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Prognostic significance of a complement factor H autoantibody in early stage NSCLC

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Abstract.
BACKGROUND: Biomarkers that predict which patients with early stage NSCLC will develop recurrent disease would be of clinical value. We previously discovered that an autoantibody to a complement regulatory protein, complement factor H (CFH), is associated with early stage, non-recurrent NSCLC, and hypothesized that the anti-CFH antibody inhibits metastasis.
OBJECTIVES: The primary objective of this study was to evaluate the anti-CFH antibody as a prognostic marker for recurrence in stage I NSCLC. A secondary objective was to determine if changes in antibody serum level one year after resection were associated with recurrence.
METHODS: Anti-CFH antibody was measured in the sera of 157 stage I NSCLC patients designated as a prognostic cohort: 61% whose cancers did not recur, and 39% whose cancers recurred following resection. Impact of anti-CFH antibody positivity on time to recurrence was assessed using a competing risk analysis. Anti-CFH antibody levels were measured before resection and one year after resection in an independent temporal cohort of 47 antibody-positive stage I NSCLC patients: 60% whose cancers did not recur and 40% whose cancers recurred following resection. The non-recurrent and recurrent groups were compared with respect to the one-year percent change in antibody level.
RESULTS: In the prognostic cohort, the 60-month cumulative incidence of recurrence was 40% and 22% among antibody negative and positive patients, respectively; this difference was significant (Gray's test, $P = 0.0425$). In the temporal cohort, the antibody persisted in the serum at one year post-tumor resection. The change in antibody levels over the one year period was not statistically different between the non-recurrent and recurrent groups (Wilcoxon two-sample test, $P = 0.4670$).
CONCLUSIONS: The anti-CFH autoantibody may be a useful prognostic marker signifying non-recurrence in early stage NSCLC patients. However, change in the level of this antibody in antibody-positive patients one year after resection had no association with recurrence.

Keywords: NSCLC, prognosis, biomarkers, autoantibodies

1. Introduction

Early stage non-small cell lung cancer (NSCLC) patients are typically treated with surgical resection [1]. However, overall 5-year survival for stage I NSCLC is $\sim$ 60%, suggesting that many patients have undetectable metastatic disease at presentation [2]. Adjuvant therapy is usually not administered due to treatment-related toxicity with only small improvements in outcomes [3,4]. The majority of these patients are followed with surveillance imaging studies [3,4]. Biomarkers that could predict which early stage patients at the time

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of diagnosis are most likely to recur would be clinically useful, as a select group of high-risk patients may benefit from more frequent surveillance or additional therapy [5].

A variety of biomarker-based approaches, whether tumor- or blood-based, have been extensively explored for prognosis in early stage NSCLC (reviewed in [6–9]). Within the tumor microenvironment, the presence of CD4+ and CD8+ T lymphocytes is associated with positive outcomes [8,10–12] and the presence of FoxP3+ T regulatory cells (or a high FoxP3+/CD3+ cell ratio) is associated with negative outcomes [8,13–15]. Additionally, intratumoral tertiary lymphoid structures (TLS) containing germinal centers are a positive prognostic indicator in NSCLC [16–20]. Blood biomarkers include circulating tumor cells [21,22], serum or extracellular vesicle (EV)-associated microRNAs [9,23–25], and serum or EV-associated proteins [7,26–28]. Autoantibodies to tumor-associated antigens have been investigated as biomarkers for lung cancer screening and classification of indeterminate pulmonary nodules [5,29]. Since TLS display features of an active humoral response and are associated with positive prognosis [17], and NSCLC patients express autoantibodies to tumor-associated antigens [17,29], we reasoned that certain autoantibodies might serve as prognostic biomarkers, most likely as part of a panel.

We previously reported autoantibodies against complement factor H (CFH) in the sera of patients with early stage, non-metastatic NSCLC [30]. Patients with stage I NSCLC had a significantly higher incidence of anti-CFH autoantibody than those with late-stage NSCLC ($P = 0.005$). Given that some studies have suggested that host antibodies may have a role in inhibiting tumor progression [31,32], we hypothesized that anti-CFH antibodies may have anti-metastatic activity. Here, using two independent sets of samples, we determine whether anti-CFH autoantibodies in stage I NSCLC patients are a prognostic marker for recurrence, and if changes in antibody levels over time after resection were associated with outcomes.

