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LACE-Bio: Validation of Predictive and/or Prognostic Immunohistochemistry/ Histochemistry-based Biomarkers in Resected Non–small-cell Lung Cancer

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Abstract

There are no validated molecular tools to allow patient selection for adjuvant chemotherapy after complete resection of non–small-cell lung cancer. Immunohistochemistry biomarkers shown in one trial to have a prognostic/predictive effect on overall survival were tested. The majority of the promising biomarkers could not be validated, and none were predictive of benefit. Immunohistochemistry assays from single trials may be misleading.

Background: Complete resection of non–small-cell lung cancer (NSCLC) offers the potential for cure after surgery and adjuvant chemotherapy. Patients may not benefit and may experience severe toxicity. There are no validated molecular tools to allow better patient selection. **Materials and Methods:** The LACE-Bio (LACE [Lung Adjuvant Cisplatin Evaluation]) project includes 4 trials (International Adjuvant Lung Cancer Trial [IALT], Adjuvant Navelbine International Trialist Association [ANITA], JBR10, and Cancer and Leukemia Group B (CALGB)-9633). Immunohistochemistry biomarkers shown in one trial to have a prognostic/predictive effect on overall survival were tested. **Results:** The majority of the promising biomarkers could not be validated; the prognostic effect of tumor infiltrating lymphocytes and β-tubulin was confirmed. Potential causes include tissue fixation, storage, the use of tissue microarrays, and varying reagent/antibody batches. **Conclusions:** Immunohistochemistry assays from single trials may be misleading and require validation before being used for patient selection. LACE-Bio-2 is evaluating potential genomic biomarkers that may allow more precise selection of patients with NSCLC for adjuvant chemotherapy in NSCLC.

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Table 1 Individual Trials in the LACE-Bio Project Where Immunohistochemical Biomarkers Were Tested for Their Prognostic and Predictive Value on Overall Survival

Trial	Biomarker	Predictive Effect[a]	Prognostic Effect[a]	Reference
IALT	ERCC1	Yes (neg)[b]	Yes (pos)[c]	40
	TIL	No	Yes (pos)	41
	FAS	No	No	42
	FASL[d]	Borderline ($P = .06$) (neg)	No	
	FAS/FASL[e]	No	Yes (pos)	
	Survivin	No	No	
	BAX[f]	Borderline ($P = .08$) (pos)	No	43
	Cyclin E/p16^{INK4}	No	No	44
	p27^{Kip1}	Yes (neg)	No	
	Cyclin D1, D3	No	No	
	Ki-67	No	No	
	p-akt	No	No	45
	p53[g]	No	No	46
	BRCA1	No	No	Unpublished
JBR10	β-tubulin	No	Yes (neg)	47
	Cyclin E	No	No	Unpublished
	p16^{INK4}	No	No	Unpublished
	p27^{Kip1}	No	No	Unpublished
	p53	Yes (pos)	Yes (neg)	48
	PTEN	No	Borderline (neg)	Unpublished
CALGB	Mucin	No	Yes (neg)	49
	p53	No	Yes (neg)	
	Bcl-2	No	No	
	Blood group antigen A	No	No	

Abbreviations: CALGB = Cancer and Leukemia Group B; IALT = International Adjuvant Lung Cancer Trial; LACE-Bio = Lung Adjuvant Cisplatin Evaluation biomarkers; Neg = negative – absence/low expression indicated better outcomes; pos = positive – presence/high expression indicated better outcomes; TIL = tumor infiltrating lymphocytes.
[a]Considered significant when P value $< .05$ for testing of individual markers. Original trial may have used a different significance level.
[b]Neg (pos) means a treatment effect in patients with negative (positive) marker.
[c]Pos (neg) means a better prognosis for patients with a positive (negative) marker.
[d]Updated OS analyses of IALT demonstrated a predictive effect of FASL with an interaction P-value = .04. We therefore performed cross-validation (see footnote n of Table 2).
[e]FAS/FASL - prognostic effect using a categorization of $< 1, \geq 1$ (= positive marker) with hazard ratio [HR], 0.75; 95% confidence interval [CI], 0.60-0.94; $P = .01$). In LACE-Bio when FAS/FASL was categorized in 3 classes and the alpha level set at 0.01 (corrected for multiple testing), there is a borderline prognostic effect ($P = .02$) and a predictive effect ($P = .05$).
[f]Not significant when originally reported using a cutoff of 20 (see footnote o in Table 2 and Supplemental Table 4 in the online version, for cutoff used for the LACE-Bio analyses).
[g]p53 analyses were performed by each group using different cutoffs prior to Lace-Bio (footnote p Table 2). For IALT, $P = .10$ for predictive effect when using 2 categories (0-20; > 20), but .08 when 3 categories were used (0-20, 21-100, 101-300).

Introduction

Lung cancer remains the leading cause of cancer-related deaths worldwide, with an overall all-stage 5-year survival of approximately 17%.[1] Poor survival rates, even following surgical resection, led several groups to investigate the benefit of adjuvant chemotherapy (ACT). Five large randomized trials[2-6] testing platinum-based ACT were included in a meta-analysis (Lung Adjuvant Cisplatin Evaluation [LACE]) (see Supplemental Table 1 in the online version) confirming the benefit of ACT.[7] LACE-Bio included trials with biobanks; The Cancer and Leukemia Group B (CALGB)-9633 trial[8] demonstrated a comparable overall survival (OS) hazard ratio (HR) (HR, 0.83; 90% confidence interval [CI], 0.64-1.08) and was also included in LACE-Bio.

The use of ACT in the postoperative setting was reasonably well-tolerated with, in general, short-lived morbidity, although there were some increases in mortality related to ACT. However, as not all patients appeared to benefit, each trial investigated biomarkers to aid selection.[9,10] JBR10 and Cancer and Leukemia Group B (CALGB)-9633 prospectively collected tissue and tumor blocks, whereas International Adjuvant Lung Cancer Trial (IALT) and Adjuvant Navelbine International Trialist Association (ANITA) did so retrospectively; Big Lung Trial (BLT) and the Italian/European Experience with Adjuvant Chemotherapy in Resectable Non–Small-Cell Lung Cancer (ALPI) investigators did not create a companion biobank. LACE-Bio, based on the LACE meta-analysis project, but including only those trials with biobanks (IALT, ANITA, JBR.10, CALGB 9633), was formed in 2008 to further investigate the utility of tissue- and protein-based biomarkers that appeared to be promising in 1 or more of the individual LACE trials. Immunohistochemistry (IHC)-based biomarkers tested in each trial are shown in Table 1.

