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Cross-validation analysis of the prognostic significance of mucin expression in patients with resected non-small cell lung cancer treated with adjuvant chemotherapy: results from IALT, JBR.10 and ANITA

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Abstract

INTRODUCTION—CALGB 9633 was a randomized trial of observation versus adjuvant chemotherapy for patients with stage IB non-small cell lung cancer (NSCLC). In CALGB 9633, the presence of mucin in the primary tumor was associated with shorter disease-free survival (DFS; hazard ratio (HR)=1.9, p=0.002) and overall survival (OS; HR=1.9, p=0.004).

METHODS—To validate these results, mucin staining was performed on primary tumor specimens from 780 patients treated on IALT, 351 on JBR.10 and 150 on ANITA. The

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CONFLICT OF INTEREST

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histochemical technique using mucicarmine was performed. The prognostic value of mucin for DFS and OS was tested in a Cox model stratified by trial and adjusted for clinical and pathological factors. A pooled analysis of all 4 trials was performed for the predictive value of mucin for benefit from adjuvant chemotherapy.

RESULTS—The cross-validation group had 48% squamous, 37% adenocarcinoma and 15% other NSCLC compared with 29%, 56%, and 15% respectively in CALGB. Among 1262 patients with assessable results, mucin was positive in IALT 24%, JBR.10 30%, ANITA 22% compared with 45% in CALGB. Histology was the only significant covariate ($p<0.0001$) in multivariate analysis with mucin seen more commonly in adenocarcinoma (56%) compared with squamous (5%) and other NSCLC (15%). Mucin was a borderline negative prognostic factor for DFS (HR=1.2 [1.0–1.5], $p=0.06$) but not significantly so for OS (HR=1.1 [0.9–1.4], $p=0.25$). Prognostic value did not vary according to histology: HR=1.3 [1.0–1.6] in adenocarcinoma vs. 1.6 [1.2–2.2] for DFS in other histology (interaction $p=0.69$). Mucin status was not predictive for benefit from adjuvant chemotherapy (test of interaction: DFS $p=0.27$; OS $p=0.49$).

CONCLUSIONS—Mucin was less frequent in the cross-validation group due to its higher percentage of squamous cell carcinomas. The negative impact of mucin was confirmed for DFS but not for OS. Mucin expression was not predictive of overall survival benefit from adjuvant chemotherapy.

Keywords

Non-small cell lung cancer; prognostic factors; mucin; predictive factors; adenocarcinoma

INTRODUCTION

Lung cancer is the leading cause of cancer-related mortality in the United States for both men and women. In 2012, it is estimated that there will be 226,000 new cases and 160,000 deaths from lung cancer [1]. The World Health Organization estimates that there will be 1.2 million new cases annually, making it the most common cancer worldwide [2]. Non-small cell lung cancer (NSCLC) makes up approximately 85% of all newly diagnosed cases of lung cancer.

One of the major advances during the last decade in the treatment of NSCLC has been the reported benefit of cisplatin-based adjuvant chemotherapy for patients with early stage disease [3–6]. Trials have suggested a survival benefit for patients with stage II and IIIA but the benefit for those with stage I has been questioned [7]. Cancer and Leukemia Group B (CALGB) 9633 was a randomized phase III study of adjuvant carboplatin and paclitaxel versus observation for patients with only stage IB NSCLC [8]. The final results demonstrated trends for superior disease-free survival and overall survival for the chemotherapy group, but these did not reach statistical significance. A retrospective subset analysis showed a statistically significant survival advantage from chemotherapy for patients with tumors ≤ 4 cm.

A number of early clinical studies have reported that mucin expression is a negative prognostic marker [9–12]. In a study of 237 patients undergoing surgical resection, The Lung Cancer Study Group found that the presence of mucin in the primary tumor was associated with worse survival [9]. Yu et al. reported that the expression of sialomucin was a negative independent predictor of early recurrence and worse survival in multivariate analysis [10]. Kwiatkowski found that the subtype of adenocarcinoma, solid tumor with mucin, was associated with worse survival [11]. We also had observed mucin to be associated with worse survival in a single institution cohort of 260 patients with stage I and II NSCLC [12]. In a laboratory companion study to CALGB 9633, the presence of mucin in

the primary tumor was a strong negative prognostic factor, where its presence was associated with shorter disease-free survival (Hazard Ratio [HR]=1.9; p-value=0.002) and overall survival (HR=1.9; p-value=0.004) [13].