2. Materials and methods

2.1. Study population and patient samples

The Duke University Institutional Review Board (IRB) approved this study. The current protocol number for this study is Pro00012914, with only minimal revisions from previous versions. The protocol is reviewed and renewed annually by the Duke IRB. Experiments were undertaken with the understanding and written consent of each subject, and the study conforms with The Code of Ethics of the World Medical Association (Declaration of Helsinki), printed in the British Medical Journal (18 July 1964). Study design, reporting, and analysis adhered to the principles summarized in ref [33] with respect to the STROBE [34] and QUIPS [35] guidelines for prognostic studies. In this report, a non-matched case-control study of autoantibody positive vs. negative patients with respect to outcome is followed by a temporal study of outcome of autoantibody positive patients as a function of change in autoantibody levels.

From our prospective database, we selected two independent groups of sequential stage I NSCLC patients: 157 patients for the prognostic cohort and 47 patients for the temporal cohort who qualified on the basis of anti-CFH autoantibody positivity, from an initial group of 203 patients tested. All patients had a new diagnosis of pathologic stage I (T1 N0 M0 or T2 N0 M0) NSCLC as defined by the International System of Staging for Lung Cancer [36]. All patients underwent surgical resection and lymph node dissection at our institution, and all but two were followed for at least 2 years or until recurrence was documented; the exceptions were two non-recurrent patients in the temporal cohort who were followed for 17 and 20 months.

A serum sample was collected from all sequential patients who consented to provide a serum sample at the time of diagnosis (March 1997 to September 2012 for the prognostic cohort and April 1998 to September 2004 for the temporal cohort) before any treatment, and again in the temporal cohort at approximately one year post-resection. Recurrence was defined as either local or distant disease, histopathologically confirmed not to be a second primary. Sera were stored in our IRB-approved -80°C repository.

2.2. Detection of anti-CFH autoantibody by ELISA

Two different ELISAs that give qualitatively similar results were used to assign anti-CFH autoantibody status: in one ELISA the immobilized target was a peptide containing the antibody's epitope; in the other ELISA, the full length protein was used. Independent evaluation demonstrated that samples defined as positive or negative in one ELISA were defined the same way in the other (data not shown).

For the prognostic cohort, serum anti-CFH autoantibody status was assessed using the peptide ELISA.

Each sample was assayed in triplicate in 96-well plates. Wells were coated with 100 μl NeutrAvidin (5 μg/ml; ThermoFisher, Waltham, MA), a biotin capture protein, and blocked the following day with PBST. Wells were then coated with either 100 μl of a synthetic biotinylated 15 amino acid peptide containing the epitope [37] of the antibody (underlined) GPPPPIDNGDITSFP(GGGK-biotin) (GenScript, Piscataway, NJ) or biotin, each at 2 μg/ml in PBST. Plates were incubated at room temperature for 30 minutes on an orbital shaker and then washed four times with PBST. The wells were loaded with 50 μl of patient sera at a 1:500 dilution in PBST and incubated at room temperature for 1 hour on an orbital shaker. Wells were washed as above and 100 μl of goat anti-human IgG gamma-chain-HRP (Chemicon) secondary antibody diluted 1:4000 in PBST was then added to each well. Plates were incubated at room temperature for 1 hour on an orbital shaker and washed as above. The captured anti-CFH antibody was visualized using ABTS/hydrogen peroxide. Absorbance was measured at 405 nm using a 96-well plate spectrophotometer.

We defined a positive autoantibody response as a mean fluorescence intensity of the synthetic peptide-coated wells that was statistically greater ($P < 0.05$) than that of the biotin-coated control wells. A two-tailed, paired student's t-test was used to make this determination.