We report here on the results of our initiative to validate potential prognostic and/or predictive biomarkers.

Materials and Methods

LACE-Bio

LACE-Bio is a collaboration between Institut Gustave Roussy (statistical lead, IALT), Canadian Cancer Trials Group (JBR10), CALGB (CALGB-9633), and the ANITA Trial Group, governed

Table 2 LACE-Bio: Summary of Analyses of Biomarkers Tested for Their Prognostic and Predictive Value on Overall Survival

Biomarker	Discovery Trial[h]	Results		Main Analysis[i] (Exploratory Analysis)	Results	
		Prognostic Effect $HR_{+/-}$ (95% CI)	Predictive Effect $HR_{CT/control}$ (95% CI) in Negative $HR_{CT/control}$ (95% CI) in Positive With *P* Value of Interaction		Prognostic Effect $HR_{+/-}$ (95% CI)	Predictive Effect $HR_{CT/control}$ (95% CI) in Negative $HR_{CT/control}$ (95% CI) in Positive With *P* Value of Interaction
ERCC1	IALT (N = 761)	0.66 (0.49-0.90)[a]	0.65 (0.50-0.86) 1.14 (0.84-1.55) *P* = .009	$CV^{pro\ +\ pred}$: C + J (N = 494)[b,c]	1.23 (0.88-1.72)	1.16 (0.64-2.10) 0.78 (0.58-1.05) *P* = .23
TIL[j]	IALT (N = 783)	0.56 (0.39-0.81)	1.00 (0.83-1.21) 1.18 (0.64-2.17) *P* = .61	CV^{pro}: A + C + J (N = 753)[c,d] (I + A + C + J: pool for predictive)	0.45 (0.23-0.85)	0.88 (0.76-1.02) 0.90 (0.49-1.64) *P* = .96
p27[k]	IALT (N = 778)	0.82 (0.61-1.10)[a]	0.66 (0.50-0.88) 1.09 (0.82-1.45) *P* = .02	CV^{pred}: A + C + J (N = 660) (A + C + J for prognostic)	0.97 (0.77-1.22)	0.87 (0.64-1.18) 0.83 (0.59-1.15) *P* = .83
	JBR10[g] (N = 311)	1.53 (0.99-2.37)[a] 1.05 (0.47-2.34)[a]	1.03 (0.68-1.55) 0.58 (0.35-0.94) 1.78 (0.67-4.76) *P* = .08			
Cyclin E[l]	IALT (N = 778)	1.01 (0.81-1.25)	0.77 (0.57-1.02) 0.94 (0.71-1.25) *P* = .33	$Pool^{pro\ +\ pred}$: I + J (N = 1081)	0.98 (0.82-1.17)	0.82 (0.65-1.03) 1.02 (0.79-1.31) *P* = .20
	JBR10[g] (N = 305)	1.22 (0.76-1.96)[a]	1.01 (0.62-1.65) 0.67 (0.41-1.09) *P* = .24			
p16[m]	IALT (N = 778)	1.09 (0.87-1.37)	0.87 (0.67-1.12) 0.82 (0.59-1.13) *P* = .78	$Pool^{pro\ +\ pred}$: I + J (N = 1081)	0.96 (0.79-1.16)	0.90 (0.73-1.13) 0.91 (0.70-1.19) *P* = .95
	JBR10[g] (N = 307)	0.98 (0.60-1.61)[a]	0.79 (0.55-1.12) 1.28 (0.73-2.23) *P* = .15			
FAS[n]	IALT (N = 772)	0.82 (0.65-1.05)	0.89 (0.70-1.13) 0.75 (0.50-1.12) *P* = .46			
FASL[n]	IALT (N = 773)	0.97 (0.77-1.22)	0.69 (0.52-0.93) 1.03 (0.78-1.37) *P* = .06	$CV^{pro\ +\ pred}$: C + J (N = 520)	1.22 (0.80-1.84)[a]	0.99 (0.73-1.34) 0.53 (0.31-0.91) *P* = .05
FAS/FASL[n]	IALT (N = 768)	0.72 (0.57-0.91) 0.89 (0.66-1.21)	0.80 (0.61-1.06) 1.13 (0.79-1.63) 0.51 (0.29-0.87) *P* = .05	$CV^{pro\ +\ pred}$: C + J (N = 507)	0.83 (0.61-1.14) 1.00 (0.72-1.40)	0.99 (0.66-1.49) 0.67 (0.42-1.07) 0.74 (0.44-1.24) *P* = .43
BAX[o]	IALT (N = 778)	1.32 (1.00-1.75)[a]	1.22 (0.94-1.58) 0.65 (0.48-0.87) *P* = .002	$CV^{pro\ +\ pred}$: C + J (N = 494)[c]	1.02 (0.75-1.37)	1.01 (0.61-1.68) 0.79 (0.58-1.08) *P* = .41

Table 2 Continued

Biomarker	Discovery Trial[h]	Results		Main Analysis[i] (Exploratory Analysis)	Results	
		Prognostic Effect $HR_{+/-}$ (95% CI)	Predictive Effect $HR_{CT/control}$ (95% CI) in Negative $HR_{CT/control}$ (95% CI) in Positive With *P* Value of Interaction		Prognostic Effect $HR_{+/-}$ (95% CI)	Predictive Effect $HR_{CT/control}$ (95% CI) in Negative $HR_{CT/control}$ (95% CI) in Positive With *P* Value of Interaction
p53[p]	IALT (N = 776)[e]	1.02 (0.82-1.26)	0.70 (0.52-0.94) 0.99 (0.75-1.30) *P* = .10	$Pool^{pro\ +\ pred}$: A + C + I + J (N = 1401)	1.00 (0.86-1.17)	0.85 (0.70-1.03) 0.94 (0.75-1.17) *P* = .53
	JBR10 (N = 253)	1.89 (1.07-3.34)[a]	1.40 (0.78-2.52) 0.54 (0.32-0.92) *P* = .05			
	CALGB (N = 250)	1.71 (1.09-2.67)	Hazard ratios not reported *P* > .05			
β-tubulin	JBR10 (N = 265)	1.39 (0.96-2.01) 1.72 (1.02-2.88)[a]	1.00 (0.57-1.75) 0.64 (0.39-1.04) *P* = .25	CV^{pro}: A + C + I + J^2 (N = 1141)[f] (A + C + I + $J^{1\ +\ 2}$: pool for predictive)	1.27 (1.07-1.51)	1.03 (0.83-1.02) 0.78 (0.64-0.96) *P* = .07
BRCA1[q]	IALT (N = 566)	1.08 (0.87-1.34)	0.92 (0.67-1.28) 1.10 (0.83-1.45) *P* = .49	$Pool^{pro\ +\ pred}$: I + J (N = 851)[d]	1.08 (0.89-1.32)	0.89 (0.66-1.19) 1.08 (0.83-1.39) *P* = .34
Mucin	CALGB (N = 192)	1.88 (1.22-2.89)		CV^{pro}: A + I + J (N = 1261)[d] (C + A + I + J: pool for predictive)	1.14 (0.92-1.41)	0.92 (0.77-1.09) 0.82 (0.63-1.07) *P* = .49

