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Mesothelin is a specific biomarker of invasive cancer in the Barrett-associated adenocarcinoma progression model

Translational implications for diagnosis and therapy

Hector Alvarez, M.D.¹, Pamela Leal Rojas, M.D.¹, Ken-Tye Yong, Ph.D.⁴, Hong Ding, Ph.D.⁴, Gaixia Xu, Ph.D.⁴, Paras N. Prasad, Ph.D.⁴, Jean Wang, M.D.³, Marcia Canto, M.D.³, James R. Eshleman, M.D., Ph.D.^{1,2}, Elizabeth A. Montgomery, M.D.¹, and Anirban Maitra, M.B.B.S.^{1,2}

¹Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, Maryland ²Department of Oncology, Johns Hopkins University School of Medicine, Baltimore, Maryland ³Division of Gastroenterology and Hepatology, Johns Hopkins University School of Medicine, Baltimore, Maryland ⁴Institute for Lasers, Photonics, and Biophotonics, Department of Chemistry, State University of New York, Buffalo, New York

Abstract

Esophageal adenocarcinoma arises in the backdrop of Barrett metaplasia-dysplasia sequence, with the vast majority of patients presenting with late stage malignancy. Mesothelin, a GPI-anchored protein, is aberrantly overexpressed on the surface of many solid cancers. Mesothelin expression was assessed in esophageal tissue microarrays (TMAs) encompassing the entire histologic spectrum of Barrett-associated dysplasia and adenocarcinoma. Mesothelin expression was observed in 24/84 (29%) of invasive adenocarcinomas, and in 5/34 (15%) lymph node metastases. In contrast, normal squamous and cardia mucosa, as well as non-invasive Barrett lesions, failed to label with mesothelin. Mesothelin was expressed in the esophageal adenocarcinoma cell line JHU-EsoAd1, but not in primary human esophageal epithelial cells. Anti-mesothelin antibody conjugated CdSe/CDS/ZnS quantum rods (QRs) were synthesized as described (Young et al, *NanoLetters*, 2007), and confocal bio-imaging confirmed robust binding to JHU-EsoAd1 cells. Anti-mesothelin antibody conjugated nanoparticles can be useful for the diagnosis and therapy of mesothelin-overexpressing esophageal adenocarcinomas.

Keywords

Barrett esophagus; mesothelin; immunohistochemistry; quantum rods; antibody conjugated nanoparticles; molecular imaging; esophageal adenocarcinoma

Corresponding author: Anirban Maitra, MBBS, Room 345, CRB II, Johns Hopkins University School of Medicine, 1550 Orleans Street, Baltimore, MD 21231, Phone 410 502 8191; Fax 410 614 0671; Email amaitra1@jhmi.edu.

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Introduction

Esophageal adenocarcinoma arises within the context of progressive histologic alterations, originating with the development of a metaplastic epithelium (Barrett esophagus) and extending through low- and high-grade dysplastic stages to frank invasive cancer^{1, 2}. The progression from high-grade dysplasia to adenocarcinoma of the esophagus occurs frequently, and approximately 50–60% of patients develop invasive cancer within three- five years of a diagnosis (on routine histologic evaluation) of high grade dysplasia^{3, 4}. Esophageal carcinoma currently has one of the most rapidly increasing tumor incidences in the United States⁵; the American Cancer Society estimates that there will be over 15,500 new cases of esophageal carcinoma in 2007⁶. Unfortunately, despite an established precursor lesion, and the availability of endoscopic screening programs, the overwhelming majority of patients presenting with esophageal adenocarcinoma will do so at a late stage, and without a pre-existing diagnosis of Barrett esophagus^{7, 8}. As a consequence, of the 15,500 estimated new patients this year, almost 14,000 (~90%) are expected to succumb to their disease⁶. Thus, there is an urgent need for developing better biomarkers for early diagnosis of esophageal cancer, as well as potent molecularly targeted therapies that can be utilized for treatment of established, advanced disease.

