Adenosine Triphosphate-Based Chemotherapy Response Assay (ATP-CRA)-Guided Platinum-Based 2-Drug Chemotherapy for Unresectable Nonsmall-Cell Lung Cancer

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BACKGROUND. The study investigated correlations between adenosine triphosphate / chemotherapy response assay (ATP-CRA) and clinical outcomes after ATP-CRA-guided platinum-based chemotherapy for unresectable nonsmall-cell lung cancer (NSCLC).

METHODS. The authors performed an in vitro chemosensitivity test, ATP-CRA, to evaluate the chemosensitivities of anticancer drugs such as cisplatin, carboplatin, paclitaxel, docetaxel, gemcitabine, and vinorelbine for chemonaive, unresectable NSCLC. The cell death rate was determined by measuring the intracellular ATP levels of drug-exposed cells compared with untreated controls. A sensitive drug was defined as a drug producing 30% or more reduction in ATP compared with untreated controls. Assay-guided platinum-based 2-drug chemotherapy was given to patients with pathologically confirmed NSCLC.

RESULTS. Thirty-four patients were enrolled. Thirty tumor specimens were obtained by bronchoscopic biopsies and 4 obtained surgically. The median age was 61 years and 27 patients had an Eastern Cooperative Oncology Group (ECOG) performance status of 0–1. The response rate was 43.8%. At a median follow-up period of 16.9 months, the median progression-free and overall survivals were 3.6 and 11.2 months, respectively. Patients were dichotomized into the platinum-sensitive (S; 20 patients) and resistant (R; 14 patients) groups. The positive/negative predictive values were 61.1% and 78.6% with a predictive accuracy of 68.8%. Although without significant differences in pretreatment parameters, the S-group showed better clinical response ($P = .036$), longer progression-free survival ($P = .060$), and longer overall survival ($P = .025$).

CONCLUSIONS. Despite using bronchoscopic biopsied specimens, ATP-CRA and clinical outcomes correlated well after assay-guided platinum-based 2-drug chemotherapy for unresectable NSCLC. There was a favorable response and survival in the platinum-sensitive vs resistant groups. ***Cancer*** **2007;109:1829–35.**

KEYWORDS: adenosine triphosphate, chemotherapy response assay, nonsmall-cell lung cancer.

Lung cancer is the leading cause of cancer-related death around the world. Nonsmall-cell lung cancer (NSCLC) comprises about 80% of all lung malignancies. More than half of NSCLC patients present with stage IIIB or IV disease and they are candidates for systemic chemotherapy.[1]

A big obstacle in cancer treatment, including NSCLC, is the heterogeneity of tumor response to chemotherapy. In this situation,

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several in vitro chemosensitivity assays have been developed to predict chemotherapy outcomes.[2–8] An in vitro chemosensitivity assay refers to any laboratory analysis that is performed specifically to evaluate whether or not tumor growth is inhibited by various chemotherapy drugs. Although an in vitro assay-guided therapy seems to be ideal, in actuality this therapy is not widely used in clinical practice because of various technical problems encountered with this assay, including the requirement of a high technical skill level, the large number of required tumor cells, and the long test turnaround time.[9]

The adenosine triphosphate (ATP)-based assay is a sensitive assay that evaluates tumor cell viability by measuring the intracellular ATP levels of drug-exposed cells and untreated controls.[6,10–13] This assay has been somewhat widely studied because its reliability was demonstrated as a sensitive measure of viability in the field of cell biology.[14,15] In this study we modified previous ATP-based assays. Our ATP-based chemotherapy response assay (ATP-CRA) adopted new methods, such as the use of ultra-low attachment culture plates to inhibit the growth of normal cells and a shortened test turnaround time.[16] We previously reported the clinical feasibility of our ATP-CRA in a study on a limited volume of tumor samples from bronchoscopic biopsies.[16] Moreover, a survival benefit with the original ATP assay-guided chemotherapy design was observed in ovarian cancer patients.[6,10,12] Therefore, we investigated correlations between the ATP-CRA results using bronchoscopic biopsied samples and the clinical outcomes after ATP-CRA-guided platinum-based chemotherapy in chemonaive patients with unresectable NSCLC.

