Aspartoacyclase is differentially expressed in non-small cell lung cancer and associates with patient survival.

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Non-small cell lung cancer (NSCLC) is the leading cause of cancer death in the United States¹. We mined published microarray data^{2,3,4,5} to identify differentially expressed genes in NSCLC. We found that the gene encoding aspartoacyclase (ASPA) was among the genes whose expression was most quantitatively different in tumors from patients with NSCLC as compared to the lung. ASPA expression was significantly decreased in NSCLC tumors as compared to the lung, and lower expression of ASPA in patient tumors was significantly associated with worse overall survival. ASPA may be important for initiation or progression of non-small cell lung cancer in humans.

Keywords: ASPA, aspartoacyclase, NSCLC, non-small cell lung cancer, systems biology of NSCLC, targeted therapeutics in NSCLC.

1 In 2016, lung cancer resulted in the death of 158,000 Americans; 81% of all patients
2 diagnosed with lung cancer will expire within 5 years⁶. Non-small cell lung cancer (NSCLC) is
3 the most common type of lung cancer, diagnosed in 84% of patients with lung cancer, and 76%
4 of all patients with NSCLC will expire within 5 years⁶. The rational development of targeted
5 therapeutics to treat patients with NSCLC can be supported by an enhanced understanding of
6 fundamental transcriptional features of NSCLC tumors. To discover genes associated with
7 NSCLC tumors in an unbiased fashion and at the systems-level, we mined independently
8 published microarray data^{2,3,4,5} to compare global gene expression profiles of NSCLC tumors to
9 that of the normal lung. We found recurrent and significant differential expression of
10 aspartoacyclase, encoded by the ASPA gene, in adenocarcinoma tumors from patients with
11 NSCLC, suggesting ASPA may be important for NSCLC tumor initiation or progression.
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17 **Methods**

18 We utilized microarray datasets GSE74706², GSE43458³, GSE33532⁴ and GSE62113⁵
19 for this differential gene expression analysis of NSCLC tumors in conjunction with GEO2R.
20 GSE74706 was generated using Agilent-026652 Whole Human Genome Microarray 4x44K v2
21 technology; for this analysis, we used $n=18$ control lung tissue and $n=10$ NSCLC tumors, and the
22 analysis was performed using platform GPL13497. GSE43458 was generated using Affymetrix
23 Human Gene 1.0 ST Array technology; for this analysis, we used with $n=30$ control lung tissue
24 and $n=80$ NSCLC tumors, and the analysis was performed using platform GPL6244. GSE33532
25 was generated using Affymetrix Human Genome U133 Plus 2.0 Array technology; for this
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analysis, we used $n=20$ control lung tissue and $n=10$ NSCLC tumors, and the analysis was performed using platform GPL570. GSE62113 was generated using Illumina HumanHT-12 WG-DASL V4.0 R2 expression beadchip technology; for this analysis, we used $n=9$ control lung tissue and $n=7$ NSCLC tumors, and the analysis was performed using platform GPL14951. All tumors utilized for differential gene expression analysis here were of the adenocarcinoma type.

The Benjamini and Hochberg method of p -value adjustment was used for ranking of differential expression but raw p -values were used to assess statistical significance of global differential expression. Log-transformation of data was auto-detected, and the NCBI generated category of platform annotation was used. A statistical test was performed to evaluate whether ASPA expression was significantly between normal lung tissue and NSCLC tumors using a two-tailed, unpaired t-test with Welch's correction. We used PRISM for all statistical analyses of differential gene expression in NSCLC tumors (Version 8.4.0)(455). For Kaplan-Meier survival analysis, we used the Kaplan-Meier plotter online tool⁷ for correlation of ASPA mRNA expression levels with overall survival in non-small cell lung cancer in $n=1925$ patients.

Results

We harnessed the power of multiple, independently published microarray datasets^{2,3,4,5} to discover in an unbiased fashion and at the transcriptome-level the most striking gene expression features of NSCLC tumors.

ASPA is differentially expressed in non-small cell lung cancers.

We found significant differential expression of the gene encoding aspartoacyclase, ASPA, in NSCLC tumors when compared to the lung² (Table 1). When sorting each of the transcripts measured based on significance of difference in expression of ASPA between NSCLC tumors and the normal lung, ASPA ranked 22 out of 34183 total transcripts (Table 1). Differential expression of ASPA in NSCLC tumors was statistically significant (Table 1; $p=3.89E-14$).

Analysis of a second microarray dataset³ again revealed significant differential expression of ASPA in NSCLC tumors of the adenocarcinoma type (Table 2). When sorting each of the transcripts measured based on significance of difference in expression of ASPA between NSCLC tumors and the normal lung, ASPA ranked 154 out of 33252 total transcripts (Table 2). Differential expression of ASPA in NSCLC tumors was statistically significant (Table 2; $p=2.22E-22$).