Mesothelin is a 40kDa GPI-anchored surface glycoprotein that is expressed on pleural and peritoneal mesothelial cells, but not in other non-neoplastic tissues⁹. Mesothelin is synthesized as a 69kDa precursor protein, which is subsequently cleaved into the membrane bound mesothelin antigen, and a 31kDa soluble fragment known as mesothelin-associated lung cancer antigen (MUC16) (MPF)¹⁰. Multiple lines of evidence have established that mesothelin is aberrantly overexpressed in certain cancers, including the vast majority (80–100%) of pancreatic and non-mucinous ovarian cancers, and in mesotheliomas^{9, 11–14}. In addition, a recent broad survey by Ordóñez of 471 archival human cancers by immunohistochemistry has found mesothelin expression in other neoplasms at a lower frequency, including in approximately a third of pulmonary adenocarcinomas, cholangiocarcinomas, colorectal and gastric adenocarcinomas¹⁵. In this study, four esophageal adenocarcinomas were also examined, and one (25%) labeled with mesothelin. However, there have been no comprehensive analyses of mesothelin expression in esophageal adenocarcinomas, specifically as it relates to the multi-step Barrett progression model. This is an important question to address for multiple reasons with immediate translational impact. *First*, mesothelin expression is a useful ancillary diagnostic marker in clinical specimens^{15–22}; *second*, surface mesothelin antigen has been utilized as a target for antibody-based cancer treatment approaches (e.g., using immunotoxins)^{10, 23, 24}; *third*, mesothelin epitopes have been identified in the context of adoptive immunotherapy using anti-cancer vaccines^{25–27}; and *fourth*, elevated mesothelin levels in circulation has emerged as a promising biomarker in cancer^{28–32}. Therefore, we undertook this study to systematically analyze mesothelin expression in the entire histologic spectrum of Barrett esophagus, dysplasia and adenocarcinoma. Our study indicates that mesothelin is a specific marker of invasive adenocarcinoma arising in the context of Barrett esophagus, and can potentially be utilized as a molecular target in the diagnosis and treatment of esophageal cancer.

Materials and Methods

Esophageal tissue microarrays (TMAs)

TMAs were created representing the full histological spectrum of lesions observed in the Barrett esophagus progression model³³. We have previously utilized these TMAs for immunohistochemical analysis of molecular alterations in esophageal adenocarcinoma^{34, 35}. Briefly, the TMAs were created from 98 patients who underwent endoscopic biopsies or surgical resection for Barrett esophagus and adenocarcinoma. Overall, 365 cores were evaluated for mesothelin labeling, including 64 cores of squamous mucosa, 86 of cardia

mucosa, 30 with non-dysplastic Barrett esophagus, 23 with low-grade dysplasia (LGD), 41 with high-grade dysplasia (HGD), 84 with adenocarcinoma, and 37 cores representing metastatic lesions (34 lymph node, 1 liver, and 2 lung metastases).

Immunohistochemistry and assessment of mesothelin labeling

Immunohistochemistry for mesothelin expression was performed on TMAs using an anti-mesothelin antibody (clone 5B2, dilution 1:20, Novocastra, Newcastle Upon Tyne, UK), as previously described^{36, 37}. Briefly, unstained 4µm sections were cut from each esophageal TMA and deparaffinized by routine techniques before placing in 200 ml Target Retrieval Solution, pH 6.0 (Dako, Envision Plus Detection Kit, Carpinteria, CA, USA) for 20 min at 100°C. After cooling for 20 min, slides were quenched with 3% H₂O₂ for 5 min, before incubating with anti-mesothelin antibody for 60 minutes, using the Dako Autostainer. Labeling was detected with the Dako Envision system as per the manufacturer's protocol. All sections were counterstained with Giles' hematoxylin. Two of the authors (HA and AM) evaluated the staining, using cytoplasmic labeling with apical membranous accentuation as the pattern of specific mesothelin labeling¹¹. TMA cores were scored as negative (<5% labeling) or positive, based on a simple two-tier classification, and tabulated by histologic subtype of the lesion.

Western blot analysis for mesothelin expression

Mesothelin expression was evaluated in the human esophageal adenocarcinoma cell line, JH-Eso-Ad1, created by one of the authors (JRE) from a patient undergoing surgical resection for esophageal adenocarcinoma at Johns Hopkins. The HeLa cell line was used as a positive control. In addition, we also obtained primary non-malignant human esophageal epithelial cells (HEEPIC, ScienCell Inc)³⁸ as a negative control to exclude mesothelin expression in the normal esophageal epithelium. A volume of 50µg of protein was separated by SDS-polyacrylamide electrophoresis and transferred to a nitro-cellulose membrane. The membrane was stained with mouse monoclonal anti-mesothelin antibody (clone K1, dilution 1:40, Invitrogen, Carlsbad, CA), and incubated with a secondary horseradish peroxidase (HRP)-conjugated antibody. The blots were then washed and developed using the enhanced chemiluminescence (ECL) detection system (GE Healthcare, Piscataway, NJ). Actin was used as loading control in these experiments. The Western blot was performed in triplicate, and densitometric comparison performed using ImageQuant software (GE Healthcare).