MATERIALS AND METHODS

Patients

ATP-CRA was performed in tumor tissue specimens obtained from patients with suspected lung malignancies. Assay-guided platinum-based 2-drug chemotherapy was given to patients with pathologically confirmed NSCLC. The eligible patients for this study were subjects from whom we had successfully obtained assay results, who presented with unresectable NSCLC, and who received at least 1 cycle of chemotherapy. The other eligibility criteria were: 1) age $\geq$18 years; 2) histologically or cytologically proven NSCLC; 3) stage IIIB or IV (American Joint Cancer Committee [AJCC] staging 2002)[17]; 4) Eastern Cooperative Oncology Group performance status (ECOG PS) $\leq$2; 5) adequate bone marrow function (neutrophils $\geq 1.5 \times 10^3/\mu L$, platelets $\geq 100 \times 10^3/\mu L$, and Hb $\geq$ 10.0 g/dL), adequate renal function (serum creatinine $\leq$ 1.5 $\times$ upper normal limit), and adequate liver function (serum bilirubin $\leq$1.5 $\times$ upper normal limit, aspartate aminotransferase [AST], and alanine aminotransferase [ALT] $\leq$1.5 $\times$ upper normal limit); 6) no previous chemo- or radiotherapy; and 7) no history of other malignancies (excluding nonmelanoma skin cancer or carcinoma-in-situ of the uterine cervix) within 5 years. This clinical trial was approved by the appropriate Institutional Review Board and all patients in the study gave written informed consent.

ATP-Based Chemotherapy Response Assay Methodology

Isolation of cancer cells, elimination of normal cells, and tissue culture

Tumor tissues stored in Hank balanced salt solution (HBSS, Gibco, Rockville, Md), containing 100 IU/mL penicillin (Sigma, St. Louis, MO), 100 μg/mL streptomycin (Sigma), 100 μg/mL gentamicin (Gibco), 2.5 μg/mL amphotericin B (Gibco), and 5% fetal bovine serum (FBS, Gibco) were delivered to the laboratory. These tissues were first washed, quantified, and minced, then incubated with extracellular matrix-degrading enzymes, such as dispase (Sigma), pronase (Sigma), and DNase (Sigma) at 37°C for 12 to 16 hours. Cells were harvested using a cell strainer (BD Falcon, Bedford, Mass). To eliminate red blood cells and dead cells, the cell suspensions were subjected to Ficoll (1.077 g/mL) gradient centrifugation at 400*g* for 15 minutes. The viability of isolated cells was tested using Trypan blue exclusion. The histological types of the tumor tissues as well as qualitative and quantitative analyses of the cancer cells were evaluated by pathologists.

ATP measurement

Separated tumor cells were diluted to 2000–20,000 viable cells/100 μL using Iscove modified Dulbecco medium (IMDM, Gibco), including 10% FBS, and seeded in triplicate to a 96-well ultra-low attachment plate (Costar, Cambridge, Mass), which restricted the growth of normal cells such as fibroblasts. In treated groups, 100 μL of chemotherapeutic agents were added to the seeded cell cultures and incubated for 48 hours in a CO_2 incubator. In the untreated control groups, 100 μL of IMDM, without chemotherapeutic agents, was added. For the purpose of quality control, a negative control group of 3–6 wells (containing only seeding medium without cells) and 2 positive control groups were included in the culture plate. Each positive control group was composed of 3 wells that contained the minimal (105 pg ATP) and median (280 pg ATP) amounts of ATP, as measured in 1000 tumor cells harvested from tissue. The final concentrations of anticancer drugs were determined by training set

experiments, which exhibited a scattered distribution of cell deaths from each patient (data not shown): cisplatin (2.5 μg/mL), carboplatin (12 μg/mL), paclitaxel (8.5 μg/mL), docetaxel (3.7 μg/mL), gemcitabine (16.9 μg/mL), vinorelbine (0.18 μg/mL), and irinotecan (4.7 μg/mL), which are all active drugs widely used as a combination with platinum in advanced NSCLC.[18] Cells from the untreated control and treated groups were lysed and the amount of ATP in the cell lysates was measured using flash type luminescence measurements on a Victor 3 multilabel counter (PerkinElmer Boston, Mass). The cell death rate for each drug was defined as the rate of ATP luminescence reduction in the treated group compared with the untreated control.

ATP-CRA-Guided Chemotherapy

A sensitive drug was defined as a drug producing 30% or more reduction in ATP compared with untreated controls. The sensitivity criterion of 30% was determined by results from a previous, retrospective study[19] that analyzed correlations between the ATP-CRA results and the outcomes of chemotherapy for advanced NSCLC. All the patients received platinum-based 2-drug chemotherapy regardless of their in vitro platinum-sensitivity. Platinum choice (cisplatin or carboplatin) was dependent on the physicians' decision. The second drug combined with platinum was chosen based on the ATP-CRA results. In the cases sensitive to no drug in vitro, the drug with the highest cell death rate was chosen.

