Spotlight on Molecular Profiling

# Asparagine synthetase is a predictive biomarker of L-asparaginase activity in ovarian cancer cell lines

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## Abstract

**We recently used RNA interference to show that a negative correlation of L-asparaginase (L-ASP) chemotherapeutic activity with asparagine synthetase (ASNS) expression in the ovarian subset of the NCI-60 cell line panel is causal. To determine whether that relationship would be sustained in a larger, more diverse set of ovarian cell lines, we have now measured *ASNS* mRNA expression using microarrays and a branched-DNA RNA assay, ASNS protein expression using an electrochemiluminescent immunoassay, and L-ASP activity using an MTS assay on 19 human ovarian cancer cell lines. Contrary to our previous findings, L-ASP activity was only weakly correlated with *ASNS* mRNA expression; Pearson's correlation coefficients were $r = -0.21$ for microarray data and $r = -0.39$ for the branched-DNA RNA assay, with just the latter being marginally statistically significant ($P = 0.047$, one-tailed). ASNS protein expression measured by liquid-phase immunoassay exhibited a much stronger correlation ($r = -0.65$; $P = 0.0014$, one-tailed). We conclude that ASNS protein expression measured by immunoassay is a strong univariate predictor of L-ASP activity in ovarian cancer cell lines. These findings provide rationale for evaluation of ASNS protein expression as a predictive biomarker of clinical L-ASP activity in ovarian cancer. [Mol Cancer Ther 2008;7(10):3123–8]**

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## Introduction

In 2008, there will be an estimated 21,650 new cases of ovarian cancer that will result in ~15,520 deaths, making ovarian cancer second among gynecologic cancers in incidence and the most lethal of the gynecologic malignancies (1). The 5-year survival of patients diagnosed with ovarian cancer is >80% when the disease is diagnosed at stage I or II. However, the majority of patients present at stage III and IV, when the malignancy has spread beyond the ovaries and the 5-year survival is <20%. There is significant room for improvement, and the era of personalized medicine promises to contribute.

One way personalized medicine will improve ovarian cancer outcome is through the identification of novel drug/gene relationships. We recently used molecular profiling and RNA interference to show that asparagine synthetase (ASNS) is a causal biomarker of L-asparaginase (L-ASP) activity in ovarian lines of the NCI-60 panel (2). L-ASP is a Food and Drug Administration-approved enzyme drug for cancer and has been used in combination with traditional chemotherapy to treat acute lymphoblastic leukemia since the early 1970s. In our previous study, small interfering RNA-mediated silencing of *ASNS* in three ovarian lines caused 3- to >500-fold potentiation of L-ASP activity, and the effect was independent of classical multidrug resistance. Those findings suggested that L-ASP might be used to treat ovarian cancer by using ASNS expression as a predictive biomarker of L-ASP efficacy.

To determine the conditions under which ASNS predicts L-ASP activity in a larger sample set, we have now measured L-ASP activity, as well as ASNS mRNA and protein expression, in an expanded set of ovarian cancer cell lines. We report that baseline ASNS protein expression is a strong univariate predictor of L-ASP activity across those lines.

## Materials and Methods

### Compounds

*Escherichia coli* L-ASP was obtained from Sigma.