Synthesis of anti-mesothelin antibody conjugated nanoparticles and confocal imaging

Our group has recently described the detailed synthesis of cadmium selenide / cadmium sulfide / zinc sulfide (CdSe/CdS/Zns) rod-shaped semi-conductor nanocrystals, also known as quantum rods (QRs)³⁹. The QRs have an average size of less than 15nm, emit in the red spectrum, and are amenable to surface functionalization (e.g., antibody conjugation), in order to ensure cell specific targeting. We have previously demonstrated the utility of these QRs for confocal and two-photon fluorescence imaging of cancer cells *in vitro*³⁹. Briefly, CdSe/CdS/ZnS QRs were synthesized in organic media, and subsequently dispersed in aqueous media using a mixture of mercaptoundecanoic acid (MUA) and lysine. Following aqueous dispersion, the QRs were conjugated with mouse monoclonal anti-mesothelin antibody (clone K1, Invitrogen) using carbodiimide chemistry. Of note, K1 is the original antibody clone that was utilized for identification of mesothelin antigen⁴⁰. For confocal imaging, the three esophageal cancer cells (BIC-1, SEG-1 and JH-Eso-Ad1) were seeded overnight in 35 mm cell culture dishes, and allowed to reach 60–70% confluence. Thereafter, the cells were incubated for 2 hours in the presence of anti-mesothelin antibody conjugated QRs, or unconjugated lysine coated QRs. The cells were then washed three times with PBS and directly imaged using a Leica TCS SP2 AOBS spectral confocal microscope (Leica Microsystems Semiconductor GmbH, Wetzlar, Germany) with laser excitation at 442nm.

Results

Immunohistochemical analysis confirmed that mesothelin expression is restricted to esophageal adenocarcinoma and related metastases, with 0/64 squamous mucosal samples, 0/86 cardiac mucosal samples, 0/30 Barrett esophagus, 0/23 LGD, and 0/41 HGD labeling with anti-mesothelin antibody. Cytoplasmic mesothelin labeling with prominent apical membranous accentuation was observed in 24/84 (29%) primary adenocarcinomas (Figure 1). Mesothelin expression was usually intense and present in >50% of neoplastic cells, and not uncommonly, secreted protein was observed within the lumina of neoplastic glands, as we have previously reported in other mesothelin-positive cancer types¹¹. A lower frequency of mesothelin labeling was observed in lymph node metastases (5/34, or 15%); the 3 liver and lung metastases were negative.

Elevated mesothelin expression was observed in the JHU-EsoAd1 cells, almost double that of levels seen in the HeLa cell line by densitometric analysis, while no mesothelin expression was observed in the non-malignant HEEPIC cells (Figure 2). At 2 hours post-incubation with anti-mesothelin conjugated QRs, optical imaging established robust binding of the red fluorescent QRs to JHU-EsoAd1 cells (Figure 3A and B), while no binding was seen upon incubation with the unconjugated QRs (Figure 3C), confirming that the cell surface mesothelin is amenable to active targeting for diagnostic and/or therapeutic purposes.