Chemotherapy was delivered according to the dosage and schedule listed in Table 2 and was continued for a maximum of 6 cycles or until the appearance of progressive disease.

Analysis of Endpoints and Statistical Considerations

The primary endpoints were a correlation between the ATP-CRA results and the clinical response and the predictive values. Response was assessed every 3 cycles or whenever needed. Tumor response was classified according to the Response Evaluation Criteria in Solid Tumors.[20] The positive predictive value (PPV) indicates how accurately the test identifies those patients who will respond to chemotherapy and the negative predictive value (NPV) indicates how accurately the test detects resistance to chemotherapy.

The secondary endpoints of our study were the correlations between the ATP-CRA results and progression-free survival (PFS) or overall survival (OS). PFS was defined as the time from commencement of chemotherapy until progression or death. OS was defined as the time from chemotherapy to death from all causes.

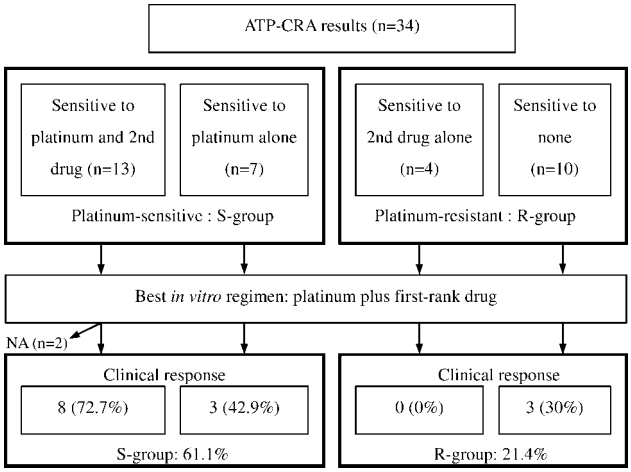

FIGURE 1. Treatment scheme and results. Second drug, the other drug combined with platinum. NA, not assessable.

All statistical calculations were carried out using the SPSS Windows program, v. 11.5 (SPSS, Chicago, Ill). All *P*-values were 2-sided and the α-value was set at 0.05. Survival was calculated using the Kaplan-Meier method. A log-rank test was used to compare survival between subgroups. Prognostic variables found by univariate analysis were used in multivariate analysis by the Cox proportional hazard regression model.

RESULTS

Patient Characteristics

From September 2003 to October 2005, 34 patients were enrolled in this study. The median age was 61 years (range, 42–74) and the ECOG PS was 0–1 in 27 patients and 2 in 7 patients. Thirty tumor specimens were obtained by bronchoscopic biopsies and the remaining 4 were obtained surgically. According to the ATP-CRA results, patients were dichotomized into the platinum-sensitive (S) and -resistant (R) groups (Fig. 1). There were no significant differences in pretreatment parameters such as ECOG PS, histology, and stage between the S- and R-groups. A detailed comparison of the patient characteristics in the S- and R-groups is summarized in Table 1.

Correlation Between ATP-CRA Results and Clinical Response

The ATP-CRA-based treatment scheme and its clinical response are shown in Figure 1. The S-group (n = 20) was comprised of 2 subgroups: sensitive to both platinum and the second drug or sensitive to platinum alone. The R-group (n = 14) was also comprised of 2 subgroups: sensitive to the second drug alone or sen-

TABLE 1
Comparison of Patient Characteristics According to ATP-CRA Results

		S-Group, n = 20		R-Group, n = 14		
	No.	No.	(%)	No.	(%)	*P*
Median age, y		61 [range, 42–74]		58 [range, 38–76]		0.742*
Sex						
Men	21	13	(65.0)	8	(57.0)	.728†
Women	13	7	(35.0)	6	(43.0)	
ECOG performance status						
0–1	27	17	(85.0)	10	(71.0)	.410†
2	7	3	(15.0)	4	(29.0)	
Histology						
Adenocarcinoma	20	11	(55.0)	9	(64.0)	.275†
Squamous cell carcinoma	13	9	(45.0)	4	(29.0)	
Large cell carcinoma	1	0	(0)	1	(7.0)	
Stage						
IIIB	12	9	(45.0)	3	(21.0)	.275†
IV	22	11	(55.0)	11	(79.0)	
Brain metastasis						
Present	7	3	(15.0)	4	(29.0)	.410†
Absent	27	17	(85.0)	10	(71.0)	

ATP-CRA indicates adenosine triphosphate/chemotherapy response assay; ECOG, Eastern Cooperative Oncology Group.