**Table 1. Summary of L-ASP pharmacology experiments**

| Cell line | Mean $EC_{50}$ (units/mL) | 95% Confidence interval | Type |
|---|---|---|---|
| 222 | 1.61 | 1.21-2.12 | Endometrioid adenocarcinoma |
| A224 | 0.36 | 0.33-0.39 | Papillary serous adenocarcinoma |
| A2780 | 0.22 | 0.20-0.24 | Papillary serous adenocarcinoma |
| A364 | 0.25 | 0.22-0.29 | Papillary serous adenocarcinoma |
| A547 | 0.19 | 0.11-0.34 | Papillary serous adenocarcinoma |
| AD10 | 0.24 | 0.22-0.27 | Papillary serous adenocarcinoma |
| CaOV3 | 5.04 | 3.77-6.74 | Papillary serous adenocarcinoma |
| IGROV1 (NCI) | 1.10 | 0.45-2.67 | Adenocarcinoma |
| OVCAR-3 (NCI) | 1.19 | 0.94-1.50 | Adenocarcinoma |
| OVCAR-3 | 0.86 | 0.56-1.32 | Papillary serous adenocarcinoma |
| OVCAR-4 (NCI) | 5.48 | 4.27-7.02 | Adenocarcinoma |
| OVCAR420 | 0.30 | 0.21-0.42 | Adenocarcinoma |
| OVCAR429 | 0.40 | 0.28-0.58 | Serous cystadenocarcinoma |
| OVCAR432 | 0.29 | 0.23-0.36 | Adenocarcinoma |
| OVCAR-8 (NCI) | 0.49 | 0.44-0.53 | Adenocarcinoma |
| OVCAR-8/ADR (NCI) | 0.20 | 0.17-0.23 | Adenocarcinoma |
| SKOV3 (NCI) | 0.18 | 0.10-0.31 | Papillary serous adenocarcinoma |
| SKOV3 | 0.29 | 0.25-0.33 | Papillary serous adenocarcinoma |
| UCI101 | 7.71 | 1-82 | Papillary serous adenocarcinoma |

### Cell Culture

222, A364, A547, A2780, AD10, CaOV3, CP70, IGROV1 (NCI), OVCAR-3 (NCI), OVCAR-3, OVCAR-4 (NCI), OVCAR-420, OVCAR-429, OVCAR-432, OVCAR-8 (NCI), OVCAR-8/ADR (NCI), SK-OV-3 (NCI), SK-OV-3, UCI101, and UCI107 cell lines were maintained in RPMI 1640 (Lonza) containing 5% fetal bovine serum and 2 mmol/L L-glutamine. All cell lines were tested for *Mycoplasma* using the MycoAlert assay (Lonza) at the commencement of this study and found to be negative. In addition, DNA fingerprints were obtained for all cell lines (Supplementary Table S1)[6] using the AmpFlSTR Identifiler PCR Amplification Kit (Applied Biosystems) according to the manufacturer's protocol. Genomically heterogeneous cell lines were defined by the presence of more than two alleles at ≥3 of the 16 markers/loci and eliminated from the study. By that criterion, CP70 and UCI107 were eliminated from the study. Table 1 shows the final list of cell lines used in the study. Their sources and characteristics have been described previously (2–4).

### Detection of *ASNS* mRNA

Transcript levels were measured in the 13 non-NCI-60 cell lines listed in Table 1 using the Affymetrix HG-U133 Plus 2.0 array according to previously reported methods (5). The data were GCRMA normalized using BRB Array Tools 3.5.0 developed by R. Simon and A.P. Lam.[7]

*ASNS* mRNA levels were additionally assayed in all 19 cell lines listed in Table 1 using the Quantigene Branched-DNA RNA Assay (probe set nucleotides 670-1,321, which recognizes all three *ASNS* transcript variants) according to the manufacturer's protocol (Panomics) as reported previously (2). *ASNS* levels were normalized to *β-actin* (*ACTB*; probe set nucleotides 48-780) levels within each sample.

### MTS Proliferation Assay

Cell proliferation was assessed using CellTiter 96 $AQ_{ueous}$ One Solution (MTS; Promega), and the L-ASP $EC_{50}$ for each cell line was calculated using GraphPad Prism 5.01 (GraphPad Software) as described previously (2).

### Detection of ASNS Protein

ASNS protein levels were determined using an electrochemiluminescent immunoassay[8] developed for the SECTOR Imager 2400 (Meso Scale Discovery). Briefly, cells were lysed with CellLytic M Lysis Reagent containing protease inhibitor cocktail (Sigma). Total protein was quantitated using the BCA Protein Assay Kit (Pierce), and 5 μg of each lysate was loaded into an avidin-coated plate, where ASNS was captured using a biotinylated anti-ASNS antibody and quantitated using a SULFO-TAG-labeled anti-ASNS antibody. Purified, recombinant human ASNS protein was used to generate standard curves, to which $1 / y^2$ weighting was applied to determine absolute ASNS protein level for each sample.