Discussion

The vast majority of patients with esophageal adenocarcinoma present with late stage disease, and current conventional therapies have not improved the prognosis of this neoplasm^{1, 2}. We undertook this study to evaluate the pattern and frequency of mesothelin expression in the multi-step progression model of Barrett esophagus, since mesothelin has garnered considerable promise as a diagnostic and therapeutic anti-cancer target in recent times. Our study demonstrates that mesothelin expression is essentially restricted to invasive adenocarcinoma, with preceding non-invasive lesions, including high-grade dysplasia, not expressing this antigen. The frequency of mesothelin labeling in esophageal adenocarcinoma in our study (24 of 84 cases, or 29%) is comparable to that observed by Ordonez (1 of 4 cases, or 25%) in his broad survey of human cancers for mesothelin expression¹⁵. Of note, we have previously demonstrated that mesothelin expression is absent in non-invasive precursor lesions of pancreatic and biliary adenocarcinomas, but is upregulated during the transition from carcinoma-*in-situ* (PanIN-3 in the pancreas) to invasive cancer^{36, 41}. Thus, our study adds to the evidence that mesothelin might play a role in the process of invasion during multi-step epithelial cancer progression. Based on our data, we propose that mesothelin expression is a highly specific, albeit not particularly sensitive, biomarker of invasive adenocarcinoma in Barrett esophagus, amenable to application in routine clinical specimens. It is likely that mesothelin expression has to be utilized in conjunction with other molecular markers of advanced disease, such as diffuse cdc2/CDK1 expression⁴², in order to enhance diagnostic sensitivity.

Despite the restricted expression of mesothelin to ~1/3rd of esophageal adenocarcinomas, it is important to note that approximately 90% of patients with this malignancy will currently succumb to their disease⁶. Exploiting the repertoire of mesothelin targeted therapeutic approaches, therefore, provides an opportunity to improve prognosis in the mesothelin-positive subset of esophageal adenocarcinomas. Using novel anti-mesothelin antibody conjugated quantum rod (QR) nanocrystals, we have confirmed the feasibility of binding the surface antigen expressed on esophageal adenocarcinoma cell lines, and analogous strategies can be used for delivery of immunotoxins^{10, 23, 24}, immunoliposomes^{43, 44}, or other nanoparticle encapsulated therapeutic payloads. The naturally fluorescent anti-mesothelin QRs, or

comparable mesothelin targeted nano-imaging devices like quantum dots^{45, 46}, can also be used in the context of imaging of esophageal adenocarcinoma *in vivo*, such as assessing for the presence or depth of invasion in the primary lesion. Immunogenic mesothelin epitopes have been utilized as cancer vaccines in preclinical models of ovarian cancer^{25, 26}, and these could be extended to esophageal cancer as well. Most recently, Phase I clinical trials were conducted with a humanized monoclonal antibody targeting mesothelin (MORab-009, Morphotek Inc.) in patients with mesothelin expressing solid cancers, and demonstrated a safe clinical profile. Antibody-based single agent or combination therapies could be evaluated in the context of mesothelin-expressing advanced esophageal adenocarcinomas⁴⁷. The availability of a reliable immunohistochemical assay for mesothelin expression in biopsy specimens would permit prior selection of patients most likely to benefit from such molecularly targeted therapies in clinical trials.

In summary, we have demonstrated mesothelin expression in a subset (~1/3rd) of esophageal adenocarcinomas, with absence of labeling in normal esophagus and non-invasive precursor lesions in the Barrett esophagus progression model. Future studies are warranted exploiting mesothelin as a molecular target in esophageal adenocarcinoma for diagnostic and therapeutic purposes.

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Figure 1. Immunohistochemical labeling of mesothelin in Barrett esophagus and associated adenocarcinomas

No mesothelin labeling is observed in normal squamous mucosa (A), or in non-dysplastic Barrett esophagus (B). Absence of labeling is also observed in low-grade and high-grade dysplasia (*not illustrated*). In contrast, robust mesothelin expression is seen in a representative example of esophageal adenocarcinoma (C). In (D), high power photomicrograph illustrates the prominent apical membrane accentuation of mesothelin labeling.

Figure 2. Expression of mesothelin antigen in esophageal adenocarcinoma cell line

Western blot analysis confirms robust mesothelin expression in the JHU-EsoAd1 esophageal adenocarcinoma cell line. The HeLa cell line is used as positive control and primary non-malignant human esophageal epithelial cells (HEPPIC) is used as negative control. Actin is used as loading control. Densitometric reading designated represents the average of three independent Western blot experiments.

Figure 3. *In vitro* labeling of JHU-EsoAd1 esophageal adenocarcinoma cell line using mesothelin targeted nanoparticles

Figures 3A and 3B demonstrate labeling of independent fields in the JHU-EsoAd1 cell line with anti-mesothelin antibody conjugated CdSe/CdS/ZnS quantum rods (QRs). In contrast, Figure 3C demonstrates no labeling is observed with unconjugated lysine coated QRs. Each of the three figures includes a red channel, bright field, and “merge” panel.