For the temporal cohort, an indirect ELISA with full-length human CFH (Complement Technology, Inc., Tyler, TX) immobilized in the wells was employed. Prior to immobilization, the CFH autoantibody epitope was exposed by incubating the CFH for 30 minutes in 15 mM Tris (2-carboxyethyl) phosphine (TCEP)/0.1 M Tris-HCl, pH 8. Following reduction, the CFH was diluted to 2 μg/ml with PBS, 100 μl was then added to the wells, followed by overnight incubation at 4°C. Negative control wells contained PBS/TCEP only. The remainder of the ELISA protocol was the same as that for the prognostic cohort.

2.3. Statistical analysis

To assess the effect of antibody status on time to recurrence, a competing risk framework was using since death and recurrence are competing risks [38]. Using SAS PROC TEST and PROC PHREG, cumulative incidence curves were generated for each stratum, and Gray's test was used to compare curves [39]. We used the SeSpPPVNPV function within the R software package timeROC to obtain performance measures of the antibody as a biomarker for time to recurrence [40].

A Wilcoxon two-sample test was used to compare the percent change in CFH at 1 year among patients with and without recurrence within the second temporal cohort.

3. Results

3.1. Study population, antibody and recurrence status

Baseline demographics and clinical characteristics, including gender, race, pathologic stage, and histology, for the 157 early-stage lung cancer patients analyzed in the prognostic cohort are reported in Table 1. Included in this study population were 95 patients with stage I NSCLC who had not developed recurrence at least 2 years after resection, and 62 patients with stage I NSCLC who did develop recurrent disease after resection. All diagnoses were confirmed by a board certified pathologist. Recurrences were diagnosed by CT-PET imaging and/or biopsy. The mean age was 67 years (SD, 8.6 years) and mean smoking history was 46.4 pack years. By ELISA, 42 (27%) of stage I NSCLC patients were antibody-positive and 115 (73%) were antibody-negative. The distribution of the 157 patients according to their antibody status, recurrence, and survival status is shown in Table 2.

For the temporal cohort, sera from 203 stage I NSCLC patients were first screened for anti-CFH antibody in serum collected at time of resection and again approximately one year later. Of the 203 patients, 47 (23%) were antibody positive, including 28 patients with stage I NSCLC who had not developed recurrence at least 2 years after resection (with the exception of two patients not followed beyond 17 and 20 months), and 19 patients with stage I NSCLC who did develop recurrence. Among patients with recurrence, time to recurrence averaged 3 years (range 0.7–8.9 years). Baseline demographics and clinical characteristics for the 47 early-stage lung cancer patients analyzed in the temporal study are reported in Table 3.

3.2. Prognostic cohort: Competing risk analysis

Cumulative incidence curves for the 157 anti-CFH early stage NSCLC patients are shown in Fig. 1. The difference between the two cumulative incidence curves is statistically significant ($P = 0.0425$). In addition, the hazard ratio associated with the cumulative incidence function is 0.52 (95% CI: 0.266, 1.016). The cumulative incidence of recurrence after 24 months was 26% (95%

Table 1
Prognostic study: Patient demographics and clinical characteristics

Characteristic	# of patients	# CFH AutoAb positive	# CFH AutoAb negative
Total number of patients	157	42 (27%)	115 (73%)
Age, mean	67.2 yr (SD, 8.6)	66.0 yr (SD, 8.8)	67.6 yr (SD, 8.5)
Gender			
Female	77 (49%)	21 (50%)	56 (49%)
Male	80 (51%)	21 (50%)	59 (51%)
Race			
Black	19 (12%)	5 (12%)	14 (12%)
White	138 (88%)	37 (88%)	101 (88%)
Histology			
BAC	5 (3%)	0 (0%)	5 (4%)
Adenocarcinoma	82 (52%)	20 (48%)	62 (54%)
Large cell	8 (5%)	6 (14%)	72 (2%)
Neuroendocrine	2 (1%)	1 (2%)	1 (1%)
NSCLC	10 (6%)	2 (5%)	8 (7%)
Squamous cell	50 (32%)	13 (31%)	37 (32%)
Stage			
IA	72 (46%)	20 48%)	52 (45%)
IB	85 (54%)	22 (52%)	63 (55%)
Recurred			
No	95 (61%)	31 (74%)	64 (56%)
Yes	62 (39%)	11 (26%)	51 (44%)