Abbreviations: A = ANITA; ANITA = Adjuvant Navelbine International Trialist Association; C = CALGB; CALGB = Cancer and Leukemia Group B; CI = confidence interval; CV = cross-validation; DFS = disease-free survival; HR = hazard ratio; I = IALT; IALT = International Adjuvant Lung Cancer Trial; J = JBR10; OS = overall survival; P = pooled; pred = predictive; pro = prognostic; TIL = tumor-infiltrating lymphocytes.

[a]Prognostic analyses performed in control arm only.

[b]See Friboulet et al[13] for a full description.

[c]The same adjustment was used both for the discovery and cross-validation analyses.

[d]To take into account mucin results, a new revised histology of all non-adenocarcinoma mucin was performed. This new revised histology was used for the analysis of the following markers: TIL, BRCA1.

[e]Prognostic and predictive analyses with different cutoffs have been used and showed similar results on IALT. For p53 with score > 100, HR, 0.98; 95% CI, 0.79-1.21; *P* = .82 for OS prognostic analysis and HR, 0.72; 95% CI, 0.55-0.94 (score ≤ 100) and HR, 1.05; 95% CI, 0.77-1.43 (score > 100) for predictive analysis (*P* = .08). A trend test (*P* = .08) for the predictive analysis of p53 was observed with the following categories: 0-20, 21-100, and > 100. For BRCA1, quartiles and trend test for pooled analysis showed similar results.

[f]A first sample from JBR.10 (J^1) was used as discovery set and a sub-sample of JBR10 trial (J^2; n = 52) was used for the cross-validation of the prognostic effect.

[g]For these 3 markers in JBR10 trial, (p27 [< 60, 60-200, ≥ 200], Cyclin E [cutoff = 20], and p16 [cutoff = 80]), a standard score was used without adjustment on baseline factors correlated to the marker of interest.

[h]The baseline factors included in the multivariable Cox model may differ between trials. For example, for JBR10 trial, RAS gene mutation, smoking history, baseline anemia, lactate dehydrogenase, comorbidity, alkaline phosphate value, and tumor size were used.

[i]We pre-specified the analysis type, prognostic, predictive, or both.

[j]TIL: intense versus not intense. The endpoints were OS, DFS, and specific disease-free survival.

[k]Filipits et al[10] used a standard score and cutoff 50. For JBR10, the cutoff of the standard score was < 60, 60-200, and > 200.We also used cutoff as a continuous variable; results were comparable for discovery and validation analysis.

[l]For IALT standard score, cutoff = 20, similar results were observed when cyclin E was analyzed as a continuous variable for the prognostic analysis. For JBR10, the cutoff = 20.

[m]For IALT standard score, cutoff = 1, similar results were observed when p16 is analyzed as a continuous variable for the prognostic analysis. For JBR10, the cutoff = 80.

[n]Cross-validation for predictive effect of FASL was performed based on an updated OS analysis of IALT (HR, 0.77; 95% CI, 0.59-1.00 and HR, 1.15; 95% CI, 0.88-1.49 in < 240, ≥ 240; interaction *P* value of .04). The alpha level was set at 0.01 to correct for multiple testing. For FAS, FASL, and their ratio, several cutoffs have been explored using quartiles or considering biomarkers as a continuous biomarker; we used the following cutoffs: 240 for FAS and FASL and < 0.8, 0.8-1.0, > 1 for the ratio. Both analyses controlled for lymphoid infiltration. Cross-validation was also performed using H-score for the ratio with similar results.

[o]The results for IALT were obtained with the same adjustment (including lymphoid infiltration) as the cross-validation. Discovery used cutoff of 90 because of unbalanced distribution in the cross-validation data set. Using this, a prognostic effect was identified, and therefore, cross-validation was performed rather than a pooled analysis. In exploratory analyses, a borderline (*P* = .06) for the predictive effect of BAX was observed when the following categories were used: (0-20, 21-100, and > 100).

[p]Each trial defined p53 score and cutoff. In ANITA and IALT trials, p53 score = intensity of staining × % of stained cells (cutoff = 100). In JBR10 trial, positive = % of tumor cells with nuclear staining of ≥ 15% for tumors showing intensity scores of 1 or more. In CALGB, p53 positive = staining intensity ≥ 2 (in any % of cells). For the pooled analysis, p53 was initially defined by a standard score (% stained cells × intensity) (cutoff = 100) and H-score (% stained cells in category × intensity, cutoff = 4). When we used the standard score as a quantitative variable for the pool analysis, the prognostic effect was HR, 0.99; 95% CI, 0.98-1.01 for OS. The p53 × treatment interaction term was *P* = .51 for OS. The analysis on discovery set included lymphoid infiltration as covariate.

[q]OS, DFS, and specific disease-free survival were the endpoints for the pooled analysis. The multivariable analysis contains lymphoid infiltration as covariate.

by a Steering Committee (LBSC). Each group maintains its own trial database and associated biobank. For IALT, blocks were collected retrospectively from each site at which 10 or more patients were enrolled, and central pathology review was conducted with collection of the following parameters: percentage of tumor cells on the sections; percentage of necrosis in the tumor bulk; pleural, lymphatic, and vessel invasion; and intensity of lymphocytic infiltration. For JBR10, a tissue and tumor bank for the trial is part of the Canadian Cancer Trials Biobank first created in 1997 (https://www.ctg.queensu.ca/public/correlative-science-tumour-bank). For CALGB 9633, specimens were collected prospectively and stored in the CALGB lung cancer biobank (https://www.calgb.org/DMZ/lctb_calgb.pdf). For ANITA, a retrospective collection was organized in a subgroup of centers.

