Cancer-specific production of N-acetylaspartate via NAT8L overexpression in non-small cell lung cancer and its potential as a circulating biomarker

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Abstract

In order to identify new cancer-associated metabolites that may be useful for early detection of lung cancer, we performed a global metabolite profiling of a non-small cell lung cancer (NSCLC) line and immortalized normal lung epithelial cells from the same patient. Among several metabolites with significant cancer/normal differences, we identified a unique metabolic compound, N-acetylaspartate (NAA) in cancer cells — undetectable in normal lung epithelium. NAA's cancer-specific detection was validated in additional cancer and control lung cells as well as selected NSCLC patient tumors and control tissues. NAA's cancer-specificity was further supported in our analysis of NAA synthetase (gene symbol: NAT8L) gene expression levels in The Cancer Genome Atlas: elevated NAT8L expression in approximately 40% of adenocarcinoma and squamous cell carcinoma cases (N=577), with minimal expression in all non-malignant lung tissues (N=74). We then showed that NAT8L is functionally involved in NAA production of NSCLC cells through siRNA-mediated suppression of NAT8L, which caused selective reduction of intracellular and secreted NAA. Our cell culture experiments also indicated that NAA biosynthesis in NSCLC cells depends on glutamine availability. For preliminary evaluation of NAA's clinical potential as a circulating biomarker, we developed a sensitive NAA blood assay and found that NAA blood levels were elevated in 46% of NSCLC patients (N=13) in comparison with age-matched healthy controls (N=21) among individuals aged 55 years or younger. Taken together, these results indicate that NAA is produced specifically in NSCLC tumors through NAT8L overexpression and its extracellular secretion can be detected in blood.

Keywords

lung cancer; N-acetylaspartate; NAT8L; blood; biomarker

Introduction

Lung cancer is the leading cause of cancer death worldwide, leading to 1.6 million deaths every year (1). The majority of lung cancer cases are diagnosed in late stages, and early-stage detection and treatment are now known to reduce mortality rates, as recently reported for non-invasive screening with low-dose CT (LDCT) scan (2). Currently, LDCT screening is recommended only for the high-risk population of smokers over 55 years of age. This limitation is due to high false positive rates (96.4%) as well as risks of radiation exposure in LDCT. For better screening methods, recent studies have attempted to use diverse biological fluid samples from patients for finding new lung cancer biomarkers (3-5). Unlike diagnostic biomarkers that are required to have high sensitivity (i.e. high true positive rates) for clinical application, screening biomarkers must have high specificity (i.e. low false positive rates) in order to avoid a large number of people without lung cancer from undergoing invasive or costly procedures for confirmation (6). For example, specificity should be at least 99.6% (false positive rate < 0.4%) for screening tests on ovarian cancer among postmenopausal women to be clinically beneficial (7). Among recent studies on new lung cancer biomarkers, only two small-scale studies identified blood markers showing cancer-specificity higher than 99% (8, 9).

In order to discover new biomarker molecules for detecting cancer cases with high specificity, a small group of recent biomarker discovery studies have paid special attention to finding unique metabolites (small metabolic compounds) produced at levels significantly higher in tumors and minimal in most non-malignant cells and tissues. These efforts are based on new insights revealed in distinct metabolism of cancer cells and the fact that metabolites offer more possibilities of non-invasive tumor detection — such as imaging — than DNA, RNA, or proteins. A few such studies showed promising results for gliomas (10, 11) and prostate cancers (12, 13). For lung cancer, previous metabolic profiling studies on cancer cell lines or tumors did not report new cancer-specific metabolites, presumably due to their focus on characterizing cancer-selective metabolic fluxes and pathways of common metabolites (14-22).

In this study, we used a metabolite profiling approach with special focus on finding uncommon metabolites produced by non-small cell lung cancer (NSCLC) cells, but not by most healthy or non-malignant cells. This approach allowed us to identify a unique metabolite, N-acetylaspartate (NAA). We then examined NAA's cancer-specificity and the mechanistic basis of its production in cancer cells. We also conducted proof-of-principle experiments with selected blood samples from lung cancer patients and controls as the first attempt to evaluate the feasibility of using NAA as one of the circulating biomarkers for lung cancer.

Materials and Methods

Metabolite extraction and mass spectrometry analysis of cell lines, media and tissues

All reagents were purchased from Sigma-Aldrich unless noted otherwise. All non-small cell lung cancer (NSCLC) cell lines have been authenticated, i.e. DNA fingerprinted for provenance with the Power-Plex 1.2wkit (Promega) and confirmed to be identical to the DNA fingerprint library maintained by ATCC and the Minna/Gazdar laboratory, and confirmed to be free of mycoplasma by e-Myco kit (Boca Scientific) (23). All NSCLC cell lines except HCC4017 were cultured with RPMI1640 (Invitrogen) supplemented with 5% fetal bovine serum (FBS)(Atlanta Biologicals). HCC4017 and patient-matched immortalized lung epithelial cells (HBEC30KT) were cultured in ACL4 medium (24) with 2% FBS, which was developed for the two lines to grow with reasonable rates under the same culture conditions. For isotope labeling experiments, RPMI1640 medium without glucose or glutamine (Invitrogen) was supplemented with glucose or glutamine whose carbons were either unlabeled or uniformly labeled with ^{13}C ([U-^{13}C]). From confluent cells, polar metabolites were extracted with methanol/water (1:1) (25) and derivatized with methoxyamine hydrochloride and N-methyl-N-(trimethylsilyl)trifluoroacetamide after adding $^{2}H_{27}$-myristic acid as internal standard.

Snap-frozen tissues (resected NSCLC tumors from University of Texas Southwestern Medical Center (UTSW) Tissue Resource collected under IRB approved protocols; non-malignant lung tissues of healthy individuals from Biochain) were homogenized in ice-cold methanol/phosphate buffer. After centrifugation, supernatants were collected and dried. Metabolites in cell culture media were extracted with methanol and dried (11). Tumor and media samples were then derivatized as with cells above.