The Lung Adjuvant Cisplatin Evaluation (LACE) Collaborative group has performed a pooled analysis of the 5 largest trials of adjuvant cisplatin-based chemotherapy in NSCLC [7]. Members from several of these trials formed the LACE biological (LACE-Bio) collaborative group. We report here the results of a cross-validation study of the prognostic significance of mucin expression in patients with resected NSCLC treated with adjuvant chemotherapy. We also report a pooled analysis on the predictive value of mucin for benefit from adjuvant chemotherapy in these patients.

MATERIALS AND METHODS

The cross-validation cohort was comprised of patients with available paraffin-embedded tissue who were treated on the International Adjuvant Lung Cancer Trial (IALT), JBR.10 and Adjuvant Navelbine International Trialists Association (ANITA) studies [3–5]. Four micron sections were cut from the primary tumor, placed on glass slides and sent to the CALGB Pathology Coordinating Office at Ohio State University, Columbus, Ohio, USA for histochemical staining for mucicarmine. All studies were performed without knowledge of treatment arm and clinical outcome. Patient consent and local Institutional Review Board (IRB) approval were obtained for all studies.

The general histochemical technique to detect mucin was used and details are published elsewhere [14]. Mucicarmine, which detects all mucins, was employed. Briefly, slides were deparaffinized and hydrated in distilled water, exposed to Mayer's hematoxylin for 10 minutes, washed in running tap water for 5 minutes and then exposed to Southgate's Mucicarmine solution at room temperature for one hour. Slides were then rinsed quickly in distilled water and exposed to Metanil yellow stain for 30–60 seconds. Slides were then dehydrated quickly in three changes of absolute alcohol until clear and then coverslipped in Permount. The mucin stain was a deep rose color. A positive result was the presence of mucicarmine positive secretory vacuoles within tumor cells or within the lumens of tumor glands and was significantly stronger than background staining. In general mucin positive tumors manifest mucin staining in multiple cells and/or glands. Slides were interpreted by one pathologist (Robin Vollmer).

At least one hematoxylin and eosin stained slide was obtained for each case for central histological review by the LACE-Bio Group and these results have been presented [15]. Using the mucin staining results, final classification of histological subtype was assigned.

A logistic regression model stratified by trial was used to determine the association between mucin and covariates. A Cox model stratified by trial and including covariates was used to examine the prognostic significance of mucin expression on overall survival (OS), the main endpoint and disease-free survival (DFS). The final Cox model was adjusted for treatment, sex, age (<55, 55–64, >64 years), histology (squamous cell carcinoma, adenocarcinoma, other), performance status (PS 1/2, PS0), T-stage (T1, T2, T3/4), and N-stage (N0, N1, N2).

A pooled analysis of all 4 trials (CALGB 9633, IALT, JBR.10 and ANITA) was performed to study the interaction between mucin prognostic value and histology and to test the predictive value of mucin for benefit from chemotherapy. The interaction between treatment (chemotherapy or no chemotherapy) and mucin status on OS and DFS was assessed in the models to determine the predictive value of mucin. A test for heterogeneity was used to compare the hazard ratios (HR) among trials. Statistical significance was set at p 0.05 using two-sided analysis for cross-validation and 0.01 for pooled analysis.

RESULTS

For the 3 LACE-Bio trials used in the cross-validation group, a comparison was made between patients with slides available for analysis and patients without slides. No significant differences were seen for age, sex, stage performances status, histology, surgery, treatment arm (chemotherapy versus observation) (data not shown).

Mucin staining was performed on primary tumor specimens from 780 patients treated on the IALT trial, 351 on JBR.10 and 150 on ANITA; 19 were not assessable, leaving a total of 1262 samples analyzed. The cross-validation group had 48% squamous cell, 37% adenocarcinoma and 15% other NSCLC compared with 29%, 56%, and 15% respectively in CALGB 9633. Mucin staining was positive in IALT 24%, JBR.10 30%, ANITA 22% compared with 45% in the CALGB trial (Table 1). Mucin positivity was noted to be higher in females, patients with lower T-stage, patients with adenocarcinoma and in patients undergoing lobectomy (Table 2). However, histology was the only covariate significantly ($p<0.0001$) related to mucin staining in multivariate analysis.