Each trial provided an anonymized dataset to the meta-analysis unit at Institut Gustave Roussy, who performed all statistical analyses based on a prospectively defined statistical analysis plan. At the time LACE-Bio was formed, a number of IHC biomarkers had already been tested for prognostic and/or predictive effects in 1 or more of the individual trials (Table 1), with the choice of the biomarker based on promising data in smaller clinical trials or from the literature. Promising biomarkers were assayed in the other trials, in general by the original laboratory. For LACE-Bio, results of all assays — even if performed by another LACE-Bio laboratory — were returned to the original trial for incorporation into the trial database, and in turn, the LACE-Bio database was updated. The LBSC identified which biomarker reports were to be published separately or grouped in the overview manuscript.

Assays and Central Pathology Review

Two pathologists (EB, MST) oversaw the conduct of all assays and the pathology review.[11] Scanned sections (Aperio Scan-Scope XT whole-slide scanning system) were reviewed independently by EB and MST, with discordant cases resolved by consensus. The original and reviewed histopathologic subtypes were recorded (for adenocarcinoma based on the International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society/World Health Organization classification of lung adenocarcinoma[12]). The majority of IHC assays were conducted in the research laboratories of EB and MST or where the original assays were performed, using pre-optimized and consistent staining protocols (see Supplemental Table 2 in the online version). A composite staining score was obtained by multiplying the percentage of positively stained cells by the intensity of staining (standard score). For some assays, a second score (H-score) of a weighted categorization of the percentage multiplied by the intensity was used.

Statistical Methods

Detailed statistical methodology is summarized in Supplemental Appendix 1 (in the online version). The primary endpoint was OS. IHC-based biomarkers were considered as binary markers (negative, $<$ cutoff; positive, $\geq$ cutoff). The cutoff was defined by the median of the distribution if not predefined (see Supplemental Table 3 in the online version).

Cross-validation was performed when the primary trial had demonstrated a prognostic and/or predictive effect; otherwise, a pooled analysis was performed combining all 4 trials. Demography and follow-up were compared for patients with and without biomarker data.

The main analysis consisted of fitting a multivariable Cox proportional hazard regression model stratified by trials. For the prognostic analyses, an 'all comers' strategy was used unless the biomarker was found to be predictive, in which case only the control arm was included. Between-trial heterogeneity also was tested. For the predictive analyses, we tested for an interaction between treatment and the biomarker. The level of statistical significance was set at 0.05 and 0.01 for cross validation and pooled analyses, respectively, and statistical analyses were conducted using SAS software.

Results

Some tumor blocks were exhausted when the original assays were performed, and thus not all patients had results. For JBR10, additional samples were obtained for β-tubulin assays and were included in the validation set.

Analyses approved by the LBSC include ERCC1,[13] tumor-infiltrating lymphocytes (TILs),[14] p53[15] (protein expression and mutations), β-tubulin,[16] and mucin,[17] p27,[18] cyclin E/p16,[18] and BAX.[19] A summary of all cross-validation or pooled analyses for OS is presented in Table 2. Results for disease-free survival (DFS) are presented in Supplemental Table 4 (in the online version).

We were unable to validate a predictive or prognostic effect for ERCC1; the analytical issues have been described in detail. We were able to confirm a prognostic effect of TILs (defined as intense staining) for both longer OS and DFS. The poorer prognosis of patients with high β-tubulin expression was confirmed, but no significant predictive effect was seen, although a marginal effect was noted in the pooled analysis (high β-tubulin HR, 0.78; 95% CI, 0.64-0.96 and HR, 0.73; 95% CI, 0.60-0.89 for OS and DFS, respectively). A marginal predictive effect for high FASL was noted; however, the effect seen in IALT was for low FASL expression to predict for better outcomes with ACT. The clinical relevance of this finding is not clear. Validation studies for other biomarkers failed to confirm any prognostic or predictive effect on OS.

Discussion

Predictive biomarkers based on IHC have proven particularly difficult to validate in the context of clinical trials,[20] although the same is true even for some genomic markers.[21] Indeed, the rationale for LACE-Bio was to validate promising results from LACE trials, especially IALT,[9] for which an extensive biomarker examination was conducted. The biomarkers tested in the original trials were chosen because they had already been shown to have a predictive or prognostic effect, or were hypothesized to be potentially relevant to ACT, including apoptosis, DNA repair, or checkpoint or immune pathways. The trials included in LACE-Bio represented unique opportunity to define and validate selective biomarkers. These trials are all randomized phase III studies with control arms of the standard of care in the early-mid 1990s — watchful waiting. They will never be repeated. LACE-Bio met all of the recommendations proposed in the literature for the investigation of biomarkers utilizing archival tissue (Table 3).[22]

Despite the use of appropriate and prospectively planned methodology, we were unable to validate or confirm virtually all of the

Table 3 Recommendations for the Use of Archival Tissue in Biomarker Development in the Context of Clinical Trials[22]

Archived tissue available from an adequate number of patients (at least two-thirds is recommended) from the pivotal trials.	☑
Analytical validity - results obtained from the archived specimens will closely resemble those that would have been obtained from analysis of specimens collected in real time.	☑
Assays should be conducted blinded to the clinical data.	☑
The analysis plan for the biomarker evaluation must be completely developed before the performance of the biomarker assays.	☑
The analysis should be focused on a single, completely defined, diagnostic classifier. In general, the analysis should not be exploratory, and practices that might lead to a false-positive conclusion should be avoided.	☑
The results must be validated in at least 1 or more similarly designed studies using the same assay techniques.	☑

findings from individual trials. Specifically, none of the putative biomarkers was found to be predictive, and only 2 were confirmed to have a prognostic effect (TIL and β-tubulin). Other biomarkers tested, including PDL-1 and EGFR, have been published separately[23-27] and, in general, failed to identify any robust predictive or prognostic biomarker, although we did observe a potential negative predictive effect of TP53/KRAS comutation,[23] a potential interaction with codon-13 KRAS mutation[27] and a correlation between tumor size[26] and chemotherapy effect, which are worthy of further exploration.