For Gas Chromatography – Mass Spectrometry (GC-MS) analysis of cell, media and tumor samples, derivatized metabolites were analyzed with Agilent 7890A/5975C using can mode at m/z 50-550. GC-MS datasets were deconvoluted with AMDIS (26) and metabolites were identified with NIST Spectral Library. Quantitative comparisons in different sample sets were made through SpectConnect (27) and were manually confirmed with Agilent's ChemStation software. In this setting, the detection limit for NAA in tissues was 2 μM.

Protein extraction and Western blot

Proteins were extracted with two cycles of freeze-thaw method in phosphate-buffered saline, and Western blot was performed on 30 μg protein extracts per sample using commercial antibodies (anti-NAT8L from Abcore and anti-β-actin from EMD Millipore). See Supplementary Info for more details on protein extraction and Western blot.

NAT8L knockdown with siRNA

100,000 cells were plated per well in six-well plates and were transiently transfected with 40 nM synthetic small interfering RNA (siRNA) targeting NAT8L or scrambled negative control siRNA (OriGene) using DharmaFECT transfection reagent (ThermoFisher) according to the manufacturer's protocol. The sequences for si(NAT8L) (human) were CGGACAUCGAGCAGUACUACAUGAA (#1), GCACUCUGCAUGACUUUAAUUCUTG (#2), and GGAAUAUACAGACAGACGUAAAGTG (#3). After 48 hrs, transfected cells were collected for further analyses.

RNA-seq data analysis of TCGA data for NAT8L

RNA-seq data in gene-level normalized read counts were downloaded from The Cancer Genome Atlas (TCGA) data portal (28). The data were logarithm transformed (base 2) and were quantile-normalized with R package for the same distribution of gene expression levels between samples.

NAA analysis of blood samples

Pooled and individual healthy plasma samples were obtained from Innovative Research (IR). Patient and age-matched control plasma samples were from the University of Texas Southwestern Medical Center(UTSW) (Dallas, TX) and the Sungkyunkwan University School of Medicine (SKK) (Seoul, Korea) under IRB-approved protocols. $^2H_3$-Methylmalonic acid (D_3-MMA) was purchased from Cerilliant (Round Rock, TX). Strong anion exchange columns (SAX, 100 mg sorbent mass and 3 mL reservoir volume) were procured from Biotage USA (Charlotte, NC). All solvents including water, methanol and acetonitrile were of HPLC grade (ThermoFisher).

Blood plasma samples were initially cleaned up with pretreatment methods as modified from those developed by Simon-Manso et al. in NIST (29). An aliquot of 200 μL blood plasma sample, with 1 μg D_3-MMA added as internal standard, was centrifuged (5000g, 15 min). The supernatant was pre-treated with methanol (800 μL, vortex for 30 seconds, and two cycles of -20°C storage and vortexing). Then, the mixture was centrifuged at 19,600g for 10 min at room temperature. The supernatant was dried under vacuum, which was

reconstituted in HPLC grade 0.6 mL water, vortexed and centrifuged at 425g for 5 minutes. The sample was then loaded onto the SAX columns, which were previously conditioned with 1 mL methanol and 1 mL HPLC grade water. The columns were washed sequentially with 1 mL HPLC grade water, 1 mL acetonitrile and 0.5 mL MTBE (Methyl-*tert*-butyl-ether). The analytes were eluted with 3 mL of 3% formic acid in MTBE and was dried under vacuum. The residue was reconstituted in 25 μL ethyl acetate, derivatized with 25 μL N-*tert*-butyldimethylsilyl-N-methyltrifluoroacetamide, at 70 °C for 20 minutes.

GC-MS analysis was carried out on the same Agilent GC-MS system as above, using 2 °C/min ramping from 70 °C to 180 °C, 1°C/min upto 185°C and at 40 °C/min till 325 °C with a final hold for 15 min. N-acetylaspartate and internal standards were targeted on selected ion monitoring mode, wherein the ions targeted for derivatives were: m/z 346 (quantifier) and 287 (qualifier) for NAA; m/z 312 (quantifier) and 354 (qualifier) for $^2H_{27}$-myrisitic acid and m/z 292 (quantifier) and 189 (qualifier) for D_3-MMA. NAA concentration was estimated through fitting the NAA peak area of m/z 346 (normalized with peak area of D_3-MMA (m/z 292)) against standard curves constructed with pooled commercial plasma containing supplemented NAA at 0 to 1 μM. In order to confirm the identities of these targets in sample GC-MS data, the ratios between peak intensities of quantifier and qualifier fragments and the retention times of these peaks were compared between standards and samples. Using these methods, the detection limit for NAA in blood plasma was 38 nM.

Statistical Analysis

The data are reported as the mean ± standard error of the mean from at least three data points generated from independent treatments. Data were analyzed by a two-tailed student's *t*-test, and p values < 0.05 were considered statistically significant using Microsoft Excel.

Results

Discovery of N-acetylaspartate (NAA) in lung cancer cells

In order to expedite the discovery of candidates for cancer-specific metabolites in lung cancer, we exploited a unique system of a non-small cell lung cancer (NSCLC) cell line and a line of immortalized bronchial epithelial cells derived from the same patient, HCC4017 and HBEC30KT, for the initial discovery. After molecular characterization, we validated the selected candidate's cancer specificity in additional NSCLC cell lines and NSCLC tumors. The mechanistic basis of this cancer specificity was further investigated with NSCLC cell lines, and its clinical potential as a circulating biomarker of lung cancer was evaluated with selected blood samples from lung cancer patients (Fig. 1).

In global metabolite profiling of HCC4017 and HBEC30KT using gas chromatography – mass spectrometry (GC-MS) methods modified from our previous work (30), we identified 194 non-redundant compounds and found that 47 of them were not detected in HBEC30KT cells but only in HCC4017 cells. We were able to positively match 17 of 47 to known compounds in the NIST spectral library, but not the remaining 30 compounds due to incompleteness of this library (see Supplementary Table S1 for the full list of these 194 compounds and their fold differences in HCC4017 vs. HBEC30KT cells). The 17 known

compounds were all common metabolites that can be detected in many healthy or non-malignant cells and tissues, which was inconsistent with our objective to identify candidates of cancer-specific lung cancer markers. Thus, we focused on characterizing the identities of the 30 unidentified compounds.