### Correlative Analysis

Pearson's correlation coefficients were computed for the relationship between $-\log_{10}$ (L-ASP $EC_{50}$) and $\log_2$(ASNS expression) using GraphPad Prism 5.01, including *P* values for the one-tailed test of significance, because we expected the correlation to be negative based on previous results (2, 6, 7).

[6] Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).
[7] http://linus.nci.nih.gov/BRB-ArrayTools.html
[8] Gunsior et al., in preparation.

## Results

### DNA Fingerprinting of the Cell Lines

All of the cell lines were microsatellite fingerprinted using the AmpFlSTR Identifiler PCR Amplification Kit. The results in Supplementary Table S1 show similar (although not identical) fingerprints for the two versions of OVCAR-3 included in the study and the same for the two versions of SK-OV-3. Technical repeats of the fingerprinting process indicated an assay variability of about one 4-bp repeat. A difference greater than one repeat was observed for just

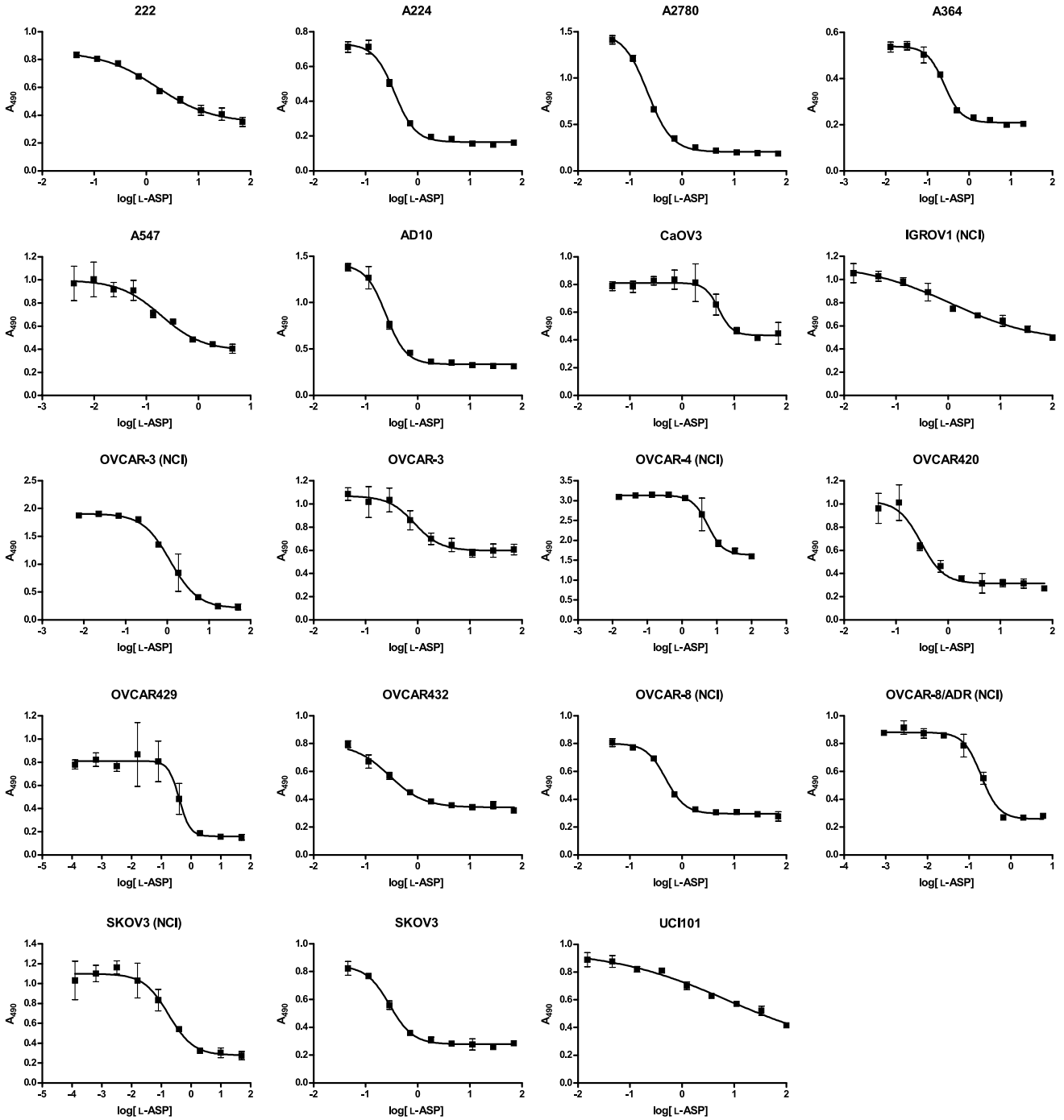


**Figure 1.** L-ASP concentration-activity curves determined by MTS assay in ovarian cancer cell lines. Nineteen indicated cell lines were seeded in 96-well plates and incubated for 48 h, treated with a range of L-ASP concentrations for 48 h, and finally assayed with MTS. Note that the axis scales differ from cell line to cell line.

1 of the 32 tested markers in OVCAR-3 and at 5 of the 32 tested markers in SK-OV-3. The data in Supplementary Table S1 therefore indicate that the two versions of each line represent the same cell line, although we cannot rule out some degree of divergence during passage. The profiles in Supplementary Table S1 for all cell lines used are available for future reference and standardization.

### L-ASP Exhibits a Wide Range of Activity in Ovarian Cancer Cell Lines

We used the MTS assay to measure L-ASP activity in 19 ovarian cancer cell lines, including 6 NCI-60 and 13 non-NCI-60 lines. The resulting L-ASP $EC_{50}$ values spanned a 43-fold range from 0.18 to 7.71 units/mL (Fig. 1; Table 1). $EC_{50}$ values for the NCI-60 and non-NCI versions of the OVCAR-3 cell line were roughly the same (1.2 and 0.9 units/mL), and this was also the case for the SK-OV-3 cell line (0.2 and 0.3 units/mL).