Prognostic value of covariates

The prognostic impact on OS of the covariates other than mucin was tested in a Cox model stratified by trial (Table 3). Women had superior overall survival compared with men ($p<0.0001$). Inferior OS was seen in patients ≥ 65 years old ($p=0.02$ when three age categories were compared), adenocarcinoma and other NSCLC compared to squamous cell carcinoma ($p<0.0001$), higher T-stage ($p<0.0001$) and higher N-stage ($p<0.0001$). Performance status ($p=0.20$), chemotherapy ($p=0.19$) and type of surgery ($p=0.57$) did not have a statistically significant impact on OS. Based on this analysis, the final Cox model was stratified by trial and adjusted for sex, age, histology, T and N-stage, and performance status. Due to the strong correlation between stage and the T, N of the TNM, stage was not included in the Cox model.

Prognostic value of mucin expression

Disease-free survival—The impact of mucin on DFS is shown in Figure 1A. Patients with mucin positive tumors had inferior DFS but this did not reach statistical significance in multivariable analysis (HR=1.2, 95% CI [1.0–1.5]; $p=0.06$). There was no variation of the prognostic impact according to trial (heterogeneity test: $p=0.46$). Analysis was repeated after exclusion of histology in the model because of its strong correlation with mucin ($r=0.5$). In this case, the HR was equal to 1.4 (95% CI, 1.2–1.7, $p<0.0001$).

Overall Survival—The impact of mucin on OS is shown in Figure 1B. There was a weak trend for worse survival in patients with mucin positive tumors (HR=1.1 [0.9–1.4]; $p=0.25$). There was no variation of the prognostic impact according to trial (heterogeneity test: OS $p=0.23$). Analysis was repeated after exclusion of histology in the model because of its strong correlation with mucin ($r=0.5$). In this case, the HR was equal to 1.3 ([1.1–1.5], $p=0.01$). The prognostic value of mucin did not differ between patients with stage I versus stage II/III (data not shown).

Interaction with histology

Interaction between mucin and histology on the prognostic value was studied. For DFS, the prognostic value did not vary according to histology: HR=1.3 [1.0–1.6]; $p=0.03$ in adenocarcinoma vs. 1.6 [1.2–2.2]; $p=0.002$ in non-adenocarcinoma (other NSCLC and squamous cell carcinoma); interaction $p=0.69$. For OS, the prognostic value did not vary according to histology: HR=1.2 [0.9–1.5]; $p=0.15$ in adenocarcinoma vs. 1.7 [1.3–2.3]; $p=0.0007$ in non-adenocarcinoma (other and squamous cell carcinoma); interaction $p=0.14$.

Predictive value of mucin expression

Because mucin had no predictive effect in the CALGB study, a pooled analysis of all 4 trials (ANITA, IALT, JBR.10 and CALGB 9633) was performed. In a multivariate Cox model, adjusted for the same covariates as in the prognostic analysis, mucin status was not predictive for the benefit of chemotherapy. The test for interaction was $p=0.27$ for DFS and $p=0.49$ for OS (Table 4). Results were unchanged after exclusion of histology in the model. Figures 2A and 2B show the forest plots of the HR of chemotherapy effect for DFS and OS with chemotherapy versus control according to mucin status and trial. Prognostic value of mucin was significant in the control group, but not in the chemotherapy group, both for OS and PFS (table 4).

DISCUSSION

Well established prognostic factors for NSCLC remain limited and include TNM stage, performance status and weight loss [16]. Many research efforts have attempted to define additional clinical as well as biological and molecular markers to further refine prognosis [11, 17–23]. While several of these factors have demonstrated prognostic value in selected series, only tumor size has been sufficiently robust across multiple studies to incorporate into the new staging system [24]. The main purpose of the current study was to cross-validate the prognostic impact of mucin expression. In addition, the LACE-Bio group looked at a number of clinical factors not related (except histology) to mucin and found a negative prognostic impact for male sex, older age, adenocarcinoma, higher T-stage and higher N-stage.

