THE UNIVERSITY OF TEXAS
MD Anderson Cancer Center

Development of a Robust Nanoimmunoassay and Immunohistochemical Assay for ASNS

Philip L. Lorenzi[1], Lindsey E. Heathcock[2], Aditya Raghunathan[2], Kenneth Aldape[2], and John N. Weinstein[1]

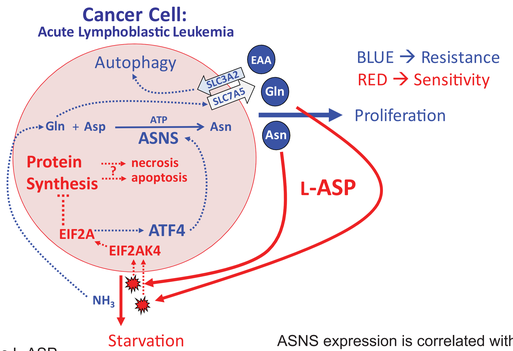
Departments of [1]Bioinformatics and Computational Biology and [2]Pathology, MD Anderson Cancer Center, Houston, TX

Abstract

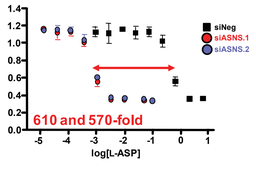
The enzyme-drug L-asparaginase (L-ASP) has been used for four decades to treat acute lymphoblastic leukemia. However, its unique mechanism of action is still poorly understood, and its clinical efficacy has proven unpredictable. Those problems have prompted a continuing search for biomarkers that predict L-ASP response. We previously found that the expression of asparagine synthetase (ASNS) is strongly negatively correlated with L-ASP anticancer activity in ovarian cancer cell lines, suggesting that L-ASP might be effective against a low-ASNS subset of ovarian cancers if salient characteristics of the cell lines reflect clinical ovarian tumors. However, quantitatively robust, single-antibody assays for ASNS expression have been absent from the literature. We therefore used a capillary-based isoelectric focusing (IEF) platform (the NanoPro 1000) to screen twelve ASNS antibodies for their specificity and sensitivity. Only two antibodies exhibited completely on-target activity (as shown by signal ablation by ASNS siRNA) and sufficient sensitivity. The on-target activity corresponded to a single band on Western blot and a single peak on the NanoPro 1000, suggesting the existence of just one ASNS protein isoform. Optimized, final NanoPro assay conditions yielded less than 8% CV, a 160-fold dynamic range, and Z′-factor of 0.82, indicating a robust assay that is amenable to high-throughput screening. We next used the best ASNS antibody to develop an immunohistochemistry (IHC) assay for ASNS. As with the NanoPro assay, optimized IHC conditions yielded a large dynamic range of staining intensity, and staining was completely ablated by ASNS siRNA. To test the hypothesis that subsets of various cancer types express very low levels of ASNS, we have initiated ASNS IHC of more than 20 tissue arrays representing a wide variety of cancer types. Using a 3-point scoring system (0 = negative, 1 = low, 2 = high), among the tumor samples assayed, 90/136 (66%) of bladder cancer, 63/133 (47%) of bone cancer, 32/149 (22%) of breast cancer, 29/115 (25%) of brain cancer, 51/168 (30%) of colon cancer, 2/85 (2%) of endocrine system cancer, 23/99 (23%) of liver cancer, 7/64 (11%) of head and neck cancer, 7/136 (5%) of lung cancer, 13/53 (25%) of lymphoma, 1/25 (4%) of bone marrow lymphoma, 2/35 (6%) of lymphoma from spleen, 9/109 (8%) of melanoma, 81/396 (21%) of ovarian cancer, 3/29 (10%) of uterine cancer, 27/73 (37%) of pancreatic cancer, 5/119 (4%) of prostate cancer, 10/125 (8%) of renal cancer, 25/138 (18%) of testicular cancer, and 8/39 (21%) of thyroid cancer were ASNS-negative (score = 0), suggesting that a subset of each cancer type may be sensitive to the drug L-asparaginase. Efforts are underway to apply the NanoPro assay to the NCI-60 cell line panel and to continue performing ASNS IHC to survey tissue arrays for ASNS expression.