We queried a third microarray dataset⁴, again finding significant differential expression of ASPA in non-small cell lung cancers of the adenocarcinoma type when compared to the normal lung. When sorting each of the transcripts measured based on significance of difference in expression of ASPA between NSCLC tumors and the normal lung, in this dataset, ASPA ranked 16 out of 25906 total transcripts (Table 3). Differential expression of ASPA in NSCLC tumors was statistically significant (Table 3; $p=1.01E-17$).

In a fourth dataset⁵, we again observed significant differential expression of ASPA in

human NSCLC tumors when compared to normal lung. When sorting each gene by significance of difference in expression between NSCLC tumors and the normal lung, ASPA ranked 8 out of 29376 total transcripts (Table 4). Differential expression of ASPA in this fourth dataset was again statistically significant (Table 4; $p=2.61E-09$).

ASPA is expressed at significantly lower levels in NSCLC tumors as compared to the lung.

We obtained exact mRNA levels for ASPA from NSCLC tumors and from the lung to directly compare ASPA expression between tumor and control lung tissue and assess for statistical significance. ASPA was expressed at significantly lower levels in NSCLC tumors as compared to the normal lung in each of the datasets queried (Figure 1: $p<0.0001$; Figure 2: $p<0.0001$; Figure 3: $p<0.0001$; and Figure 4: $p<0.0001$). We calculated a fold change of 0.7512 ± 0.1054 (Table 2), 0.6246 ± 0.0566 (Table 3) and 0.7567 ± 0.0486 (Table 4) in ASPA expression when comparing NSCLC tumors to the lung.

ASPA expression in NSCLC tumors correlates with overall survival.

We performed Kaplan-Meier survival analysis using ASPA mRNA expression in NSCLC tumors coupled with paired overall survival data from each patient to determine whether ASPA tumor expression was correlated with patient outcomes in NSCLC. We found that patients whose tumors expressed lower levels of ASPA possessed significantly shorter overall survival than patients with high tumor expression of ASPA (Figure 5). Median overall survival of patients in the low expression cohort was 56.7 months, while median OS in patients in the high ASPA expression cohort was 107 months (Table 5); this difference in median OS based on

ASPA tumor expression in NSCLC was statistically significant (Figure 5; logrank p -value: 2.3×10^{-10} ; hazard ratio: 0.6 (0.52-0.71)).

Thus, blind comparative transcriptome analysis of non-small cell lung cancers revealed differential expression of ASPA as among the most significant transcriptional features of NSCLC tumors, and ASPA expression was significantly correlated with patient outcomes, as patients with lower tumor expression of ASPA possessed significantly worse overall survival.

Discussion

Aspartoacyclase is a 58 kilodalton enzyme with most prominent expression in the white matter of the brain, which functions in hydrolysis of N-acetylaspartate (NAA) into L-aspartate and acetate⁸, whose mutation results in Canavan Disease, a neurodegenerative disease resulting in demyelination of the central nervous system and brain vacuolization, and fatality in childhood^{9,10}. N-acetylaspartic acid is present in high amounts in the brain, including specifically in oligodendrocytes, and fibroblasts from patients with Canavan Disease display a marked deficiency in aspartoacyclase catalytic activity. Patients with Canavan Disease feature spongy degeneration in the cerebrum¹¹, and in a mouse model of CD, total acetate levels in the brain are reduced and the synthesis of six different types of myelin-associated lipids is reduced, suggesting that in the central nervous system¹², NAA functions to supply acetyl moieties for the synthesis of lipids to support myelination⁸. In addition to the brain, ASPA gene expression can also be detected in the kidneys, lung and in the liver¹³. In macrophages from Gata6-deficient

mice, ASPA is among the genes most significantly down-regulated, suggesting that Gata6 either

1 directly or indirectly supports the expression of ASPA¹⁴. In tissue macrophages, ASPA
2 expression was highest in peritoneal F4/80 macrophages with modest expression also observed
3 in macrophages from the red pulp of the spleen¹⁴. Another metabolite whose levels are linked to
4 ASPA is acetyl-CoA; in brown adipocytes, depletion of aspartoacyclase results in a deficit of
5 acetyl-CoA and interestingly, a global decrease in histone H3 acetylation, including H3K27ac
6 and H3K9ac¹⁵. ASPA depletion results in loss of the brown adipocyte phenotype as assessed by
7 decreased lipolysis and thermogenic gene expression, together linking NAA and the ASPA
8 metabolic pathway to epigenetic control of cell-type specific differentiation¹⁵. There is limited
9 information describing deregulation of ASPA or the NAA metabolite in human cancer. In
10 oligodendroglioma, one study reported decreased expression of ASPA in high-grade
11 oligodendrogliomas, with low tumor expression of ASPA correlating with increased mortality¹⁶.
12 A study performing metabolic profiling in non-small cell lung cancer cell lines found increased
13 levels of NAA in lung cancer cells which was undetectable in normal lung epithelium, with
14 validation in some NSCLC patient tumors¹⁷. Expression of the NAA synthetase, NAT8L, was
15 significantly increased in 40% of 577 adenocarcinoma and squamous cell carcinoma tumor
16 tissues, and NAA blood levels were elevated in 46% of 13 NSCLC patients tested¹⁷.