Table 2
Prognostic study: Grouping by anti-CFH antibody, recurrence, and survival status

Anti-CFH antibody status	Progression free survival status*	Frequency	Percent	Cumulative frequency	Cumulative percent
Negative	Censored Event/Alive	59	37.58	59	37.58
Negative	Uncensored Event/Recurred	51	32.48	110	70.06
Negative	Censored Event/Dead	5	3.18	115	73.25
Positive	Censored Event/Alive	28	17.83	143	91.08
Positive	Uncensored Event/Recurred	11	7.01	154	98.09
Positive	Censored Event/Dead	3	1.91	157	100.00

*Patient status at last follow-up.

Table 3
Temporal study: Patient demographics and clinical characteristics

Characteristic	# of patients
Total number of patients	47
Age, mean	67.4 years (SD, 8.2)
Gender	
Female	24 (51%)
Male	23 (49%)
Race	
Black	5 (11%)
White	41 (87%)
American indian/alaska native	1 (2%)
Histology	
BAC	1 (2%)
Adenocarcinoma	25 (53%)
NSCLC	3 (6%)
Squamous	17 (36%)
Adenosquamous	1 (2%)
Recurred	
No	28 (60%)
Yes	19 (40%)

CI: 19%, 35%) among antibody negative patients, and 17% (95% CI: 7%, 29%) among antibody positive patients. Long-term (after approximately 60 months), the cumulative incidence was 40% (95% CI: 30%, 49%) and 22% (95% CI: 11%, 35%) among antibody negative and positive patients respectively. Performance parameters of the anti-CFH antibody as a biomarker are presented in Table 4.

3.3. Temporal cohort: Change in serum anti-CFH antibody level

The change in serum anti-CFH antibody level between diagnosis and one year after diagnosis was computed. The distribution of change within each recurrence group is shown in Fig. 2. The comparison of the two groups using an exact Wilcoxon two-sample test is not statistically significant ($p = 0.4670$).

Table 4
Performance measures of anti-CFH antibody as a biomarker for time to recurrence

Time (mo)	Cases	Survivors	Other events	Censored	Se	Sp	PPV (%)	NPV (%)
24	34	112	3	8	20.37	72.32	16.96	74.81
60	53	73	5	26	16.52	67.12	21.16	56.93

Abbreviations: Se, sensitivity; Sp, specificity; PPV, positive predictive value; NPV, negative predictive value. Predictive accuracy measures were estimated at cutpoint $c = 0.5$ using inverse probability of censoring weighting (IPCW) in timeROC [40], with $n = 157$, with competing risks. Number of positive $(X > c) = 42$; number of negative $(X \leqslant c) = 115$. Method used for estimating IPCW: marginal.

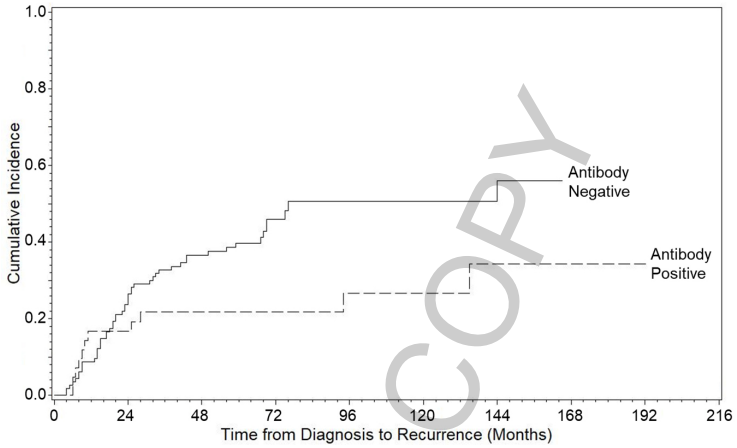

Fig. 1. Cumulative incidence functions according to anti-CFH antibody status.