Background

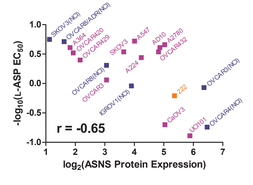
Rationale for L-ASP Therapy



ASNS knockdown potentiates L-ASP activity in ovarian cancer cell lines



ASNS expression is correlated with L-ASP activity in ovarian cancer lines



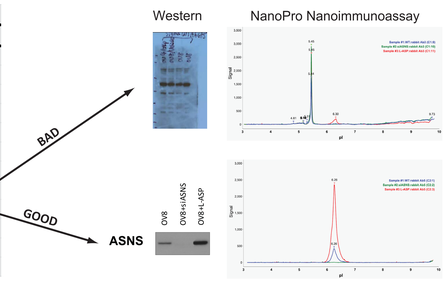
Methods

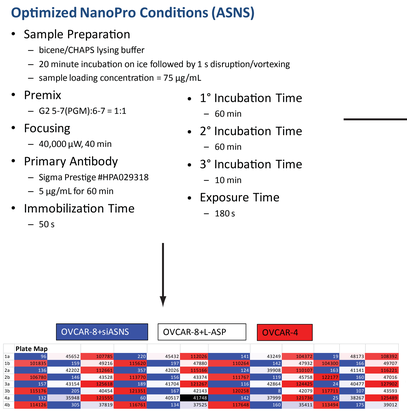
1. **Nanoimmunoassay Development: STEP 1 - Model Selection.** Based on our previous work, we chose the OVCAR-8 and OVCAR-4 cell lines as models for ASNS assay development; the former expresses low/medium levels of ASNS, and the latter expresses high levels of ASNS.
2. **Nanoimmunoassay Development: STEP 2 - Antibody Screen.** OVCAR-8 and OVCAR-4 cells treated with *ASNS* siRNA and/or L-ASP were lysed in bicene/CHAPS buffer (Protein Simple) for 20 min on ice and used to screen 12 ASNS antibodies (**Table 1**) by Western blot and NanoPro 1000 assay (Protein Simple). NanoPro assays were conducted using 3-10 premix, isoelectric focusing at 30,000 uW for 40 min, a UV immobilization time of 80 s, primary and secondary antibody incubations for 1 h, and tertiary antibody incubation for 10 min.
3. **Nanoimmunoassay Development: STEPS 3-6 - Optimization.** Following selection of Sigma antibody #HPA029318 as the most specific and sensitive primary antibody for ASNS, the following NanoPro assay parameters were optimized: premix and focusing conditions, UV immobilization time, lysate concentration (i.e., total protein loading), primary antibody concentration, primary antibody incubation time, and exposure.
4. **Characterization of Nanoimmunoassay Robustness.** Robustness of the optimized ASNS NanoPro assay was assessed by determining dynamic range, limit of detection, and Z'-factor for 96 samples divided equally among the following three types of control lysates: OVCAR-8 + siASNS (negative control), OVCAR-8 + L-ASP (positive control), and OVCAR-4 (positive control). Since the OVCAR-4 lysates yielded greater ASNS signal intensity than did the OVCAR-8 + L-ASP lysates, the former was chosen as the positive control for subsequent analyses.
5. **Immunohistochemistry.** OVCAR-8 cells were treated with *ASNS* siRNA or L-ASP, harvested and fixed in 10% formalin, paraffinized, and sectioned onto slides. Staining conditions were optimized for the Sigma HPA029318 antibody. Tissue microarrays (US Biomax) were deparaffinized in methylcyclohexane and rehydrated in decreasing concentrations of ethanol:water. After antigen retrieval (35 min at 99°C in sodium citrate buffer, pH 7.3), slides were cooled for 20 min at room temperature and rinsed in 1x DPBS. Endogenous peroxidase was quenched with 3% hydrogen peroxide for 10 minutes, then rinsed in 1x DPBS. Slides were incubated with a 1:50 dilution of ASNS antibody (Sigma #HPA029318) for 30 minutes. Slides were washed in 1x DPBS. Anti-rabbit conjugated to peroxidase-labeled polymer (Dako EnVision™+ Dual Link System-HRP) was used to incubate samples for 30 minutes. Slides were rinsed with 1x DPBS and incubated with 3,3'diaminobenzidine (DAB). Slides were then rinsed in water, and Mayer's hematoxylin was used for counterstaining. Slides were again rinsed in water, and the tissues were dehydrated in increasing concentrations of ethanol and methylcyclohexane and then coverslipped in permanent mounting medium (Pertex).