While most of these 30 compounds showed mass spectra significantly different from those of the closest hits from the spectral library (see Supplementary Table S1 for the closest hits), one compound's mass spectrum was very closely matched to that of an uncommon metabolite, methylmalonylglycine (thus was tentatively named as "methylmalonylglycine-like (MMG-L)") (see Fig. 2A for MMG-L's chromatographic peak and Fig. 2B for its mass spectrum and library search result; Peak #105 in Supplementary Table S1). This similarity allowed us to determine MMG-L's exact identity, with a combination of chemical synthesis of methylmalonylglycine and high-accuracy mass spectrometry coupled with liquid chromatography, as N-acetylaspartate (NAA) (see Supplementary Info and Supplementary Figs. S1, S2, and S3 for details). This study is the first to report its detection in lung cancer cells, which is supported by recent reports of NAA detection in ovarian and prostate cancers (31, 32).

Validation of cancer specificity of NAA and its biosynthetic enzyme NAT8L in lung cancer

In order to characterize the prevalence and the dynamic range of NAA production in lung cancer cells with diverse genetic backgrounds, NAA levels were then examined in eight additional NSCLC cell lines (see Supplementary Table S2 for clinical information and common oncogenic mutations for all cell lines used in this study). All examined cancer cell lines produced NAA but HBEC30KT or another immortalized normal lung cells (HBEC34KT) did not (Fig. 3A). We then investigated NAA's potential as a secreted marker of lung tumors with culture media used in growing NSCLC cell lines. NAA levels in conditioned media from confluent cells were compared across 11 NSCLC cell lines and HBEC30KT. These lines included four from above and eight additional NSCLC lines (HCC2450, H522, H2347, H1819, H2126, H1568, H1993, and HCC1195), which allowed us to expand the range of intracellular and secreted NAA levels in NSCLC cells. The results show that intracellular NAA levels were reasonably well correlated with secreted NAA levels (R^2=0.62) (Fig. 3B), indicating that NAA is produced and secreted into extracellular space by lung cancer cells.

To evaluate the cancer selectivity of NAA in NSCLC tumors, we first analyzed NAA levels in 11 resected lung tumor samples from NSCLC patients and 5 lung tissues from non-cancer individuals with GC-MS (See Supplementary Table S3 for clinical information of these lung tissue samples). NAA was detected in 10 of 11 samples between 4.5 and 56.7 μM (12.6 ± 4.6 μM), but not in any of 5 non-malignant lung tissues (Fig. 3C). Despite the small sample sizes, this preliminary result was consistent with the cell line results above in terms of NAA's cancer-selective detection in NSCLC.

To further examine the cancer-specificity of NAA in a larger number of lung tumors, we took advantage of the RNA-seq data in TCGA (The Cancer Genome Atlas) database (28) and the known dependence of NAA biosynthesis on NAA synthetase (gene symbol: NAT8L) (33). NAT8L's gene expression levels were analyzed for 577 NSCLC tumors and

74 non-malignant lung tissues in TCGA database. Among 55 lung adenocarcinoma (AD) tumors with patient-matched non-malignant lung tissues, 24 tumors (44%) showed significant elevation of NAT8L expression above baseline (Fig. 3D: ** = greater than two-fold elevation over all non-malignant lung tissues and p<0.01:). Similar results were obtained from 16 lung squamous cell carcinoma tumors (SC) vs. patient-matched control tissues (elevated expression in 6 tumors (38%)) and all 577 NSCLC tumors (overexpression in 154 of 355 AD tumors (43%) and 89 of 222 SC tumors (40%)) (Supplementary Figs. S4 and S5). Taken together, these data suggest that NAA production in lung cancer may be not only selective but also specific to cancer cells, as compared with non-malignant lung cells.

Functional involvement of NAT8L in NAA production of lung cancer cells

Our TCGA data analysis above indicated the cancer-specific involvement of NAT8L in NAA production of lung tumors, which prompted us to examine NAT8L's functional roles in NAA biosynthesis at the cellular level. NAT8L protein expression levels in most of eight selected NSCLC cell lines were significantly higher than in HBEC30KT cells (Supplementary Fig. S6). NAT8L's functional involvement in NAA production of NSCLC cells was examined through siRNA-mediated knockdown experiments. Suppression of NAT8L with si(NAT8L)#1 led to selective reduction of intracellular NAA levels (by 72%) as compared with direct precursors of NAA synthesis (pyruvate, as a surrogate for acetyl-CoA, and aspartate)(34) and their directly-related metabolites (Fig. 4A). The si(NAT8L) #1 was chosen for this experiment because it showed the most efficient protein-level knockdown in H1299 cells (by 68%) out of three independent siRNAs for NAT8L (Fig. 4B). This dependence of NAA on NAT8L was also observed in another NSCLC cell line (HCC4017) (Supplementary Fig. S7). NAT8L suppression also caused selective decreases in extracellular NAA levels in comparison with two major nutrients in culture medium (glutamine and glucose) and two secreted metabolites (lactate and alanine) related to NAA (Fig. 4C).

Glutamine dependence of NAA production in lung cancer cells

In order to further characterize the mechanism of NAA biosynthesis in lung cancer cells, we examined which major nutrients provide carbon sources for NAA through ^{13}C-labeling experiments (35). When uniformly ^{13}C-labeled ([U-^{13}C]) glucose or glutamine were used in culture medium for H1299 lung cancer cells, acetyl group of NAA was labeled primarily with ^{13}C from glucose (as indicated by relative enrichment of "M+2" peak) while aspartate group of NAA was labeled mainly with ^{13}C from glutamine (as indicated by relative enrichment of "M+4" peak) (Fig. 5A). This result is consistent with glutamine's contribution to aspartate through glutaminolysis, commonly observed in many cancer cells including lung cancer (36).

Then, we investigated whether glucose or glutamine is required in NAA production in NSCLC cells. When glucose was removed from the culture medium, intracellular NAA levels were decreased by approximately 40%, concomitant with 30% decrease in free palmitic acid (Fig. 5B). Interestingly, NAA production was completely abolished when glutamine was removed from the medium whereas little change was observed in free palmitic acid levels. Moreover, NAA levels in selected NSCLC cell lines showed

significantly better correlation with intracellular glutamine levels (R^2=0.68) than glucose levels or NAT8L protein amounts (R^2<0.1) (Supplementary Fig. S8).