Although the assays and analyses that contributed to this work were carefully and prospectively planned, there were some inherent limitations. Only 2 of the trials prospectively created a biobank, and so fixation methods may have differed between specimens and participating sites; some specimens were processed with Bouin solution, thought to result in better nuclear definition but described to result in issues with later IHC.[28] All 4 trials were initiated 20 years ago, and antigenicity is known to degrade over time with cut slides and even formalin-fixed paraffin-embedded blocks.[29] To mitigate this, in most instances, slides were cut from blocks less than 6 weeks before an assay. We were not always able to use the same batch or lot of the antibody used for the initial studies, given the time differences between when the original assays were done and when the cross-validation assays were performed. The impact of these factors has been examined exhaustively for ERCC1 and effectively demonstrates issues with IHC and validation.[13] These results led to the cancellation of at least 1 phase III study (Tailored Postsurgical Therapy in Early-Stage NSCLC [TASTE]). In addition, heterogeneity between trials and in their respective patient populations cannot be ruled out.[11]

Issues with biomarker standardization, validation, reproducibility, and analysis are well described.[30] Tissue collection and assays variability may lead to non-reproducible results, including acquisition (time before processing), fixation (fixatives, timing), antigen retrieval,[31] storage of blocks, slide preparation, storage and time to assays, varying reagents and differing batch lots of antibodies (including concentration[32]), scoring (especially manual), and cutoffs and the definition of what constitutes a positive test. These issues remain even with the use of tissue microarrays.[33] Only recently have guidelines for analytical validation been published,[34] which attempt to reduce inter-laboratory variation in analytic validation. Further, IHC typically is more useful qualitatively with binary assessment of a biomarker rather than quantification, or evaluation as a continuous variable, especially when manual scoring is used.[35] Finally, in the original IALT-Bio analyses, the significance was set at 0.01. If it had been set at $P = .005$, to better account for multiple testing, none of the predictive results would have been significant. Therefore, from a statistical point of view, the absence of validation is not surprising.

Our results are also consistent with other published data, including genomic-based, such as the Spanish Customized Adjuvant Treatment trial (SCAT), and other LACE-Bio analyses, where putative predictive biomarkers could not be validated.[27,36]

The development of new companion biomarkers[37] in a research laboratory for use in a clinical trial adds further challenges to validation for all assays. Indeed, it appears to be more challenging to develop a companion diagnostic than a new cancer therapeutic. A number of new recommendations and guidelines have been developed, none of which was in place when our trials were conducted. Similar initiatives have attempted to develop quantitative IHC methodology using digitization. These recommendations should be incorporated prospectively into modern oncology trials.

We have now implemented LACE-Bio2, investigating the utility of genomic-based biomarkers, funded in part by a National Institutes of Health RO-1 grant (http://grantome.com/grant/NIH/R01-CA165958-02). Initial results suggest chromosomal instability and high nonsynonymous TMB (> 8 mutations/Mb) may have a prognostic effect, and we have identified new candidate prognostic markers.[38,39]

Conclusions

LACE-Bio attempted to validate putative biomarkers identified in the pivotal ACT trials but were unable to validate any that could be used for the selection of patients for ACT. We strongly recommend that investigators prospectively follow the published guidelines and recommendations for biomarkers in clinical trials, and only consider testing of new IHC-based biomarkers when there is a strong rationale and robust supporting data including a well-validated and reproducible assay.

Clinical Practice Points

- Not all patients with completely resected NSCLC benefit from ACT. Identifying patients who do not need chemotherapy by developing predictive biomarkers will prevent unnecessary toxicity and reduce health care costs.
- IHC-based tests were hoped to be predictive for outcomes based on previous publications. We pooled results from 4 large adjuvant trials with biobanks, but were unable to identify any IHC-based biomarker that was predictive for outcomes, even if they had appeared promising in the original publication.
- Although predictive biomarkers are critically important to develop to help guide the selection of patients for ACT after complete resection of NSCLC, IHC-based biomarkers, especially

from single trials, are misleading. Additional research, including the incorporation of genomic-based biomarkers, is required to further elucidate this important question.

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Disclosure

J.-C.S. is a full-time employee of Medimmune (from 2017) and has received consultancy fees from Astrazeneca, Abbvie, Bristol Myers Squibb, GSK, Genentech-Roche, Merus, Merck, Sanofi, Servier, Pierre Fabre, and Pharmamar. The remaining authors have stated that they have no conflicts of interest.

Supplemental Data

Supplemental tables accompanying this article can be found in the online version at https://doi.org/10.1016/j.cllc.2018.10.001.

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Supplemental Appendix 1

Statistical Methods

All statistical analyses were approved by the LACE-Bio Steering Committee. Standard methods were used, and a prospective decision was made whether to use the original (assigned at randomization) or central review pathologic subtype.

The primary endpoint was overall survival, defined as the time from randomization to the date of death whatever the cause. Secondary endpoints included disease-free survival (time from randomization to the time of the first event [recurrence, death]) and for some markers, specific disease-free survival (time from randomization to cancer-related event). Patients with no events were censored at the date of their last follow-up.

Immunohistochemistry-based biomarkers were considered as binary markers (negative: $<$ cutoff, positive: $\geq$ cutoff). If a predefined cutoff was not available, or there was a low prevalence, the cutoff was defined by the median of the distribution; quartiles were also used for some analyses.

Our general strategy when evaluating the prognostic and predictive value of a biomarker was to perform either a cross-validation analysis or a pooled analysis. When a biomarker had been shown to have a significant prognostic and/or predictive effect in one trial, then a cross-validation was performed in the 3 other trials. When only a marginal effect was observed (ie, no statistically significant association but *P*-value close to alpha level), we performed a pooled analysis combining all 4 trials. For cross-validation, we used the predefined cutoff reported from the initial trial where possible. Whatever the approach, the statistical analysis includes several common and specific steps which are described below.

Not all patients had biomarker data available, as in some patients, the block had been exhausted, or available tissue sections contained no tumor. As a first step, we compared the demographic characteristics (gender, age [$<$ 55, 55-64, $\geq$ 65 years], T stage [1, 2, 3/4], N stage [0, 1, 2], tumor stage [I, II, III], performance status [0, $\geq$ 1], histology [squamous cell carcinoma, adenocarcinoma, other], type of surgery [pneumonectomy, other]) of patients with a successful biomarker assay with those without in order to detect potential bias. We also compared follow-up (estimated using the Schemper method) and the number of deaths and events.

Next, we evaluated the correlation between the biomarker and clinical covariates. This was performed in both the training and the validation set in a cross-validation analysis. After univariate and multivariable logistic regression stratified on trials, covariates significantly associated with the biomarker were included in the survival model.