### L-ASP Activity Is Weakly Correlated with *ASNS* mRNA Expression

We reported previously a correlation between L-ASP activity and *ASNS* gene expression in the ovarian cancer cell lines that comprise the NCI-60 ovarian subpanel, and *ASNS* RNA interference showed that the relationship is causal (1). In the current study, however, microarray analysis of 13 non-NCI ovarian cell lines yielded a weak, statistically non-significant L-ASP/*ASNS* Pearson's correlation of $r$ = -0.21 (Fig. 2A), and branched-DNA RNA analysis corroborated that finding, also yielding $r$ = -0.21 (the 13 data points are represented in Fig. 2B). The branched-DNA assay exhibited a much larger dynamic range than the microarray, but both data sets were strongly correlated with each other ($r$ = 0.74; $P$ = 0.0037; Supplementary Fig. S1). Inclusion of data from 6 NCI-60 ovarian cell lines (19 total cell lines) significantly improved the branched-DNA correlation to $r$ = -0.39 ($P$ = 0.047; Fig. 2B).

### L-ASP Activity Is Strongly Correlated with ASNS Protein Expression

ASNS protein levels for the 19 ovarian cell lines were determined by immunoassay and strongly correlated with L-ASP activity ($r$ = -0.65, $P$ = 0.0014; Fig. 2C). Protein expression data from the 13 non-NCI cell lines alone yielded a Pearson's correlation of $r$ = -0.49 ($P$ = 0.045; the 13 data points are represented in Fig. 2C). Analyses of the relationship between protein and mRNA expression of ASNS (immunoassay versus microarray and immunoassay versus b-DNA) yielded strong Pearson's correlations of 0.65 ($P$ = 0.016) and 0.75 ($P$ = 0.0002), respectively (Supplementary Figs. S2 and S3). Although mRNA and protein were strongly correlated, ASNS protein expression measured by immunoassay was the strongest predictor of L-ASP activity in ovarian cancer cell lines.