Since NAA can also be a degradation product of another unique metabolite, N-acetylaspartylglutamate (NAAG)[34], we further examined whether NAAG is present in lung cancer cells. NAAG level in HCC4017 cells was below our detection limit by LC-MS (1,000 fold less than NAA: see Supplementary Fig. S9), indicating that NAAG is not likely to be an alternative precursor for NAA in lung cancer cells. Therefore, the enzyme for producing NAA from NAAG (gene symbol: FOLH1) was not examined for its involvement in NAA metabolism of lung cancer cells.

Preliminary evaluation of NAA's potential as a circulating marker of lung cancer

We then investigated the feasibility of using NAA as a blood biomarker for NSCLC tumors. NAA has been undetectable in blood of healthy individuals, but it is detected in blood of patients suffering from a rare neurological disease called Canavan disease [37], which is thought to be caused by excessive accumulation of NAA in brain and cerebrospinal fluid (CSF) of these patients due to their genetic defects of NAA-degradation enzyme, aspartoacylase. By roughly comparing 17 μM NAA in blood [37] with 380 μM NAA in CSF of these patients [38] (0.8 μM in CSF of healthy individuals [39]) and NAA concentration of lung tumors in our data (4.5 ~ 14.9 μM in 9 of 10 samples: Fig. 3A), we suspected that the majority of NAA blood levels in lung cancer patients may be approximately 0.2 μM or lower.

Since previously reported methods for NAA blood assay (detection limit: 0.35 μM) [37] were not sensitive enough to cover this concentration range, we developed a sensitive method by taking advantage of NAA's chemical property and optimizing the mass spectrometry condition. NAA is negatively charged in blood (pKa = 3.1), so it can be selectively enriched with strong anion exchange columns out of complex plasma samples. In addition, GC-MS methods used above can be modified for maximum sensitivity and minimum background noise for NAA detection through adjustment of gas chromatography program and application of 'selected ion monitoring' mode in mass spectrometry. Combining these two approaches allowed us to improve the limit of detection for blood NAA by nearly 10-fold — to approximately 0.038 μM (= 38 nM) — with 12% coefficient of variation (Supplementary Fig. S10).

To evaluate the cancer-specificity of the NAA blood assay, which depends heavily on their levels in individuals without cancer, we first examined NAA levels of blood plasma samples from 40 healthy individuals (ages 31-73 years) (see Supplementary Table S4 for clinical information of all plasma samples: in addition to 29 samples from the University of Texas Southwestern Medical Center (UTSW) (Dallas, TX) (N=15) and the Sungkyunkwan University (SKK) (N=14), 11 samples from a commercial source (Innovative Research (IR)) were included in order to bridge the age-diversity gap in control samples). In agreement with previous reports, 21 samples from individuals aged 55 years or younger showed low to no detectable NAA blood levels (less than 50 nM) (Fig. 6). Importantly, three of the 21 control samples were from individuals with non-cancer lung abnormalities (benign pulmonary nodule, pulmonary fibrosis, and inactive tuberculosis). However, unexpectedly, the majority

of 19 plasma samples from older individuals (56-73 years) showed increased NAA blood levels: 62 ± 19 nM (Supplementary Fig. S11).

Thus, our efforts to evaluate the clinical feasibility of the NAA blood assay were focused on samples from lung cancer patients at 55 years or younger. Six of 13 NSCLC patients (46%) showed NAA levels over 60 nM threshold (120 ± 20 nM, $p<0.01$) including four of nine NSCLC patients at stage 1A, compared with 21 age-matched controls (Fig. 6). None of the lung cancer patients in this age group were treated with chemo- or radio-therapy except the patient for "CA6" blood sample (collected after chemo and radiotherapy: NAA blood level=157 nM). These results are consistent with the highly cancer-specific overexpression of NAT8L in lung tumors (approximately 40% of all lung tumors) observed in the TCGA data analysis shown above. In contrast, NAA blood levels in the older age group (over 55 years: N=21) did not show significant differences from age-matched controls discussed above (N=19): 47 ± 14 nM (up to 190 nM; $p=0.5$).

Discussion

In this study, we used a metabolic profiling approach based on gas chromatography – mass spectrometry (GC-MS) with the specific objective to identify candidates of cancer-specific markers. GC-MS was chosen as the analytical method because of the richness of the available spectral libraries (approximately 200,000 unique compounds in the NIST spectral library (Electron Ionization))(40) despite the limitation of requiring derivatization of extracted metabolites before analysis. Among 194 non-redundant compounds identified in the GC-MS analysis of the isogenic pair of lung cancer and non-malignant cells (HCC4017 and HBEC30KT), we identified a new candidate of cancer-specific metabolite in lung cancer by focusing our efforts on characterizing the exact identities of 30 compounds that were detected only in cancer cells but could not be positively identified with library search.

To characterize these 30 unidentified compounds, we followed a commonly used approach: (1) come up with candidate identities, (2) obtain the corresponding synthetic compounds, and (3) compare their chromatographic retention times and mass spectra with those of compounds from original samples. Because this process is laborious and time-consuming, we prioritized the 30 unidentified cancer-selective compounds based on their similarities to the top hits from the library searches. The high spectral similarity of MMG-L to methylmalonylglycine in the NIST library allowed us to successfully characterize MMG-L as N-acetylaspartate (NAA).

NAA has been detected primarily in brains and cerebrospinal fluids, but not in other tissues or blood of healthy individuals (34, 37, 39, 41). Although NAA was recently reported as one of the potential markers of ovarian or prostate cancers (31, 32), our study is the first to report its production in lung cancer cells and lung tumors and elucidate the mechanistic basis — cancer-specific overexpression of NAA's biosynthetic enzyme, NAA synthetase (gene symbol: NAT8L) (33). We demonstrated that NAT8L plays a direct and specific role in NAA biosynthesis and extracellular secretion by lung cancer cells, using glucose and glutamine as major carbon sources. NAT8L has not been reported in previous proteomic studies of any cancer, including lung cancer cells or lung tumors (42-44).