Finally, the main analysis consisted in fitting a multivariable Cox proportional hazard regression model stratified by trials. Patients with missing information (clinical or biomarker) were excluded. Overall, the percentage of incomplete cases was lower than 3%. The assessment of the proportional hazard hypothesis was tested by introducing a time-dependent interaction with the marker or using the Schoenfeld residuals. When this hypothesis was rejected for some covariates, a Cox model stratified on these covariates (except the biomarker) was used. Where possible we used the same adjustments as had been used in the original analyses. However, after we demonstrated that tumor-infiltrating lymphocytes was associated with overall survival, we included tumor-infiltrating lymphocytes as an adjustment factor in all subsequent analyses (for example, BRCA1).

For the prognostic analyses, an 'all comers' strategy was used unless the biomarker was found to be predictive, in which case only the control arm was included. The between-trial heterogeneity was tested by including an interaction term between the biomarker and the trials and reported by a forest plot. For the predictive analyses, we tested for an interaction between treatment and the biomarker. The hazard ratio and 95% confidence intervals of the treatment effect in patients with negative ($<$ cutoff) and positive ($\geq$ cutoff) biomarker as well as the hazard ratios of the biomarker in adjuvant chemotherapy and observation arms were reported. The heterogeneity of the predictive effect between trials was evaluated with a 3-order interaction (treatment $\times$ biomarker $\times$ trial).

If we were able to validate the original prognostic and/or predictive value of the biomarker, we also combined data from all trials in order to provide a global and more accurate estimate of the effect, especially where there was a low prevalence of the biomarker (providing there was no heterogeneity among trials) and to explore interactions with histology. Sensitivity analyses were also performed categorizing the biomarker into tertiles or quartiles and exploring the functional form of the biomarker using the martingale residuals.

The level of statistical significance was set at 0.05 and 0.01 for cross validation and pooled analysis, respectively, and statistical analyses were conducted using SAS software.

Supplemental Table 1 Trials Included in the LACE Meta-analysis Project and LACE-Bio

Trial Name	Inclusion Criteria	Chemotherapy (No. Cycles, Dose of Cisplatin by Cycle, Daily Dose × No. Doses for Other Drugs)	Radiotherapy	Inclusion Period	No. Patients Randomized	No. Patients With at Least 1 Biomarker
JBR10	pT2pN0 or pT1-2pN1	4 cycles, cisplatin 50 mg/m^2/wk × 2 vinorelbine 25 mg/m^2/wk × 16	No	1994-2001	482	414
Adjuvant Lung Cancer Project Italy[a]	Stage I, II, IIIA	3 cycles, cisplatin 100 mg/m^2 mitomycin 8 mg/m^2 × 3 or vindesine 3 mg/m^2 × 6	Optional after chemotherapy	1994-1999	1088	0
Adjuvant Navelbine International Trialist Association 01 (ANITA)	Stage I, II, IIIA	4 cycles, cisplatin 100 mg/m^2 vinorelbine 30 mg/m^2 × 16	Optional for pN+ after chemotherapy	1994-2000	840	152
International Adjuvant Lung Trial (IALT)	Stage I, II, III	3-4 cycles, cisplatin 100 or 120 mg/m^2 or 80 or 100 mg/m^2 vindesine 3 mg/m^2 × 6-8, or vinblastine 4 mg/m^2 × 6-8, or vinorelbine 30 mg/m^2/wk × 13 etoposide 100 mg/m^2 × 9-12	Optional according to pN after chemotherapy	1995-2001	1867[b]	783
Big Lung Trial[a]	Stage I, II, III	3 cycles, cisplatin 50-80 mg/m^2 vindesine 3 mg/m^2 × 6, or vinorelbine 30 mg/m^2 × 6, or mitomycin 6 mg/m^2× 3 and ifosfamide 3 g/m^2 × 3, or mitomycin 6 mg/m^2× 3 and vinblastine 6 mg/m^2 × 3	Optional after chemotherapy	1995-2001	307	0
CALGB 9633[c]	Stage IA, IB, IIB, IV	4 cycles carboplatin AUC 6 paclitaxel 200 mg/m^2	No	1996-2003	344	250

Abbreviations: AUC = Area under the curve; CALGB = Cancer and Leukemia Group B; LACE-Bio = Lung Adjuvant Cisplatin Evaluation-Biomarkers.
[a]Tumor blocks/slides were not available for BIG or ALPI (The Italian/European Experience with Adjuvant Chemotherapy in Resectable Non—Small-Cell Lung Cancer), which were therefore not included in LACE-Bio.
[b]Blocks were only collected from larger sites.
[c]Not included in LACE.
Adapted from Pignon JP, Tribodet GV, Scagliotti G, et al. Lung adjuvant cisplatin evaluation: a pooled analysis by the LACE Collaborative Group. J Clin Oncol *2008; 26:3552-9.*

Supplemental Table 2 Summary of Analytic Methods Used in Original and Cross-validation Assays (Only Biomarkers of Interest Were Subject to Cross-validation)

Biomarker	Trial	Clone/Technique Used	Assays Done
ERCC1	IALT	Clone 8F1, Cat. MS-671-P, Lab Vision NeoMarkers, Fremont, CA	IALT-lab
	JBR10, CALGB	Clone 8F1, Neomarkers	IALT-lab
TIL	IALT	Hematoxylin and eosin	Reviewed by MST, EB
	JBR10, CALGB, ANITA	Hematoxylin and eosin	
$p27^{Kip1}$	IALT	Clone 57; dilution 1:200, Transduction Laboratories, Lexington, KY	IALT-lab
	JBR10	Clone 57, dilution 1:1000; Transduction Laboratories, Lexington, KY	JBR10-lab
	ANITA, CALGB	SX53G8, DAKO	IALT-Lab
Cyclin E	IALT	Clone 13A3, mouse monoclonal, dilution 1:15, Novo Castra	IALT-lab
	JBR10	Clone 13A3, mouse monoclonal, dilution 1:50, Novo Castra	JBR10-lab
$p16^{INK4}$	IALT	Clone 16P07, mouse monoclonal; dilution 1:400, Neomarkers	IALT-lab
	JBR10	Clone Ab-7, 1:200 dilution, Neomarkers	JBR10-lab
FAS	IALT	C20 rabbit polyclonal antibody, SC-715, dilution 1:200, Santa Cruz	IALT-lab
	JBR10, CALGB		IALT-lab
FASL	IALT	N20 rabbit polyclonal antibody, SC-834, dilution 1:30, Santa Cruz	IALT-lab
	JBR10, CALGB		IALT-lab
BAX	IALT	Rabbit polyclonal SC-493, dilution 1:50, Santa Cruz	IALT-lab
	JBR10, CALGB	Bax N20 (Dako) 1/100	IALT-lab
p53	IALT, ANITA	Mouse, M 7001 clone D07, dilution 1:75, Dako	IALT-lab
	JBR10		JBR10-lab
	CALGB	Clones 240 and 1801, Labvision Corporation, Fremont, CA	CALGB-lab
BRCA1	IALT	Mouse monoclonal clone 8F7 ref AB94, dilution 1:400, ABCAM	IALT-lab
	JBR10		IALT-lab
β-tubulin	JBR10	TUBB3 clone TUJ1	JBR10-lab
	IALT, CALGB JBR10[a]	TUBB3 clone TUJ1	JBR10-lab
Mucin	CALGB	Mucicarmine	CALGB-lab
	JBR10, IALT, ANITA	Mucicarmine	CALGB-lab