* *P* value was determined by Mann-Whitney test.

† *P* values by chi-square test or Fisher exact test.

sitive to none of the drugs. Among the 4 subgroups the subgroup sensitive to both drugs had the highest response rate, 72.7%.

Two patients with platinum-sensitive tumors could not be assessed for response because of an early dropout due to patient refusal. The overall clinical response rate was 43.8% (14/32). The clinical response rate was higher in the S-group (61.1% vs 21.4% in the R-group; $P = .036$; Fig. 1). The PPV and NPV were 61.1% (11 responders/18 patients from the S-group) and 78.6% (11 nonresponders/14 patients from the R-group), respectively, with a predictive accuracy of 68.8%.

Administered Chemotherapy and Clinical Response for Each Chemotherapy

Of the 6 drugs tested, cisplatin induced the highest mean cell death rate (42.6%) and sensitive rate (73.5%), which means the frequency of cell death rate was $\geq$30%. Details of the mean cell death rates and sensitive rates of each drug are shown in Figure 2.

A total of 112 cycles of chemotherapy were administered with a median cycle of 3 (range, 1–6): a median of 3.5 (range, 1–6) for the S-group and a median of 2.5 (range, 1–4) for the R-group. The selected regimens were platinum plus paclitaxel (12 patients), vinorelbine (12 patients), or gemcitabine (8 patients). Cisplatin was administered in 23 patients (14 from the S-group vs 9 from the R-group) and carboplatin in 9 (4 from the S-group vs 5 from the R-group). No significant differences were observed in the kinds of selected regimens between the S- and R-groups (Fisher exact test, $P = .372$ for second drugs, $P = .435$ for platinums). The clinical response rates according to each regimen were all higher in the S-group than in the R-group. The kinds of chemotherapy administered and response rates according to each regimen are summarized in Table 2.

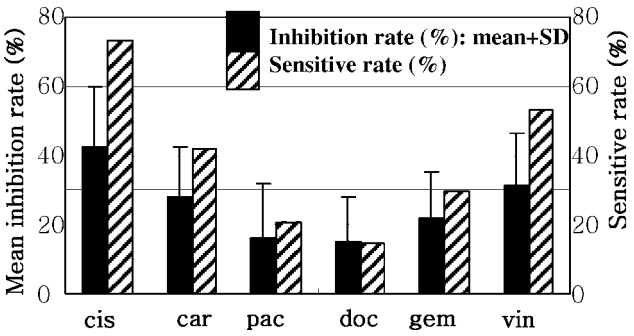

FIGURE 2. Mean cell death rate and sensitive rate of each drug. cis, cisplatin; car, carboplatin; pac, paclitaxel; doc, docetaxel; gem, gemcitabine; vin, vinorelbine; SD, standard deviation.

TABLE 2
Kinds of Chemotherapy Administered and Response Rate for Each Chemotherapy

	S-Group		R-Group		Total	
Platinum*/Second Drug	No.	RR	No.	RR	No.	RR
Platinum(D2)/Paclitaxel (175 mg/m^2, D1) q 3 wk	7	71.4%	5	40.0%	12	58.3%
Platinum(D2)/Vinorelbine (30 mg/m^2, D1,8) q 3 wk	8	37.5%	4	0%	12	25.0%
Platinum(D1)/Gemcitabine (1000 mg/m^2, D1,8) q 3wk	3	100%	5	20.0%	8	50.0%

RR indicates response rate; D, day; q, every; wk, weeks.

Two patients were not assessable for response.

* Cisplatin, 75 mg/m^2; carboplatin, area under the curve (AUC) of 5.