The results in this study indicate that NAA's roles in lung cancer cells may be regulatory rather than metabolic. In brain, NAA's major role is to provide acetate in the synthesis of fatty acids and other lipids of myelin (45, 46), after being synthesized from acetyl-CoA and aspartate via NAT8L in neuronal cells and transported to oligodendrocytes for cleavage to acetate and aspartate via aspartoacylase (34). However, in lung cancer cells, the synthesis of palmitic acid (the most abundant fatty acid) was not affected significantly upon reduction of NAA synthesis after NAT8L suppression. This discrepancy can be explained by the low NAA levels in lung tumors (average 14 μM, approximately 500-fold lower than those in brains). Also, we report for the first time that NAA's biosynthesis in lung cancer cells may depend on glutamine as the major carbon source for the aspartate moiety of NAA. Glutamine's contribution to aspartate through the glutaminolysis pathway is well established in cancer cells, where glutamine is successively converted to glutamate (via glutaminase), α-ketoglutarate (via glutamate dehydrogenase or alanine/aspartate transaminases), oxaloacetate (via citric acid cycle), and to aspartate (via aspartate transaminase) (47, 48). Thus, our data suggest that NAA's role in these cells may be related to regulation of utilizing glutamine and other related nutrients. More questions about the specific roles that NAT8L or NAA play in proliferation or survival of cancer cells and the underlying mechanisms should be addressed in future studies.

We also explored the clinical feasibility of using NAA as one of the circulating biomarkers for lung tumors. Our data support that NAA molecules are produced by a significant subset of lung cancer cells and tumors in a cancer-specific manner, are secreted into extracellular space, and can be detected in blood with the sensitive methods that we developed. Our preliminary evaluation suggests that NAA alone may be a blood biomarker for a limited subset of NSCLC tumors within the age group of 55 years old or younger who do not have conditions that can cause excessive accumulation of NAA (such as Canavan Disease). Although this age group represents only 11% of total lung cancer cases, their cases appear to behave more aggressively, with fewer stage I cases and more stage IV cases at diagnosis (49, 50). It is also noteworthy that, although NAA's capability to distinguish individuals with benign lung conditions from patients with malignant lung tumors were not thoroughly investigated in this study, 21 non-cancer individuals at 55 years of age or younger (all of which showed negative NAA blood assay results) included three with non-cancer lung abnormalities.

The most significant limitation of this preliminary evaluation is that it is not clear how much of NAA in lung cancer patients' blood is derived from lung tumors. One of the ways to examine this issue is to analyze NAA levels in blood and tumor samples of these patients collected before and after surgical removal of their lung tumors. As an initial effort, we analyzed NAA levels in six pairs of such blood samples from lung adenocarcinoma patients (before vs. after surgery). In partial support of the argument that lung tumors contribute to increasing blood NAA levels, two of six pre-surgery samples showed positive NAA levels while the two corresponding post-surgery samples (as well as four other post-surgery samples) had no detectable NAA (data not shown).

An important consideration in potential clinical application of our findings is that NAA blood levels in the healthy individuals older than 55 years seemed to be comparable to those

in lung cancer patients. To our understanding, blood NAA in these old healthy individuals is most likely derived from brain and/or cerebrospinal fluid (CSF) through blood-brain barrier (BBB) leakage. For younger healthy individuals, NAA concentrations decrease dramatically from brains (5 to 8 mM) to CSF (0.8 μM) (19–58 years) (39) and no NAA has been detected in blood samples (27–50 years). These stark differences are thought to be caused by tight barriers between brain and CSF as well as between CSF and blood in healthy individuals under 50 years old. On the other hand, increased permeability of blood-brain barrier (in particular, blood-CSF barrier) has been reported in old healthy individuals by three independent studies. Healthy 60–87 year-olds have shown increased BBB permeability as compared with healthy 21–50 year-olds approximately by two to three-fold (51, 52), which has been recently confirmed in a high-resolution MRI study on healthy 55–91 year-olds vs. healthy 23–47 year-olds (53). These findings indicate that, in order to further examine how much of the NAA from lung tumors contribute to the NAA detected in blood samples of target population, future studies should include control samples from individuals with neurological conditions associated with BBB leakage.

Also, an important clinical question that our study started to address but has not fully answered is how many lung cancer patients within the 55-and-under age group are likely to produce NAA in their lung tumors and show detectable NAA levels in blood. Our large-scale analysis on 577 NSCLC tumors in the TCGA database indicated that approximately 40% of patient tumors may overexpress NAT8L. In fairly good agreement, this percentage was close to 46% (6 of 13 total) or 44% (4 of 9 in stage 1A) of lung cancer patients at 55 years or younger showing NAA blood levels above threshold. In order to determine NAA's sensitivity in detecting lung tumors with statistical reliability, a significantly larger number of patient blood samples collected before cancer treatment should be analyzed, based on recommendations by Pepe *et al.*(6, 54). In addition, control samples properly matched for source, age, sex, and smoking history should be used for estimating the cancer-specificity of the NAA blood assay more accurately. For example, the samples from the age group of 55 years or younger in our proof-of-principle experiments were not properly matched for source, sex and smoking history (sources - UTSW and SKK for Cancer, UTSW, SKK, and IR for Control; female percentages - 61% in Cancer and 43% in Control; smoker percentages - 46% in Cancer and 38% in Control).

Finally, two technical limitations of our study are that the reproducibility of the NAA blood assay and the effect of blood samples' storage time on the assay results were not completely addressed. We showed the intra-assay variability to be 12% in terms of coefficient of variation, but intra-subject, inter-assay and inter-laboratory variabilities need to be evaluated in future studies. We observed that the NAA blood assay can be applied to archived samples stored at -80°C for up to 7 years because NAA blood levels in patient samples stored for 0~3 years (53 ± 28 nM: from SKK) were not significantly different from those stored for 3~7 years (75 ± 36 nM: from UTSW, p=0.63).