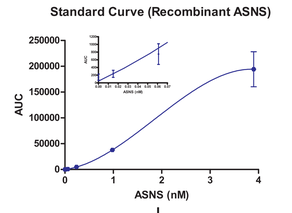
Results

Table 1: ASNS Antibodies

Vendor	Catalog #	Species
(custom)	custom (KIAH)	rabbit polyclonal
(custom)	custom (TDPS)	rabbit polyclonal
BD Biosciences	63797 (custom)	mouse monoclonal
BD Biosciences	63798 (custom)	mouse monoclonal
Novus	H00000440-B01P	mouse polyclonal
Novus	H00000440-D01P	rabbit polyclonal
Protein Tech	14681-1-AP	rabbit polyclonal
Sigma	A6485	rabbit polyclonal
Sigma	HPA029318	rabbit polyclonal
Sigma	A6610	rabbit polyclonal
Sigma	WH0000440M1	mouse monoclonal
US Biological	A3825-12	rabbit monoclonal







ASNS NanoPro Assay Robustness

Limit of Detection (LOD): 15 pM
Limit of Quantitation (LOQ): 54 pM
Dynamic Range (LOQ to linear max): 54 pM to 3 nM (60-fold)
Z'-factor: $1-\frac{3(SD_{neg}+SD_{pos})}{\mu_{pos}-\mu_{neg}} = 0.82$

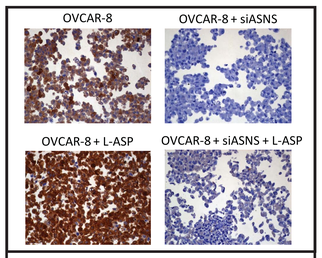


Figure 1. ASNS IHC of siRNA- and L-ASP-Treated OVCAR-8 Cells. WT cells express low/medium levels of ASNS, L-ASP induces ASNS up-regulation, and siRNA ablates and suppresses the signal.

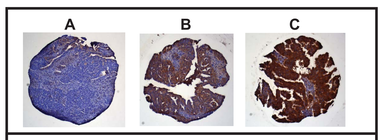
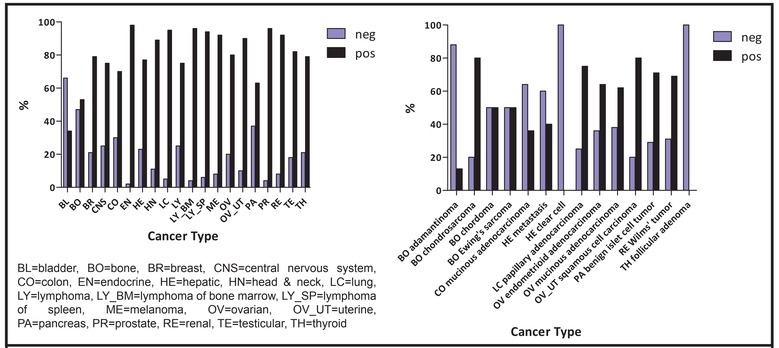


Figure 2. Representative ASNS IHC Scoring. Tissue microarrays were stained and scored using a 3-point scoring system (0=negative, 1=low, 2=high). Ovarian cancers scored as (A) 0, (B) 1, and (C) 2 are illustrated here.



BL=bladder, BO=bone, BR=breast, CNS=central nervous system, CO=colon, EN=endocrine, HE=hepatic, HN=head & neck, LC=lung, LY=lymphoma, LY_BM=lymphoma of bone marrow, LY_SP=lymphoma of spleen, ME=melanoma, OV=ovarian, OV_UT=uterine, PA=pancreas, PR=prostate, RE=renal, TE=testicular, TH=thyroid

Figure 3. ASNS IHC of Tissue Microarrays. (A) Twenty tissue microarrays representing the indicated cancers were stained and scored as in Figure 2. Blue bars indicate percentage of samples scored as 0, and black bars indicate those scored as 1-2. (B) Subsets of specific cancers that exhibited enrichment of ASNS-negative scores.

Conclusions

- A robust NanoPro assay for ASNS has been developed and is suitable for high-throughput screening
- A large percentage of bladder, bone, and pancreatic cancer types do not express ASNS and, hence, may be sensitive to L-ASP treatment

Future Directions

- Use NanoPro assay to measure ASNS expression across the NCI-60 cell line panel and assess correlation with L-ASP anticancer activity
- Use reverse-phase protein arrays (RPPA) to screen clinical samples from The Cancer Genome Atlas (TCGA) and other sources

Acknowledgements

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