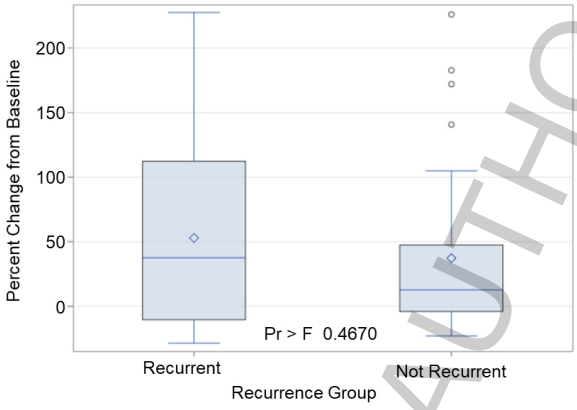

Fig. 2. Box-and-whiskers plot displaying distribution of percent change for the recurrent and non-recurrent groups. An analysis of variance was used to compare percent change from baseline among patients in the recurrent and non-recurrent groups. A percent change greater than 0 is an indication that the follow-up assessment at 1 year is greater than the baseline assessment at time of resection.

4. Discussion

While a variety of strategies have been used to develop prognostic markers for lung cancer, there remains no consistent method to determine which patients with early stage disease will recur following resection. A biomarker or panel of markers that could make this distinction at the time of diagnosis would potentially improve outcomes, as these select patients may benefit from adjuvant therapy.

In studying the host immune response to NSCLC [13,20,30,41], and in light of reports that suggest tumor rejection can be initiated by antitumor antibodies [31,32], we hypothesized that some patients have developed specific autoantibodies against tumor cells that could potentially be used for both diagnostic and therapeutic purposes. We previously reported an antibody against CFH that is associated with early stage, non-metastatic disease [30]. We cloned the antibody from a single human B cell, and have shown it causes complement dependent cytotoxicity of tumor cells, inhibits tumor growth, and modulates the adaptive immune response, lending mechanistic relevance to the association of the anti-CFH antibody with non-recurrence [42].

Here we explored the potential of this anti-CFH antibody as a biomarker to predict time to recurrence in patients with early stage NSCLC. We were able to stratify our group of 157 Stage I patients with either non-recurrent or recurrent disease on the basis of anti-CFH antibody positivity at the time of diagnosis. Possible confounding factors not addressed in this study that might affect recurrence are post-surgical treatment, if

any, concurrent other disease, or immune status. Possible sources of bias include attrition bias, in which patients who did not return to the clinic for follow-up visits were eliminated from the study, and outcome bias, in which variations in measurement intervals among patients may have differentially altered time to recurrence measurements.

While antibody titers over time have been studied in infectious diseases, temporal anti-tumor antibody levels are not well understood. It is noteworthy that in this study the antibody does persist in the blood at least one year after resection of the tumor. However, minor changes in antibody level at one year are not associated with recurrence. As more studies characterize anti-tumor antibodies, their functional consequences will be better understood and their diagnostic potentials will be realized.

Although discrimination by the anti-CFH antibody alone showed statistical significance, it is expected that other autoantibodies with prognostic value could be found that augment it in order to identify which patients will recur and potentially benefit from additional therapy. In NSCLC, as well as other cancer types, intratumoral TLS are associated with a favorable prognosis [19]. TLS, like follicles of secondary lymphoid organs, are organized assemblies of tumor antigen-presenting dendritic cells, activated and proliferating T cells, and antibody-producing plasma B cells. Within the germinal centers of intratumoral TLS, B cells are activated, proliferate, hypermutate and express IgG and IgA antibodies, and many of these antibodies are tumor-antigen specific [17,43]. Intratumoral germinal centers are more prevalent in stage I NSCLC than in later stages [20], and may be a rich source of additional tumor targeting antibodies. This approach of mining the humoral response to tumors is just beginning to be explored and has the potential to transform diagnostic and therapeutic options.

Author contributions

Conception: EFP, JEH, EBG, and MJC
Interpretation or analysis of data: JEH
Preparation of the manuscript: EFP, EBG, and JEH
Performance of experiments: RTB, RG, and MJC
Supervision: EFP and MJC

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