In conclusion, we discovered NAA's cancer-specific production in NSCLC tumor cells and characterized NAA's dependence on the elevated expression of its biosynthetic enzyme (NAT8L) and the availability of glutamine. Our preliminary evaluation of NAA's clinical potential as a circulating marker for lung tumors suggests that it may be useful in identifying

approximately 40% of lung cancer patients among a narrowly-defined target population: those under 55 years of age without neurological conditions associated with NAA accumulation or blood-brain barrier leakage. Future work should include (1) in-depth investigation into elucidating the physiological roles of NAA molecule and NAT8L enzyme in lung tumors and (2) rigorous evaluation of the clinical potential and benefit of using NAA as one of the circulating biomarkers of lung tumors in a defined target population.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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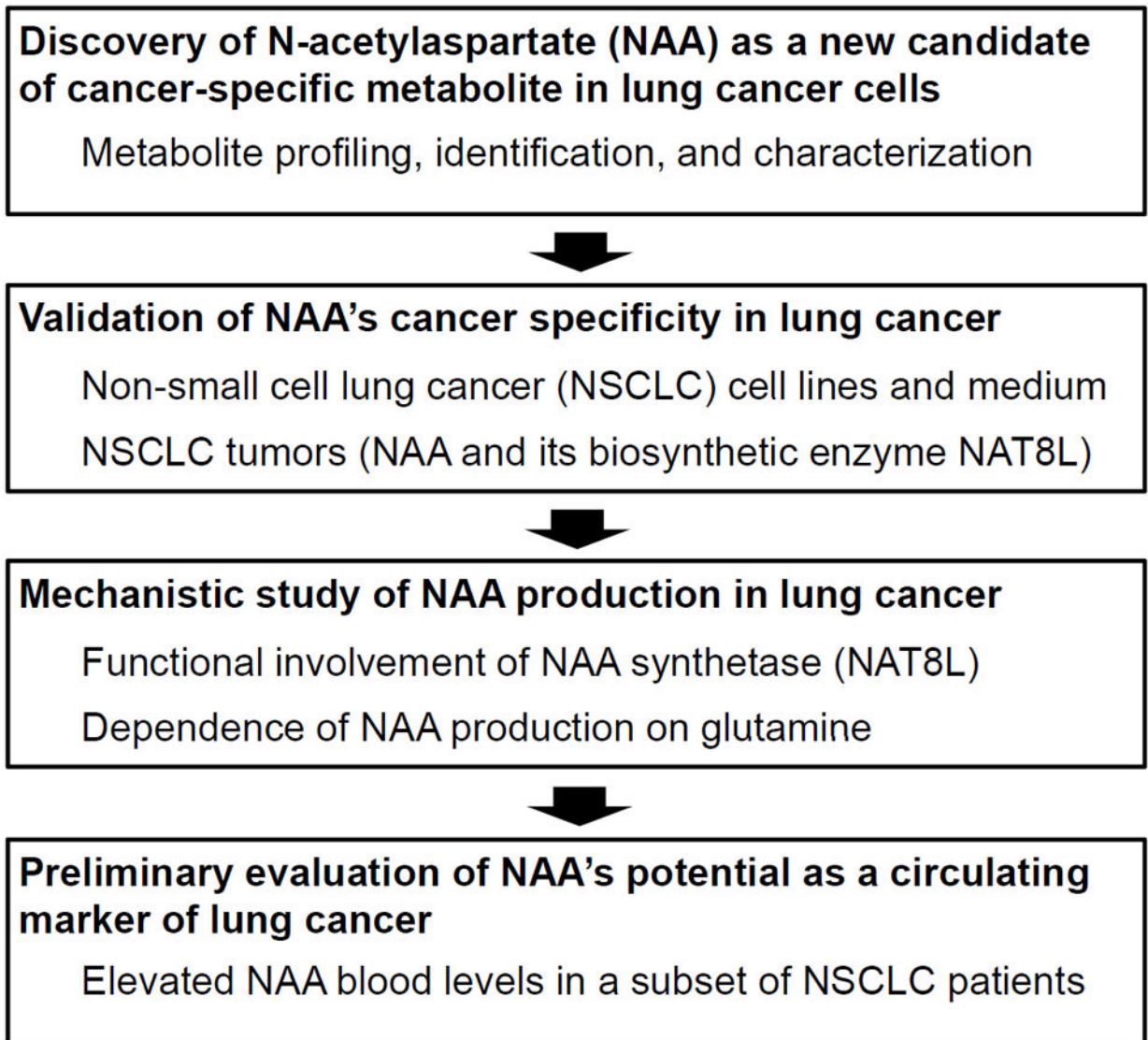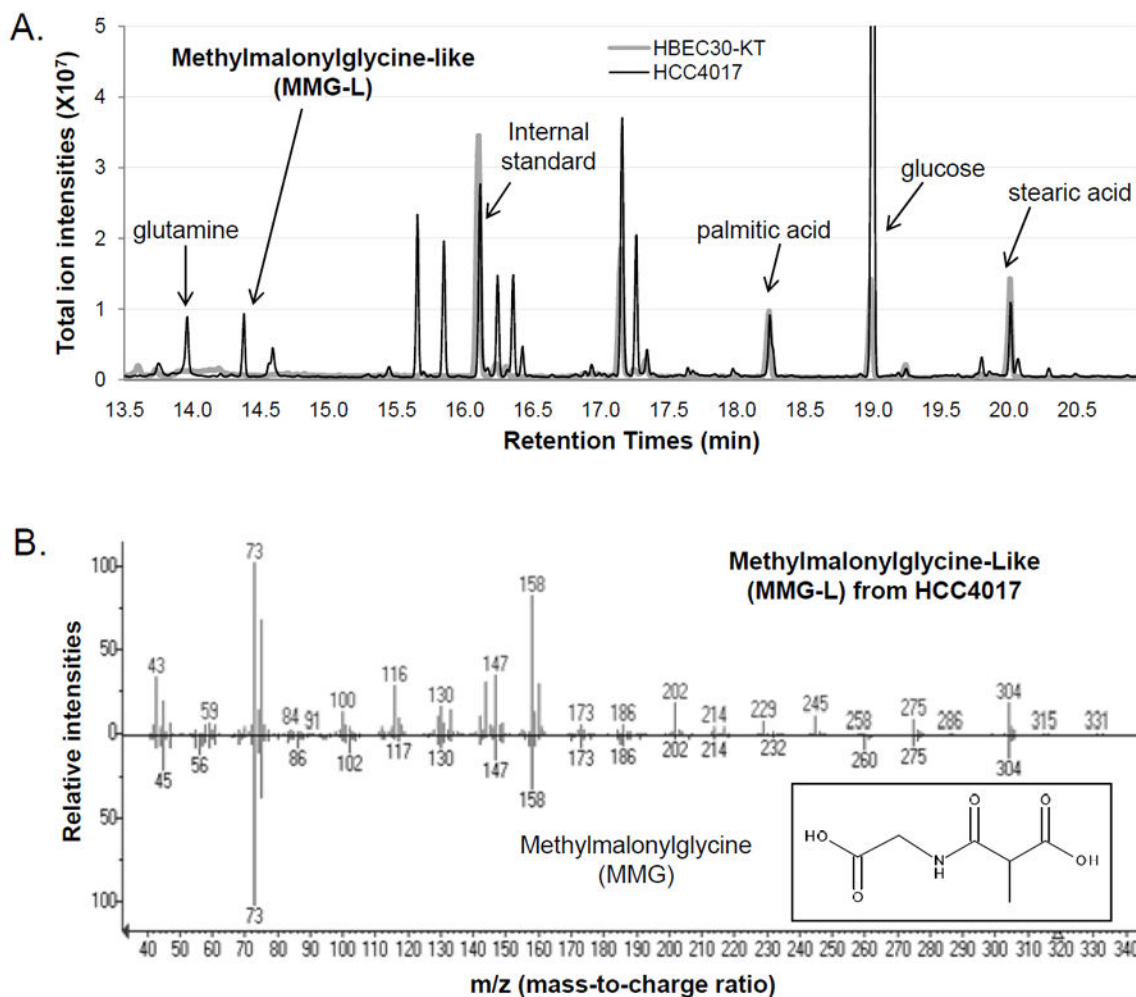
Discovery of N-acetylaspartate (NAA) as a new candidate of cancer-specific metabolite in lung cancer cells

