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Circulating histocompatibility antigen (HLA) gene products may help differentiate benign from malignant indeterminate pulmonary lesions

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ABSTRACT

Background: This study explores the potential diagnostic utility of soluble Human Leukocyte Antigen (sHLA) molecules differentially released by lung adenocarcinoma and benign lung lesions.
Methods: Conditioned media from the NSCLC cell lines H358 and H1703 were immunoblotted for soluble isoforms of major histocompatibility complex (MHC) class I (ABC) and II (DRB1, DMB, and DQ) antigens. Sera from 25 patients with benign and 25 patients with malignant lesions were similarly evaluated to appraise the potential diagnostic value.
Results: Higher concentrations of soluble HLA class I molecules were observed in conditioned medium for the highly-invasive H1703 cell line, relative to the more indolent H358 cells. Evaluation of these markers against a cohort of 50 cases demonstrated that patients with malignant lesions possess higher levels of HLA class I and II molecules relative to those with benign lesions ($p < 0.05$), with exception to the primary isoform, DQA1, which was suppressed in malignancies. An analysis of biomarker performance via ROC analysis revealed promising performance (AUC > 0.75) for DMB and the 26 kDa isoform of DQ in distinguishing lesion pathology.
Conclusions: Soluble HLA molecules may have diagnostic value for early-stage NSCLC. Validation studies are currently underway using sera from a lung cancer screening cohort.

1. Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide [1]. According to the American Cancer Society, approximately 220,000 patients will be diagnosed with lung cancer and 160,000 will die of the disease in 2017 [1]. This has led to a variety of efforts to identify early-stage lung cancer, prime among which has been low-dose computed tomography (LDCT) lung cancer screening. Although the National Lung Screening Trial (NLST) demonstrated a 20% relative reduction in lung cancer mortality with annual LDCT, there was a high rate of false positive screens [1,2]. Accordingly, the International Association for the Study of Lung Cancer (IASLC) and the Strategic CT Screening Advisory Committee has called for the increased use of blood-based diagnostics to augment LDCT screening [3].

Human leukocyte antigens (HLA) are a key immune mechanism through which self- versus non-self-recognition occurs [4,5]. There are limited reports describing decreased expression of major histocompatibility complex (MHC) class I or class II antigens on the surface of tumor cells [6–8]. It has been proposed that shedding of these HLA molecules into the peripheral circulation may be a key mechanism by which tumor cells circumvent host immune surveillance [6–8]. That is, if the HLA molecules are not present on the tumor cell surface, the host immune system will fail to recognize the tumor cell as foreign and mount an immune response [9]. We hypothesized that patients with malignant and benign lung lesions could be differentiated by comparing serum concentrations of the 'secretome' of soluble HLA molecules.

Abbreviations: NSCLC, non-small cell lung cancer; HLA, human leukocyte antigens; LDCT, low-dose computed tomography; MHC, major histocompatibility complex; ATCC, American Type Culture Collection; RPMI, Roswell Park Memorial Park; SDS-PAGE, standard denaturing polyacrylamide gel electrophoresis; LOH, loss of heterozygosity

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2. Materials and methods

2.1. Cell lines and media

Human adenocarcinoma cell lines H358 and H1703 were cultured at 37 °C in a humidified CO_2 incubator in RPMI-1640 containing 10% fetal bovine serum (FBS), 0.3 g/L glutamine, and antibiotics (100 units/mL penicillin/100 units/mL streptomycin/10 μg/mL gentamicin sulfate). Cultures were primary acquisitions from ATCC and all experiments performed within 10 passages of acquisition.

2.2. Collection of conditioned media

Cultures of the H358 and H1703 cells were individually grown to near-confluence (85–90%) and incubated for 3 days in RPMI-1640 containing 2.5% FBS, 0.3 g/L glutamine, and 100 units/mL penicillin and streptomycin, and 10 μg/mL gentamicin sulfate. After 3 days, the cell-conditioned media was collected and centrifuged for 10 min at 1,000 RCF. The resulting supernatant was albumin depleted using the Albumin Depletion Kit (Pierce Thermo Scientific), per manufacturer recommended protocols. The resulting supernatant was stored at −80 °C until further processing.

2.3. Patient specimens

A total of 50 serum specimens (25 patients with a benign nodule and 25 patients limited to $T_{1\text{-}2}N_0M_0$ adenocarcinoma (7th edition staging [10])) were obtained from the Rush University Biorepository Core. Patients were selected to fit the 'high risk' criteria for lung cancer screening (55–80 years old and a minimum 30 pack year smoking history and are either active smokers or had quit within 15 years of evaluation) and were approximately matched in terms of age, gender, and smoking history. All specimens were obtained with full written informed consent under a protocol approved by our Institutional Review Board (IRB). All samples were coded with only basic demographic and clinical parameters provided to the study personnel for the purposes of this study. All serum specimens were albumin in the same manner as mentioned in the previous section.