Previous studies have suggested a negative impact on survival for mucin expression in NSCLC [9–13, 25]. Overexpression of mucin *in vitro* has been shown to decrease tumor cell aggregation, promote tumor cell invasion, and block lymphocyte targeting, facilitating metastasis by escape from immune-surveillance [10]. More recent studies suggest that mucin plays a role in cell signaling pathways, thus impacting cell growth, proliferation and apoptosis [26]. Because previous studies suggested that mucin was a negative prognostic factor, we sought to validate these findings in a large independent cohort of patients. With regard to the major goal of the study, the negative impact of mucin was confirmed (borderline) for DFS but not for OS. Furthermore, in the pooled analysis of all 4 trials (IALT, JBR.10, ANITA and CALGB 9633), there was no predictive value for mucin on benefit from adjuvant CT.

Interestingly, when histology was removed from the statistical model, mucin expression was strongly prognostic for both OS (HR=1.3; $p=0.01$) and DFS (HR=1.4; $p<0.0001$). And in the pooled analysis, mucin was strongly prognostic in the non-chemotherapy control group (Table 4). The explanation for the loss of prognostic value when histology is added to the model is the strong correlation between mucin expression and adenocarcinoma ($p<0.0001$). In fact, it has been suggested that mucin expression, like TTF-1, strongly suggests a diagnosis of adenocarcinoma in the setting of small biopsies or solid tumors [27]. In one study of undifferentiated NSCLC in small biopsies, mucin showed sensitivity of only 23% but specificity of 100% [28]. In the Cox model for OS (Table 3), histology was a strong prognostic factor with a HR of 1.4 for adenocarcinoma versus squamous cell carcinoma and a HR of 1.6 for “other histology” versus squamous cell carcinoma (<0.0001). Once histology is accounted for, mucin appears to add little additional prognostic impact.

One limitation of the study was that mucin staining was less frequent in the validation group (26% versus 45% in the CALGB cohort) due to its higher percentage of squamous cell carcinomas. The percentage of patients with squamous cell carcinoma was higher in the cross-validation group, 48% compared to only 29% in the CALGB study. Since only 5% of

squamous cell carcinomas expressed mucin (compared to 56% of adenocarcinomas), this limited the power of the present analysis.

Studies of the bronchioloalveolar carcinoma subtype of adenocarcinoma (BAC), have suggested that mucinous tumors and nonmucinous tumors are distinct entities [29]. Nonmucinous BAC presents more frequently as a ground-glass opacity and frequently harbours *EGFR* mutations. Mucinous BAC presents more frequently as a pneumonic-type infiltrate and more often harbours *K-ras* mutations [30]. In the proposed new classification of lung adenocarcinomas, newer language for BAC includes adenocarcinoma in situ and minimally invasive adenocarcinoma (formerly BAC) [27]. Mucin staining is recommended to aid in histological subtyping. Using the new system, Russell et al, reported poorer survival for the subtype of adenocarcinoma, solid with mucin-predominant and invasive mucinous adenocarcinoma [31]. Finally, Sartori et al. suggested a significant correlation for mucin-producing adenocarcinomas with *K-ras* mutations with a corresponding lack of *EGFR* mutations [32]. As has been noted with interpretation of the Iressa Pan-Asian Study (IPASS), it may be that molecular selection is more important than clinical and pathological factors [33]. In light of recent studies showing differing molecular profiles depending on mucin status, the LACE-Bio consortium plans to study the relationship between mucin, *K-ras* and *EGFR* mutations.

CONCLUSION

Mucin staining was less frequent in the cross-validation group due to its higher percentage of squamous cell carcinomas. The negative impact of mucin was confirmed for DFS but not for OS. Mucin expression was not predictive of overall survival benefit from adjuvant chemotherapy.

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Figure 1A.

Figure 1B.

Figure 1.

Figure 1A. Disease-Free survival curves for mucin positive vs mucin negative (n= 1261 patients, same population as in the multivariate analysis)

Figure 1B. Overall survival curves for mucin positive vs mucin negative (n= 1261 patients, same population as in the multivariate analysis)

Figure 2A.

Figure 2B.

Figure 2.