Progression-Free and Overall Survival According to ATP-CRA Results

Four patients were excluded from the PFS analysis because of an early dropout by patient refusal (n = 2) or an unclear date of progression (n = 2). At a median follow-up duration of 16.9 months (range, 4.3–32.2), 28 (93.3%) of the 30 patients experienced disease progression. The median PFS was 3.6 months (95% confi-

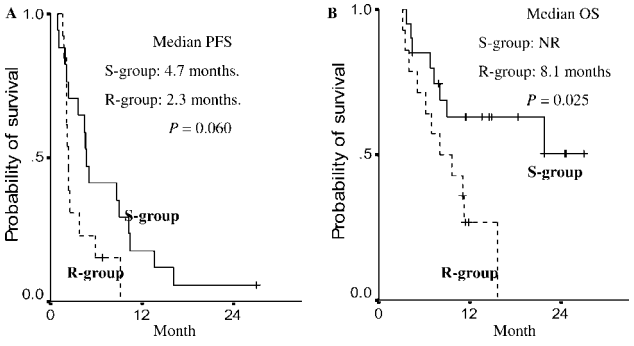

FIGURE 3. Survival according to the adenosine triphosphate/chemotherapy response assay (ATP-CRA) results. (A) Progression-free survival (PFS). (B) Overall survival (OS). NR, not reached.

dence interval [CI]: 0.9–6.3), with a 6-month PFS rate of 30.0%. All of the 34 patients were included in the OS analysis. Within the same follow-up duration, 19 (55.9%) patients died: 18 of cancer-progression and 1 as the result of a traffic accident. The median OS was 11.2 months (95% CI: 4.2–18.2), with a 1-year OS rate of 47.7%.

According to the ATP-CRA results, the S-group showed longer PFS (4.7 vs 2.3 months in the R-group; $P = .060$; Fig. 3A) and OS (not-reached vs 8.1 months in the R-group; $P = .025$; Fig. 3B).

Predictors for Clinical Response and Prognosticators for Progression or Survival

The significance of using the ATP-CRA results as a predictor for clinical response was evaluated by comparing these results to other pretreatment parameters, such as age, sex, ECOG PS, stage, histology, and brain metastasis. Using univariate analysis, we found that ECOG PS ($P = .010$) and the ATP-CRA results ($P = .036$) were significant.

The significance of using the ATP-CRA results as a prognosticator for PFS or OS was also evaluated by comparing these results to pretreatment parameters. Table 3 summarizes the significant factors, as determined by univariate analysis. Multivariate analysis identified the following independent prognostic factors (Table 3): ECOG PS for PFS (hazard ratio [HR] = 3.38; $P = .011$), histology for PFS (HR = 2.48; $P = .049$), and ECOG PS for OS (HR = 2.95; $P = .051$).

Because the ECOG PS is a significant response predictor and a prognosticator for progression or survival, a subset analysis was performed according to the ECOG PS. In the subset of patients with an ECOG PS of ≤ 1 the S-group still had a significantly better response rate (73.3% vs 26.7%; $P = .049$), median PFS (5.0 months vs 2.3 months; $P = .028$), and median OS (not-reached vs 9.7 months; $P = .006$) than the R-group.

Poststudy Therapy

Docetaxel was administered most commonly as a second-line chemotherapy: 71.4% (10/14 progressed patients) in the S-group and 66.7% (8/12 progressed patients) in the R-group. Other chemotherapies were delivered to 3 patients from the S-group and 2 from the R-group. Local therapy alone was given to 1 patient from the R-group and supportive care alone was given to 1 from the S-group.

DISCUSSION

The current standard of care for patients with advanced NSCLC is chemotherapy with a platinum-based doublet as a first-line agent,[18] indicating that none of the platinum-based doublets is superior to the others.[21,22] Under these circumstances, determining the best in vitro drug before the start of chemotherapy for advanced NSCLC may be ideal. However, for advanced NSCLC only a few in vitro chemosensitivity assays have been tested in clinical trials. Until now, assay success rates or predictive accuracies were not satisfying.[3,4] Therefore, in this study ATP-based CRA was prospectively investigated for the first time for selecting the best in vitro regimen to treat NSCLC.

The S- and R-groups that were dichotomized by ATP-CRA represented different survival (PFS or OS) as well as clinical response rates after ATP-CRA-guided chemotherapy. These clinical outcomes were more favorable in the S-group. Although our study was not randomized in order to compare in vitro assay-guided chemotherapy to empirical chemotherapy, this result is notable in that 2 subgroups classified by ATP-CRA showed significantly different survival rates. There were fewer stage IV, brain metastasis, and ECOG PS-2 patients in the S group, leading to more cycles of chemotherapy administered. This may have produced the survival difference. However, the differences in the pretreatment clinical factors were very small and were not statistically significant between the 2 subgroups. Moreover, even after excluding the influence of the ECOG PS, which was identified as an independent prognosticator in this study, a survival difference was still maintained, according to the ATP-CRA results.