Abbreviations: ANITA = Adjuvant Navelbine International Trialist Association; CALGB = Cancer and Leukemia Group B; IALT = International Adjuvant Lung Cancer Trial; TIL = tumor-infiltrating lymphocytes.
[a]Additional samples.

Supplemental Table 3 Cutoffs Used for the Statistical Analyses

Biomarker	Original	Cross-validation/Pooled Analyses
ERCC1	1; Median H score	H score > 1 + quartiles
TIL	Intense/not intense (minimal, mild, or moderate)	Intense/not intense (minimal, mild, or moderate)
p27	50; Median standard score	50; Median standard score
Cyclin E	40; Median standard score	40; Median standard score
p16	1; Standard score	1; Standard score
FAS	240; Standard score	240; Standard score
FASL	240; Median standard score	240; Median standard score
FAS/FASL	< 0.8, 0.8-1.0, > 1.0; Standard score	< 0.8, 0.8-1.0, > 1.0; standard score
BAX	20; Standard score	90[a]
p53	20; Standard score > 100	4; Semi quantitative cutoff (0-1-2-3-4)[a] intensity (0, 1, 2, 3) P53 score as continuous variable as exploratory analysis
β-tubulin	176; Median standard Score	180, also quartiles and test for trends
BRCA1	160; Standard score	160; Standard score
Mucin	Any positive staining	Any positive staining

[a]Unbalanced in CALGB (Cancer and Leukemia Group B) + JBR10 so 90 was used.

Supplemental Table 4 LACE-Bio: Summary of Analyses of Biomarkers Tested for Their Prognostic and Predictive Value on Disease-free Survival

Biomarker	Discovery Trial[h]	Results		Main Analysis[i] (Exploratory Analysis)	Results	
		Prognostic Value $HR_{+/-}$ (95% CI)	Predictive Value $HR_{CT/control}$ (95% CI) in Negative $HR_{CT/control}$ (95% CI) in Positive With *P* Value of Interaction		Prognostic Value $HR_{+/-}$ (95% CI)	Predictive Value $HR_{CT/control}$ (95% CI) in Negative $HR_{CT/control}$ (95% CI) in Positive With *P* Value of Interaction
ERCC1	I (N = 761)	0.65 (0.49-0.88)[a]	0.65 (0.50-0.85) 1.12 (0.83-1.51) *P* = .008	$CV^{pro\ +\ pred}$: C + J (N = 494)[b,c]	1.35 (0.97-1.87)	1.08 (0.61-1.92) 0.68 (0.52-0.90) *P* = .15
TIL[j]	I (N = 783)	0.59 (0.42-0.83)	0.97 (0.81-1.17) 1.29 (0.72-2.33) *P* = .37	CV^{pro}: A + C + J (N = 753)[c,d] (I + A + C + J: Pool for predictive)	0.44 (0.24-0.78)	0.84 (0.73-0.97) 0.84 (0.48-1.48) *P* = .99
p27[k]	I (N = 778)	1.05 (0.86-1.28)	0.71 (0.54-0.94) 1.02 (0.78-1.06) *P* = .07	CV^{pred}: A + C + J (N = 660) (A + C + J for prognostic)	1.00 (0.80-1.24)	0.79 (0.59-1.06) 0.78 (0.58-1.06) *P* = .96
	J[g] (N = 311)	1.72 (1.03-2.86)[a] 1.33 (0.55-3.20)[a]	1.18 (0.72-1.94) 0.39 (0.21-0.73) 1.49 (0.54-4.11) *P* = .01			
Cyclin E[l]	I (N = 778)	Not done	Not done	$Pool^{pro\ +\ pred}$: I + J (N = 1081)	0.92 (0.77-1.09)	0.77 (0.62-0.95) 0.95 (0.74-1.20) *P* = .21
	J[g] (N = 305)	1.11 (0.70-1.76)	0.93 (0.58-1.49) 0.46 (0.26-0.79) *P* = .06			
p16[m]	I (N = 778)	Not done	Not done	$Pool^{pro\ +\ pred}$: I + J (N = 1081)	0.90 (0.75-1.08)	0.86 (0.70-1.06) 0.82 (0.64-1.06) *P* = .79
	J[g] (N = 307)	0.94 (0.53-1.68)[a]	0.78 (0.52-1.18) 1.08 (0.55-2.12) *P* = .43			
FAS[n]	I (N =)	Not done	Not done			
FASL[n]	I (N = 773)	0.90 (0.74-1.11)	0.76 (0.58-0.98) 1.14 (0.89-1.47) *P* = .03	$CV^{pro\ +\ pred}$: C + J (N = 520)	0.95 (0.71-1.28)	0.83 (0.62-1.10) 0.52 (0.31-0.85) *P* = .11
FAS/FASL[n]	I (N = 768)	0.73 (0.59-0.90) 0.84 (0.64-1.12)	0.93 (0.72-1.19) 1.10 (0.80-1.51) 0.57 (0.35-0.94) *P* = .10	$CV^{pro\ +\ pred}$: C + J (N = 507)	0.84 (0.62-1.13) 1.08 (0.79-1.48)	0.78 (0.53-1.15) 0.65 (0.42-1.01) 0.68 (0.42-1.10) *P* = .81
BAX[o]	I (N = 778)	1.44 (1.10-1.87)[a]	1.19 (0.93-1.52) 0.60 (0.45-0.80) *P* = .0004	$CV^{pro\ +\ pred}$: C + J (N = 494)[c]	0.98 (0.74-1.30)	0.87 (0.54-1.40) 0.71 (0.53-0.96) *P* = .48
p53[p]	I (N = 776)	Not done	Not done	$Pool^{pro\ +\ pred}$: A + C + I + J (N = 1401)	1.00 (0.87-1.16)	0.82 (0.68-0.98) 0.87 (0.70-1.09) *P* = .64

