/angiogenic factors	EGFR, bFGF, HGF, VEGF, NGF, IGFBP-1
Cancer antigens	CA 125, CA 15-3, CEA, AFP, CA 19-9, CA 72-4
Apoptotic proteins	sFas, sFasL, FasL
Proteases	Kallikrein 8, MMP-1,2,3,7,8,9,12,13
Adhesion molecules	sICAM, sVCAM, sE-selectin
Other markers	Aesothelin, tPAI-1, active PAI-1, ULBP-1, ULBP-2, ULBP-3, MICA, angiostatin, SCC
Adipokines	Adiponectin, leptin, resistin

IL, Interleukin; *TNF*, tumor necrosis factor; *TNFR I*, tumor necrosis factor type I receptor; *G-CSF*, granulocyte-colony stimulating factor; *MIP*, macrophage inflammatory protein; *MIF*, macrophage inhibitory factor; *EGFR*, epidermal growth factor receptor; *bFGF*, basic fibroblast growth factor; *HGF*, hepatocyte growth factor; *VEGF*, vascular endothelial growth factor; *NGF*, nerve growth factor; *IGFBP*, insulin-like growth factor binding protein; *CA*, cancer antigen; *CEA*, carcinoembryonic antigen; *AFP*, alpha-fetoprotein; *FasL*, Fas ligand; *MMP*, matrix metalloproteinase; *ICAM*, intercellular adhesion molecule; *VCAM*, vascular cell adhesion molecule; *PAI*, plasminogen activator inhibitor; *ULBP*, UL16-binding protein; *MICA*, major histocompatibility complex class I-related chain A; *SCC*, squamous cell carcinoma.

Results

A total of 9 proteins were able to individually segregate at least 1 EAC sample from the GERD group (Figure 1). Fas ligand (FasL) was the marker associated with the highest sensitivity (15/18 EAC; 83.3%) and specificity (14/14 GERD; 100%) using cutoff boundaries. In the CellCorrect arrays, 11 autoantibodies were able to individually distinguish at least 1 EAC patient from GERD (Figure 2). The best performing autoantibody was NY-ESO-1, which was able to detect 3 EAC samples, 1 of which was unique to those detected by FasL. Therefore, when combined, these 2 markers were associated with 88.9% sensitivity and 100% specificity.

Discussion

The incidence of EAC has been increasing at dramatic rates over the past several decades. Additionally, the majority of patients with EAC continue to have advanced, unresectable disease on initial examination, resulting in dismal 5-year survivals that have only modestly improved since the 1970s. These observations underscore the need for developing better methods of early EAC detection. In the present study, we hypothesized that a combination of platforms that analyze cancer-related proteins as well as autoantibodies to tumor-associated antigens in sera would provide a technically simple and rapid method of identifying potential biomarkers of EAC.

TABLE 2. Discriminatory autoantibodies

Tumor-associated antigen	Biological role
Adapter-related protein complex 1 gamma 2 subunit (AP1G2)	Protein complex assembly, intracellular protein transport, endocytosis, vesicle-mediated transport
Nascent-polypeptide-associated complex alpha (NACA)	Transcription, protein biosynthesis, protein transport
Nischarin (NISCH)	Intracellular signaling cascade, Rac protein signal transduction, actin cytoskeleton organization and biogenesis, negative regulation of cell migration
Serologically defined colon cancer antigen 3 (SDCCAG3)	Protein trafficking, presentation of tumor necrosis factor receptor on surface
UTP14, U3 small nucleolar ribonucleoprotein, homolog A (UTP14A)	Ribosome biogenesis
Zinc finger protein 292 (ZNF292)	Regulation of transcription, DNA-dependent
Nuclear factor related to kappa B binding protein (NFRKB)	Transcription from RNA polymerase II promoter, inflammatory response
v-abl Abelson murine leukemia viral oncogene homolog 1 (ABL1)	Regulation of progression through cell cycle, S-phase–specific transcription in mitotic cell cycle, mismatch repair, regulation of transcription, DNA-dependent
Golgi autoantigen, golgin subfamily a,2 (GOLGA2)	Protein binding
LIM and senescent cell antigen-like domains 1 (LIMS1)	Cell–matrix adhesion, cell–cell adhesion, cell aging
Cancer/testis antigen B (NY-ESO-1)	Biological process unknown