## Discussion

Based on strong negative correlation of L-ASP activity with baseline *ASNS* gene expression in ovarian cancer cell lines of the NCI-60 (6, 7), we previously used RNA interference to show that L-ASP activity is causally related to ASNS expression (2). Here, we describe studies in an expanded set of ovarian cell lines that exhibited a wide range of sensitivity to L-ASP (Fig. 1; Table 1). The shallow Hill Slopes observed in the 222, IGROV1, and UCI101 cell lines (Fig. 1) suggested that additional factors may be involved in the response to L-ASP, but we nevertheless sought to determine whether ASNS expression alone could serve as a biomarker of L-ASP activity in this diverse collection of ovarian cancer cell lines.

We used three different assays to assess ASNS expression: microarray, branched-DNA RNA assay, and electrochemiluminescent immunoassay. Contrary to some observations (2, 7–9) but consistent with others (10), microarray and branched-DNA analyses resulted in weak L-ASP/*ASNS* mRNA correlations in the 19 cell lines studied (Fig. 2A and B). Because our previous studies indicated a strong negative L-ASP/*ASNS* mRNA correlation in the NCI-60 ovarian subset using multiple microarray platforms, the absence of strong correlations here is attributable to the non-NCI-60 lines. One sufficient explanation is that the previously observed correlation was statistical coincidence in the first place. It was based on 7 lines, one of which (OVCAR-8/ADR) was a drug-resistant version of another (OVCAR-8). Hence, there were only 6 independent lines. As stated previously, the negative correlation represented a trend, but it was not statistically significant after Bonferroni correction for the multiple tissue of origin subsets in the NCI-60 panel.

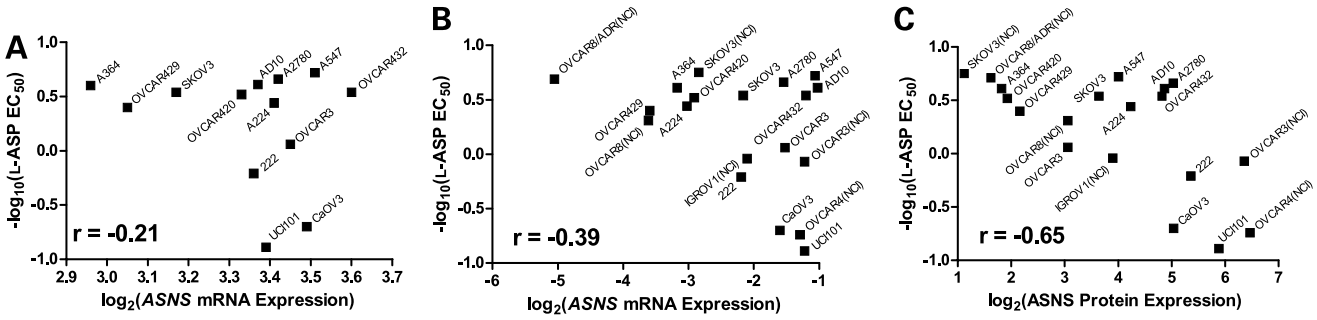


**Figure 2.** Correlation of L-ASP activity with ASNS expression. *ASNS* mRNA expression was determined using Affymetrix U133 Plus 2.0 microarrays (**A**) and a branched-DNA RNA assay (**B**). ASNS protein expression was determined using a Meso Scale Discovery electrochemiluminescent immunoassay (**C**).