Metabolite profiling, identification, and characterization

Validation of NAA's cancer specificity in lung cancer

Non-small cell lung cancer (NSCLC) cell lines and medium

NSCLC tumors (NAA and its biosynthetic enzyme NAT8L)

Mechanistic study of NAA production in lung cancer

Functional involvement of NAA synthetase (NAT8L)

Dependence of NAA production on glutamine

Preliminary evaluation of NAA's potential as a circulating marker of lung cancer

Elevated NAA blood levels in a subset of NSCLC patients

Figure 1. Schematic of the workflow in this study

Figure 2. Discovery of NAA in lung cancer cells
(A) In global metabolic profiling on lung cancer cells (HCC4017) and patient-matched control lung cells (HBEC30KT), an unidentified metabolite MMG-L (later characterized as NAA) was detected only in HCC4017 lung cancer cells, and is shown along with a few common metabolites of high abundance and the internal standard ($^2H_{27}$-myristic acid). Only a selected range of chromatographic retention times is shown on the X-axis for clarity. (B) The GC-MS mass spectrum of MMG-L from HCC4017 lung cancer cells (top panel) is compared with that of the top hit in search against NIST spectral library, methylmalonylglycine (MMG) (bottom panel). The fragment at m/z=245 is detected only for MMG-L while the fragment at m/z=260 is detected only for MMG.
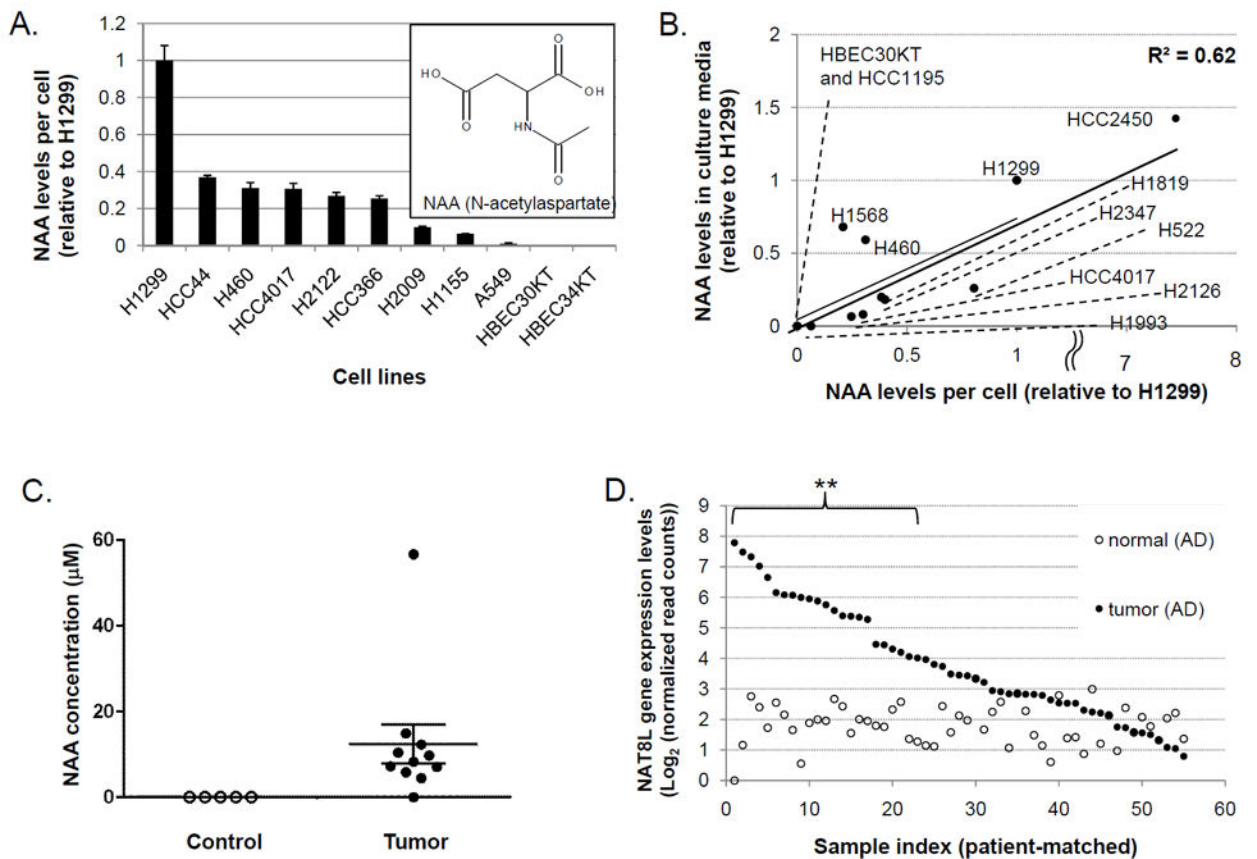
Figure 3. Validation of NAA's cancer specificity in lung cancer