2.4. Protein concentration

All albumin-depleted samples (serum or cell-condition media) were concentrated using the ProteoExtract Protein Precipitation kit (EMD Millipore). Following resuspension in PBS containing 50 μL/mL Mammalian Protease Inhibitor Cocktail (Sigma), protein concentrations were determined through standard BCA assays (Thermo Scientific).

2.5. Immunoblotting of soluble antigens

Protein samples were resolved using standard denaturing polyacrylamide gel electrophoresis (SDS-PAGE) using Criterion 4–20% Tris-HCl gels (BioRad). Proteins were then transferred onto a nitrocellulose membrane using standard overnight "wet" transfer protocols. After being blocked for 1 h in 2% bovine serum albumin (Sigma), membranes were probed with the following monoclonal antibodies against HLA gene products: class I: HLA-ABC [clone EMR8-5; 33 and 41 kDa]; class II: HLA-DRB1 [clone EPR6148; 30 kDa], HLA-DQ [clone TAL 4.1; 28 kDa], HLA-DMB [clone EPR7982; 29 kDa], HLA-DQA1 [clone EPR7300; 28 kDa and 33–35 kDa] were obtained from Abcam (Cambridge, MA, USA). The resulting immunoblots were developed using ECL Prime Western Blotting Detection Reagent (GE Healthcare Amersham) and visualized/images captured using a Versadoc MP 4000 imaging system (BioRad). Densitometry was performed with global background subtraction using the Quantity One software. Representative images of blots for each of the soluble HLA gene products tested are provided online as Supplemental Materials or at https://figshare.com/s/1d858cd5563acb58d381 (DOI https://doi.org//10.6084/m9.figshare.5998574).

2.6. Statistical methods

Mann-Whitney Rank Sum (2-sided) test was performed between the average protein density of HLA-ABC, HLA-DQ, and HLA-DRB1 bands detected in conditioned media of both non-invasive and invasive cell lines. For serum samples from patients with benign and $T_{1\text{-}2}N_0M_0$ (stage I) NSCLC samples, pixel densities were compared using the sum for the two distinct bands detected for HLA-ABC, HLA–DQA1 or the single detected band for HLA-DRB1, HLA-DQ and HLA-DMB. Replicates for these evaluations were performed in no less than triplicate measures with only sets possessing ≤20% CV permitted to be contrasted in this manner. Distributions of the study findings for the patient samples were plotted as box and whisker plots with significance indicated. Classifications via receiver operator curve (ROC) analysis were conducted in context of pathologically-confirmed benign or $T_{1\text{-}2}N_0M_0$ (stage I) NSCLC disease. All analyses were accomplished using SPSS v11.0 (IBM).

3. Results

3.1. Soluble HLA molecules in conditioned media from NSCLC cell lines

All HLA-specific protein bands were originally optimized using commercial antibodies (Abcam) with conditioned media from the H358 and H1703 cell lines. For HLA-ABC, the commercial antibodies were found to detect two HLA-ABC specific bands at 33 and 41 kDa. Densitometry values of these two HLA-ABC specific bands for the indolent (H358) and invasive (H1703) cell lines from two experiments were measured and compared, with representative immunoblots illustrated in Fig. 1. Increased levels of class I HLA were observed in the conditioned media from the H1703 cultures (approximately 4- and 7-fold increase for 41 kDa protein, approximately 3- & 8-fold increase for 33 kDa protein) in separate experiments. *Mann-Whitney Rank sum* tests were performed on the densities of the HLA-ABC proteins detected in the conditioned media of H358 and H1703 cells. Please note that these initial investigations were intended to help us appreciate the size of the soluble HLA molecules to search for in the patient specimens and approximate the sorts of differences we would expect to find with changing tumor phenotype. Therefore, we did not rigorously examine these findings with statistical tests as we do in the remainder of the study.

The conditioned media were also tested for two class II HLA proteins, namely HLA-DQ and HLA-DRB1. The levels of 28 kDa protein representing HLA-DQ in the conditioned media of H358 and H1703 cells were compared by densitometry. There was a 1.9- and 3-fold increase in HLA-DQ expression in the conditioned medium of invasive cell line H1703 compared to that of indolent cell line in two separate experiments. The HLA-DRB western blot data was unfit to be analyzed due to high background in repeated experiments.

3.2. Clinical-demographic parameters for the patient cohorts

All demographic parameters are provided in Table 1 for the patients comprising the cohort tested in this study.

3.3. Increased presence of circulating MHC class I (ABC) antigens in sera of patients with lung adenocarcinoma

HLA class I antigens were mostly detected as two discrete protein isoforms in the serum (33 and 41 kDa) with some other very low abundance isoforms also observed. Both of the MHC class I ABC monoclonal antibody reactive proteins (i.e. 33 and 41 kDa) were considered for analysis. The density of the 41 kDa bands on the western blot showed higher levels of HLA-ABC in the sera from lung