Another possible explanation for the difference in correlation between the NCI-60 and non-NCI-60 cell lines is passage number, which was as high as 170 for non-NCI-60 cell lines but <30 for the NCI-60 lines. Because the L-ASP/*ASNS* correlation is stronger for NCI-60 lines, which may somewhat more closely reflect the tumors from which they were derived, it is tempting to speculate that even stronger L-ASP/*ASNS* correlations would be obtained from primary ovarian cancer cells. Studies of primary acute lymphoblastic leukemias, however, have shown poor L-ASP/*ASNS* correlations (8, 11), yet L-ASP is an approved chemotherapy for acute lymphoblastic leukemia. Taken together, the cumulative evidence suggests that *ASNS* mRNA is not a robust biomarker of L-ASP activity. The shapes of the L-ASP versus *ASNS* expression plots, nevertheless, suggest a stronger trend than the Pearson correlations indicate. Figure 2A and B indicates a strong ''7'' shape, implying that (*a*) an upper limit of *ASNS* detection has been reached; (*b*) above some threshold level, *ASNS* expression is no longer the limiting factor that determines sensitivity to L-ASP (other factors are involved); and/or (*c*) certain cell types are outliers. To refute the first hypothesis, in other studies using the same branched-DNA RNA assay, we have measured *ASNS* mRNA levels 6.5-fold greater than the highest value measured in this study, suggesting that the data presented here are indeed below the threshold of the assay. The second hypothesis, on the other hand, is supported by the observation that high *ASNS* cell lines (data points on the vertical arm of the ''7'') do not necessarily express high ASNS protein levels (Supplementary Figs. S2 and S3). That is, above a threshold *ASNS* mRNA level, ASNS protein expression may be the limiting factor that determines sensitivity to L-ASP as suggested by Fig. 2C. There may also be additional limiting factors that have not yet been determined. The third hypothesis may also be true; certain cell types may indeed be outliers.

We next determined the L-ASP/ASNS correlation at the protein level. Because ASNS protein is responsible for the synthesis of asparagine, one would expect ASNS protein expression to be more directly related to L-ASP activity than is *ASNS* mRNA expression. We therefore expected the protein level L-ASP/ASNS correlation to be stronger than the mRNA level correlation and that was indeed the case. ASNS determination by immunoassay yielded an L-ASP/ASNS Pearson correlation of $r = -0.65$ ($P = 0.0014$, one-tailed; Fig. 2C). Because the L-ASP/ASNS correlation was already the focus of attention based on our prior results, no multiple comparisons correction was necessary. Hence, cells that express low ASNS protein levels are more sensitive to L-ASP treatment probably because they produce less asparagine and are therefore more dependent on extracellular asparagine to meet metabolic demands. It is worth noting that L-ASP activity was measured using an MTS assay, which reflects cellular metabolic activity and, for L-ASP, does reflect cell death as measured by trypan blue exclusion (data not shown). Also note that a strong correlation suggests predictive ability, not causality. We previously showed causality using small interfering RNA targeted to *ASNS* (2).

Given that *in vivo* correlations are likely to be weaker than those observed *in vitro*, it is reasonable to ask whether the $r = -0.65$ correlation is strong enough to warrant further research on ASNS as a biomarker for therapy of ovarian cancers with L-ASP. Consider the list of currently Food and Drug Administration-approved, clinical biomarkers.[9] As one example, epidermal growth factor receptor is a valid biomarker of erlotinib (NSC 718781) activity in lung cancer (12, 13), yet mining of the NCI-60 microarray data from the Affymetrix U133 platform yielded an erlotinib/*epidermal growth factor receptor* Pearson's correlation of $r = 0.58$ in 6 of the lung cancer cell lines from the NCI-60.[10] Epidermal growth factor receptor is also a valid biomarker of gefitinib (NSC 715055) activity in lung and colorectal cancers, and we computed gefitinib/*epidermal growth factor receptor* correlations of 0.57 and 0.37 in the lung and colon NCI-60 subsets, respectively.[10] Bearing in mind that the L-ASP/*ASNS* mRNA correlation in the ovarian subset is $r = -0.86$ (2) and that we found the L-ASP/ASNS protein correlation to be $r = -0.65$, we believe this report provides rationale for evaluation of ASNS protein expression as a predictive biomarker of clinical L-ASP activity.

In conclusion, we have shown that ASNS protein expression measured by immunoassay strongly predicts L-ASP activity in ovarian cancer cell lines. These findings provide rationale for clinical evaluation of ASNS protein as a predictive biomarker of L-ASP activity in ovarian cancer.

## Disclosure of Potential Conflicts of Interest

P.L. Lorenzi and J.N. Weinstein: ownership interest in NIH patent based on previously published work. No other potential conflicts of interest were disclosed.

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