Figure 1. Dot plots of discriminatory cancer-related proteins. Dot plots of 9 proteins that were able to individually segregate at least 1 EAC sample from GERD. *Black diamonds* represent GERD and *white squares* represent EAC. *TNF-RI*, Tumor necrosis factor receptor I; *IL-2R*, interleukin-2 receptor; *IGFBP*, insulin-like growth factor binding protein; *AFP*, alpha-fetoprotein; *MMP*, matrix metalloproteinase; *HGF*, hepatocyte growth factor; *FasL*, Fas ligand; *PAI*, plasminogen activator inhibitor.

We analyzed more than 100 proteins and autoantibodies in sera taken from patients with EAC or GERD and found that 20 of these markers were able to individually distinguish at least 1 cancer sample from the reflux cohort. As a preliminary analysis, we combined the most discriminative protein (FasL) and autoantibody (NY-ESO-1) and found that this combination was associated with 88.9% sensitivity and 100% specificity. Indeed, FasL has been shown to be overexpressed in several types of cancers¹⁰⁻¹³ and likely represents a mechanism whereby tumor cells induce apoptotic death in Fas-expressing lymphocytes, thereby avoiding immune destruction.¹⁴ Similarly, NY-ESO-1 is an antigen that has been demonstrated to be overexpressed in a multitude of cancers.¹⁵ In fact, its specificity has drawn much attention to its potential role in developing a cancer vaccine.

The major limitation of this study is the relatively low sample number. A more accurate measure of the sensitivity and specificity of these markers will require prospective testing with larger patient cohorts. The main focus of this article, however, is the scientific and clinical appeal of biomarker array systems rather than the validation of specific markers. Moreover, these technologies allow the analysis of hundreds of proteins within a few hours and, therefore, provide a fertile ground for the identification of biomarkers that may have discriminative capability in cancer. Furthermore, once potential biomarkers have been validated, this technology would allow for the integration of multiple markers into a single platform. This concept is clinically relevant inasmuch as technical feasibility and time requirement are important components of determining the cost effectiveness of a screening or risk

Figure 2. Dot plots of discriminatory autoantibodies. Dot plots of 11 autoantibodies that were able to individually segregate at least 1 EAC sample from GERD. *Black diamonds* represent GERD and *white squares* represent EAC. *AP1G2*, Adapter-related protein complex 1 gamma 2 subunit; *NACA*, Nascent-polypeptide-associated complex alpha; *NISCH*, nischarin; *SDCCAG3*, serologically defined colon cancer antigen 3; *UTP14A*, U3 small nucleolar ribonucleoprotein, homolog A; *ZNF292*, zinc finger protein 292; *NFRKB*, nuclear factor related to kappa B binding protein; *ABL1*, v-abl abelson murine leukemia viral oncogene homolog 1; *GOLGA2*, golgi autoantigen, golgin subfamily a,2.

stratification tool. Moreover, a subset analysis within patients with EAC could be performed to identify those markers associated with localized or advanced disease. This could reduce the necessity for invasive staging procedures and could also have a role in guiding adjuvant therapies.

In the setting of EAC, the development of a screening tool for at-risk patients could be of significant value, espe-

cially in patients with Barrett esophagus with associated high-grade dysplasia, in whom treatment guidelines remain controversial. Those patients whose serum markers are suggestive of underlying malignancy could undergo potentially curative esophagectomy, whereas those patients without these markers could be spared major surgery. This study highlights potential platforms that could be of great value

both in identifying potential biomarkers and in developing panels that are routinely used to assess cancer risk in the clinical setting.

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