The overall response rate (43.8%) and the overall survival duration (11.2 months) reported here are similar to results from advanced NSCLC studies with similar regimens to ours.[21,22] In 2004 a meta-analysis reported that in vitro chemotherapy sensitivity and resistance assays should not yet be used in clinical practice because no survival benefit was found. However,

TABLE 3
Multivariate Analysis of Prognosticators for Progression-Free and Overall Survival

		Progression-free survival		Overall survival	
Variables		**HR (95% CI)**	***P***	**HR (95% CI)**	***P***
ECOG PS	(0–1 vs 2)	3.38 (1.32–8.66)	.011	2.95 (1.00–8.74)	.051
Histology*	(Squamous vs adenoca.)	2.48 (1.00–6.17)	.049	—	—
ATP-CRA	(S-group vs R-group)	—	—	0.46 (0.17–1.30)	.143

ATP-CRA indicates adenosine triphosphate/chemotherapy response assay; ECOG, Eastern Cooperative Oncology Group; PS, performance status; HR, hazard ratio; CI, confidence interval; squamous, squamous cell carcinoma; adenoca, adenocarcinoma.

* Large cell carcinoma was excluded because of the small number (n = 1).

this analysis did find a higher response rate by assay-guided therapy and encouraged those assays to be tested widely in clinical trials.[23] In spite of the meta-analysis, some newer cell death assays (cell viability assays), such as the ATP-assay, have shown a high correlation between assay results and survival, as well as response,[6,10,12] supporting our finding that the ATP-CRA results and the clinical outcomes in this study correlated well.

It is well known that an anchorage-independent culture method, such as an agar underlayer, inhibits the growth of fibroblasts but allows tumor cells to survive and proliferate.[24] An ultra-low attachment plate is another anchorage-independent culture method. This was adopted in our ATP-CRA and inhibited fibroblast proliferation better than an agar underlayer in our preclinical study (data not shown). This method might have partially contributed to the positive correlation obtained in our study.

Moreover, in our previous feasibility study of this assay,[16] ATP-CRA had the advantages of a short test turnaround time of 7 days and a high assay success rate of 88.9%, despite using specimens obtained by bronchoscopic biopsies. For the in vitro chemosensitivity assay the procurement of specimens is another important issue. It is very encouraging that bronchoscopic biopsy, which is generally the least invasive biopsy procedure for lung cancer, could be utilized for this ATP-CRA. This fact is also supported by other reports,[14,15] which demonstrated that the ATP-based assay was sensitive enough to test cell viability with very small specimens. A short test turnaround time in our assay resulted from cutting the incubation time down to 48 hours, as reported in other studies,[25,26] with rising drug concentrations. These technical advantages would allow our ATP-CRA to be easily applied in clinical trials and perhaps eventually in clinical practice.

In order to determine the value of a diagnostic test in a specific patient it is important to know predictive values of the in vitro test in an individual patient. Our assay showed a PPV of 61.1% and an NPV of 78.6%. The fact that the NPV was higher than the PPV indicates that our ATP-CRA can detect drug resistance better than chemosensitivity, being consistent with previous in vitro assay studies.[27] Because patients' tumors may have an intraindividual heterogeneity depending on different biopsy sites with regard to chemosensitivity, in the current study the PPV, indicating an accuracy for detecting chemosensitivity, was not high. Conversely, a drug resistance result obtained from a single tumor sample for a specific drug is evidence of the presence of a nonresponsive tumor population in at least 1 site, alerting the physician to avoid such an agent.[27]

The limitation of this study was that we did not consider interactions between the 2 drugs and individual pharmacokinetic variations. We also did not compare the ATP-CRA-guided chemotherapy to empirical chemotherapy for advanced NSCLC. Our study also had a very small number of patients. Although it is not possible to make definitive conclusions, there is a good possibility that this technique may predict drug resistance. Good correlations between the ATP-CRA results and clinical outcomes are encouraging.

In conclusion, despite using bronchoscopic biopsied specimens, the ATP-CRA results and the clinical outcomes correlated well after assay-guided platinum-based 2-drug chemotherapy for unresectable NSCLC. This showed a favorable response and survival in the platinum-sensitive vs resistant groups. Randomized trials that compare ATP-CRA-guided chemotherapy to empirical chemotherapy in advanced NSCLC are warranted.

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