23 We discovered significantly decreased and differential expression of aspartoacyclase in
24 adenocarcinoma tumors from patients with NSCLC, and found that ASPA tumor expression was
25 correlated with overall survival in patients with NSCLC . ASPA may be relevant to the biology
26 of NSCLC, the most common type of the most lethal cancer in the United States and worldwide.

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Rank	ID	p-value	t	B	Gene
22	A_23_P164436	3.89E-14	-1.36E+01	22.25157	ASPA

Table 1: ASPA is differentially expressed in NSCLC tumors.

The rank of differential expression relative all transcripts measured, probe ID, *p*-value of global differential expression, t, a moderated t-statistic, B, the log-odds of differential expression between the groups compared, and gene are listed in this chart.

Rank	ID	p-value	t	B	FC	Gene	Gene name
154	8003814	2.22E-22	-12.259108	40.369827	0.7512 ± 0.1054	ASPA	aspartoacylase

Table 2: ASPA is differentially expressed in NSCLC tumors.

The rank of differential expression relative all transcripts measured, probe ID, *p*-value of global differential expression, t, a moderated t-statistic, B, the log-odds of differential expression between the groups compared, fold change of ASPA expression in NSCLC tumors as compared to the lung, gene and gene name are listed in this chart.

Rank	ID	p-value	t	B	FC	Gene	Gene name
16	206030_at	1.01E-17	-17.606794	30.3636041	0.6246 ± 0.0566	ASPA	aspartoacylase

Table 3: ASPA is differentially expressed in NSCLC tumors.

The rank of differential expression relative all transcripts measured, probe ID, *p*-value of global differential expression, *t*, a moderated *t*-statistic, *B*, the log-odds of differential expression between the groups compared, fold change of ASPA expression in NSCLC tumors as compared to the lung, gene and gene name are listed in this chart.

Rank	ID	p-value	t	B	FC	Gene
8	ILMN_1692824	2.61E-09	-12.1182398	11.238104	0.7567 ± 0.0486	ASPA

Table 4: ASPA is differentially expressed in NSCLC tumors.

The rank of differential expression relative all transcripts measured, probe ID, *p*-value of global differential expression, *t*, a moderated *t*-statistic, *B*, the log-odds of differential expression between the groups compared, fold change of ASPA expression in NSCLC tumors as compared to the lung, and gene are listed in this chart.

Figure 1: ASPA is expressed at significantly lower levels in NSCLC tumors when compared to the lung.

The mRNA expression level of ASPA is graphically represented in the lung (left) and in NSCLC tumors of the adenocarcinoma type (right) with mean mRNA expression values marked and the result of a statistical test evaluating significance of difference in ASPA expression between NSCLC tumors and the lung, a *p*-value, listed above.

Figure 2: ASPA is expressed at significantly lower levels in NSCLC tumors when compared to the lung.

The mRNA expression level of ASPA is graphically represented in the lung (left) and in NSCLC tumors of the adenocarcinoma type (right) with mean mRNA expression values marked and the result of a statistical test evaluating significance of difference in ASPA expression between NSCLC tumors and the lung, a *p*-value, listed above.

Figure 3: ASPA is expressed at significantly lower levels in NSCLC tumors when compared to the lung.

The mRNA expression level of ASPA is graphically represented in the lung (left) and in NSCLC tumors of the adenocarcinoma type (right) with mean mRNA expression values marked and the result of a statistical test evaluating significance of difference in ASPA expression between NSCLC tumors and the lung, a *p*-value, listed above.

Figure 4: ASPA is expressed at significantly lower levels in NSCLC tumors when compared to the lung.

The mRNA expression level of ASPA is graphically represented in the lung (left) and in NSCLC tumors of the adenocarcinoma type (right) with mean mRNA expression values marked and the result of a statistical test evaluating significance of difference in ASPA expression between NSCLC tumors and the lung, a *p*-value, listed above.

Figure 5: ASPA expression in NSCLC tumors significantly correlates with overall survival.

Depicted in this Kaplan-Meier plot is the probability of overall survival for $n=1925$ total patients stratified into two groups, based on low or high expression of ASPA in patient tumors. The log rank p -value denoting statistical significance of difference in overall survival when comparing the two groups, as well as hazard ratio for this comparison is listed above. Listed below is the number of patients at risk (number of patients alive) per interval, after stratification based on ASPA expression; in the first interval, number at risk is number of patients alive; in each subsequent interval, number at risk is the number at risk less those who have expired or are censored.

Low expression cohort (months)	High expression cohort (months)
56.7	107

Table 5: ASPA expression in NSCLC tumors significantly correlates with patient survival.

The median overall survival of NSCLC patients based on stratification into low or high expression of ASPA in tumors is listed in this chart.