Figure 2A. Forest plot of hazard ratios on disease-free survival with chemotherapy vs. control according to Mucin status and trial

Figure 2B. Forest plot of hazard ratios on overall survival with chemotherapy vs. control according to mucin status and trial

Table 1

Description of mucin marker from each trial

Trial reference	MUCIN marker		Total
	Negative	Positive	
ANITA	111 78%	32 22%	143
IALT	589 76%	189 24%	778
JBR.10	240 70%	101 30%	341
Total Validation Set	940 74%	322 26%	1262
CALGB Training Set	115 55%	94 45%	209

Table 2

Correlation between mucin status and covariates

	p-value *	Mucin negative		Mucin positive	
		Nb of patients N=940	%	Nb of patients N=322	%
Sex					
Male	0.0001	762	77	225	23
Female		178	65	97	35
Age (in years)					
< 55	0.22 (0.12)	261	71	105	29
55–64		410	76	132	24
> 64		269	76	85	24
Stage					
I	0.17 (0.13)	352	71	142	29
II		368	76	115	24
III		220	77	65	23
N of TNM					
0	0.84 (0.74)	441	74	157	26
I		322	75	109	25
2		177	76	56	24
T of TNM					
I	0.02 (0.006)	115	70	50	30
2		644	73	234	27
3 / 4		181	83	38	17
Histology					
Squamous cell carcinoma	<0.0001	579	95	33	5
Adenocarcinoma		202	44	260	56
Other		159	85	29	15
Type of surgery					
Lobectomy/Other	<0.0001	577	71	241	29

	p-value *	Mucin negative Nb of patients N=940	%	Mucin positive Nb of patients N=322	%
<i>Pneumnectomy</i>		363	82	81	18
WHO PS ***					
0	0.51	486	74	172	26
1 / 2		454	75	148	25

*
test for trend in parenthesis

**
data missing for one patient in the mucin positive group

Table 3

Multivariate prognostic values of variables other than mucin on overall survival (n= 1261 patients, one patient excluded because of missing covariate).

Characteristic	HR [95% CI]	p-value
Sex		
women vs men	0.6 [0.5;0.8]	<0.0001
Age		
55–64 vs <55	1.2 [1.0;1.4]	0.02
65 vs <55	1.4 [1.1;1.7]	
Histology		
adenocarcinoma vs squamous	1.4 [1.1;1.7]	<0.0001
Other vs squamous	1.7 [1.3;2.1]	
T-stage		
T2 vs T1	1.3 [1.0;1.8]	<0.0001
T3/4 vs T1	2.1 [1.6;2.9]	
N-stage		
N1 vs N0	1.5 [1.3;1.9]	<0.0001
N2 vs N0	2.7 [2.2;3.3]	
Performance status		
1,2 vs 0	1.1 [0.9;1.3]	0.20
Chemotherapy		
Yes vs No	0.9 [0.8;1.1]	0.19

Table 4

Pooled analysis of the predictive value of mucin for benefit from chemotherapy (n=1453 patients; 11 patients excluded because of missing covariate).

DISEASE-FREE SURVIVAL			
	Chemotherapy group (No events / No patients)	Control group (No events / No patients)	Hazard ratio for event CT vs. no CT [95% CI]
Mucin negative n=1048	281 / 533	286 / 515	0.9 [0.8 ; 1.1] p=0.17
Mucin positive n=412	117 / 205	136 / 207	0.8 [0.6 ; 1.0] p=0.03
Hazard ratio for event positive vs. negative [95% CI]	1.2 [1.0 ; 1.6] p=0.10	1.4 [1.1 ; 1.8] p=0.002	Test for interaction Mucin*treatment p=0.27

OVERALL SURVIVAL			
	Chemotherapy group (No deaths / No patients)	Control group (No deaths / No patients)	Hazard ratio for death CT vs. no CT [95% CI]
Mucin negative n=1048	252 / 533	256 / 515	0.9 [0.8 ; 1.0] p = 0.33
Mucin positive n=412	103 / 205	113 / 207	0.8 [0.6 ; 1.1] p = 0.14
Hazard ratio for death positive vs. negative [95% CI]	1.2 [0.9 ; 1.6] p=0.14	1.4 [1.1 ; 1.8] p=0.02	Test for interaction mucin*treatment p=0.49