Supplemental Table 4 Continued

Biomarker	Discovery Trial[h]	Results		Main Analysis[i] (Exploratory Analysis)	Results	
		Prognostic Value $HR_{+/-}$ (95% CI)	Predictive Value $HR_{CT/control}$ (95% CI) in Negative $HR_{CT/control}$ (95% CI) in Positive With *P* Value of Interaction		Prognostic Value $HR_{+/-}$ (95% CI)	Predictive Value $HR_{CT/control}$ (95% CI) in Negative $HR_{CT/control}$ (95% CI) in Positive With *P* Value of Interaction
	J (N = 253)	Not done	Not done			
	C (N = 250)	1.56 (1.02-2.37)	HRs not reported $P > .05$			
β-tubulin	J (N = 265)	1.52 (1.05-2.22) 1.92 (1.16-3.18)[a]	0.78 (0.44-1.37) 0.45 (0.27-0.75) $P = .15$	CV^{pro}: A + C + I + J^2 (N = 1141)[f] (A + C + I + J^{1+2}: Pool for predictive)	1.30 (1.11-1.53)	0.96 (0.79-1.18) 0.73 (0.60-0.89) $P = .05$
BRCA1[q]	I (N = 566)	1.03 (0.83-1.27)	0.87 (0.63-1.19) 1.07 (0.81-1.41) $P = .32$	$Pool^{pro+pred}$: I + J (N = 851)[d,e]	1.01 (0.84-1.22)	0.76 (0.58-1.00) 1.01 (0.79-1.29) $P = .12$
Mucin	C (N = 192)	1.88 (1.26-2.81)		CV^{pro}: A + I + J (N = 1261)[d] (C + A + I + J: Pool for predictive)	1.21 (0.99-1.48)	0.89 (0.76-1.05) 0.75 (0.59-0.97) $P = .27$

Abbreviations: A = ANITA; ANITA = Adjuvant Navelbine International Trialist Association; C = CALGB; CALGB = Cancer and Leukemia Group B; CI = confidence interval; CV = cross-validation; DFS = disease-free survival; HR = hazard ratio; I = IALT; IALT = International Adjuvant Lung Cancer Trial; J = JBR10; N = number of patients used in the multivariable statistical analysis; OS = overall survival; P = pooled; pred = predictive; pro = prognostic; TIL = tumor-infiltrating lymphocytes.

[a]Prognostic analyses performed in control arm only.

[b]See Friboulet et al[13] for a full description.

[c]The same adjustment was used both for the discovery and cross-validation analyses.

[d]To take into account mucin results, a new revised histology of all non-adenocarcinoma mucin was performed. This new revised histology was used for the analysis of the following markers: TIL, BRCA1.

[e]Prognostic and predictive analyses with different cut-offs have been used and showed similar results. For BRCA1: quartiles and trend test showed similar results with, however, a *P*-value of trend test = .09.

[f]A first sample from JBR10 (J^1) was used as discovery set and a sub-sample of JBR10 trial (J^2; n = 52) was used for the cross-validation of the prognostic effect.

[g]For these 3 markers in JBR10 trial, (p27 [< 60, 60-200, ≥ 200], Cyclin E [cutoff = 20], and p16 [cutoff = 80]), the reported results correspond to the standard score but without adjustment on baseline factors correlated to the marker of interest. In addition, different possible scoring systems (average intensity, average percentage, 4-tier score) were explored in this trial.

[h]The baseline factors included in the multivariable Cox model may differ between trials. For example, for JBR10 trial, RAS gene mutation, smoking history, baseline anemia, lactate dehydrogenase, comorbidity, alkaline phosphate value, and tumor size were used.

[i]We pre-specified the analysis type: prognostic value, predictive value, or both.

[j]TIL: intense versus not intense. The endpoints were OS, DFS, and specific disease-free survival.

[k]Filipits et al[10] used a standard score and cutoff = 50. For JBR10, the cutoff of the standard score was < 60, 60-200, and > 200. We also used cutoff as a continuous variable; results were comparable both for discovery and validation analysis.

[l]For IALT, standard score used cutoff = 20; similar results were observed when Cyclin E was analyzed as a continuous variable for the prognostic analysis. For JBR10, the cutoff = 20.

[m]For IALT, standard score used cutoff = 1; similar results were observed when p16 was analyzed as a continuous variable for the prognostic analysis. For JBR10, the cutoff = 80.

[n]Cross validation for predictive effect of FASL was performed based on an updated DFS analysis of IALT (HR, 0.76; 95% CI, 0.58-0.98 and HR, 1.14; 95% CI, 0.89-1.47 in < 240, ≥ 240; interaction *P* value of .03). The alpha level was set at 0.01 to correct for multiple testing. For FAS, FASL, and their ratio, several cutoffs have been explored using quartiles or considering biomarkers as a continuous biomarker; we used the following cutoffs: 240 for FAS and FASL and < 0.8, 0.8-1.0, > 1 for the ratio. Both analyses controlled for lymphoid infiltration. Cross-validation was also performed using H-score for the ratio with similar results.

[o]The results on IALT were obtained with the same adjustment (including lymphoid infiltration) as the cross-validation. Discovery set used a cutoff of 90, because of unbalanced distribution in the cross-validation data set; using this cutoff of 90, a prognostic effect was identified and therefore cross-validation was performed rather than a pooled analysis.

[p]Each trial defined p53 score and cutoff. In ANITA and IALT trials, p53 score = intensity of staining × % of stained cells (cutoff = 100). In JBR10 trial, positive = % of tumor cells with nuclear staining of ≥ 15% for tumors showing intensity scores of 1 or more. In CALGB, p53 positive = staining intensity ≥ 2 (in any % of cells). For the pooled analysis, p53 was initially defined by a standard score (% stained cells × intensity) (cutoff = 100) and H-score (% stained cells in category × intensity, cutoff = 4). When we used the standard score as a quantitative variable for the pool analysis, the prognostic effect was HR, 0.99; 95% CI, 0.98-1.01 for DFS. The p53 × treatment interaction term was *P* = .65 for OS. The analysis on discovery set included lymphoid infiltration as covariate.

[q]OS, DFS, and specific disease-free survival were the endpoints for the pooled analysis. The multivariable analysis contains lymphoid infiltration as covariate.