(A) NAA levels in nine NSCLC cell lines, HBEC30KT, and HBEC34KT relative to its average level in H1299 cells. Error bars indicate standard errors of normalized NAA levels (N=3).

(B) Intracellular and extracellular NAA levels in 11 NSCLC cell lines and HBEC30KT. Data shown are averages of triplicate data. Error bars are omitted for clarity.

(C) NAA concentrations in control lung tissues ("Control", N=5) and NSCLC tumors ("Tumor", N=11). Horizontal lines and error bars indicate means and standard errors of tissue NAA concentrations.

(D) Gene expression levels of NAT8L, the key enzyme for NAA biosynthesis, in 55 adenocarcinoma (AD) lung tumors as compared with patient-matched non-malignant lung tissues.
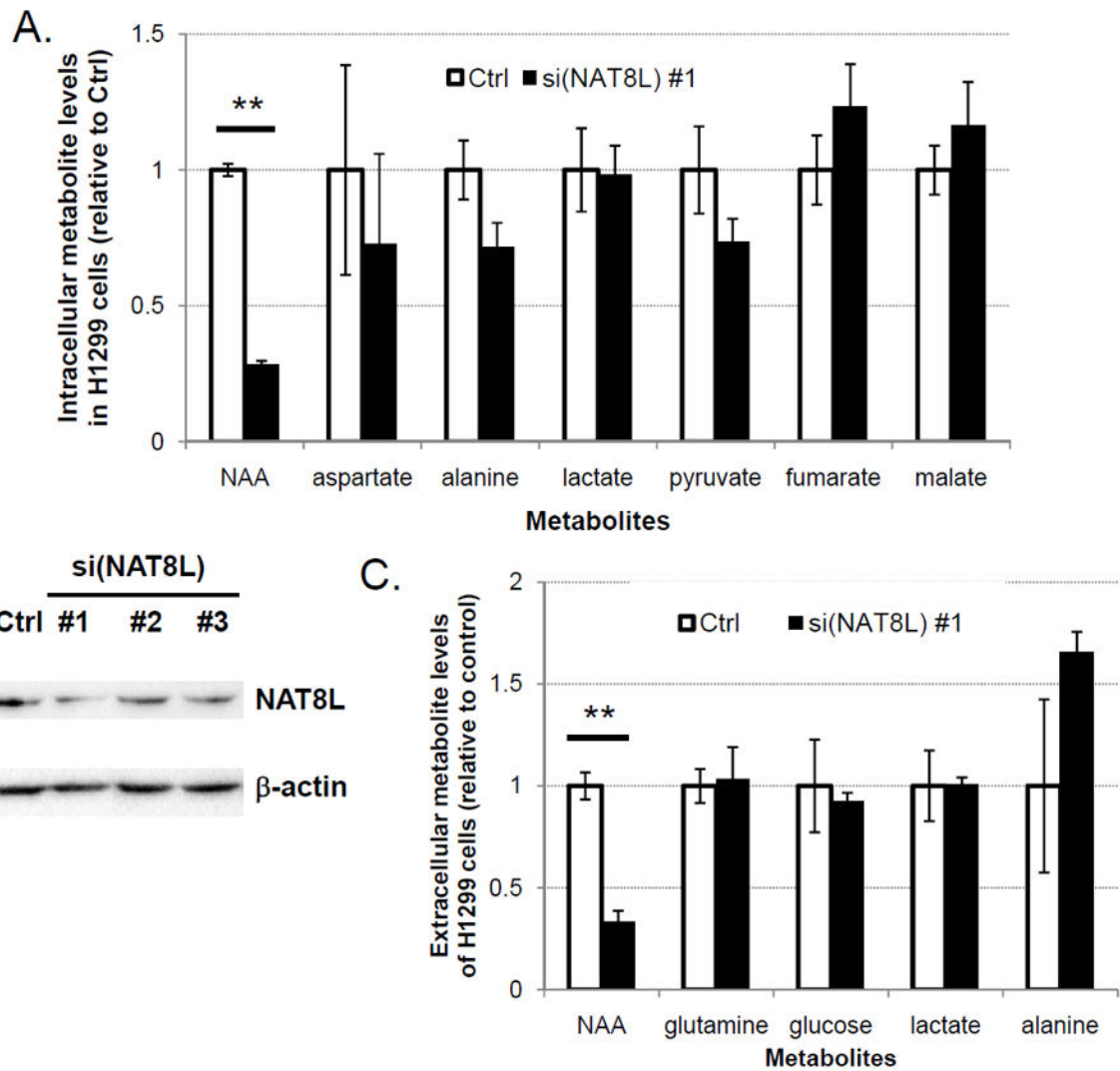
Figure 4. Functional involvement of NAT8L enzyme in NAA biosynthesis of lung cancer cells

(A) Relative levels of NAA, its precursors and related metabolites in H1299 cells under non-targeting control (Ctrl) or si(NAT8L) #1 conditions. Error bars indicate standard errors (N=3) (** = $p<0.01$).

(B) NAT8L protein levels in H1299 cells treated with the control siRNA (Ctrl) or three independent NAT8L siRNAs.

(C) Relative Levels of NAA, glutamine, glucose, lactic acid, and alanine in culture medium of H1299 cells under the control (Ctrl) or si(NAT8L) #1 conditions. Error bars indicate standard errors (N=3) (** = $p<0.01$).
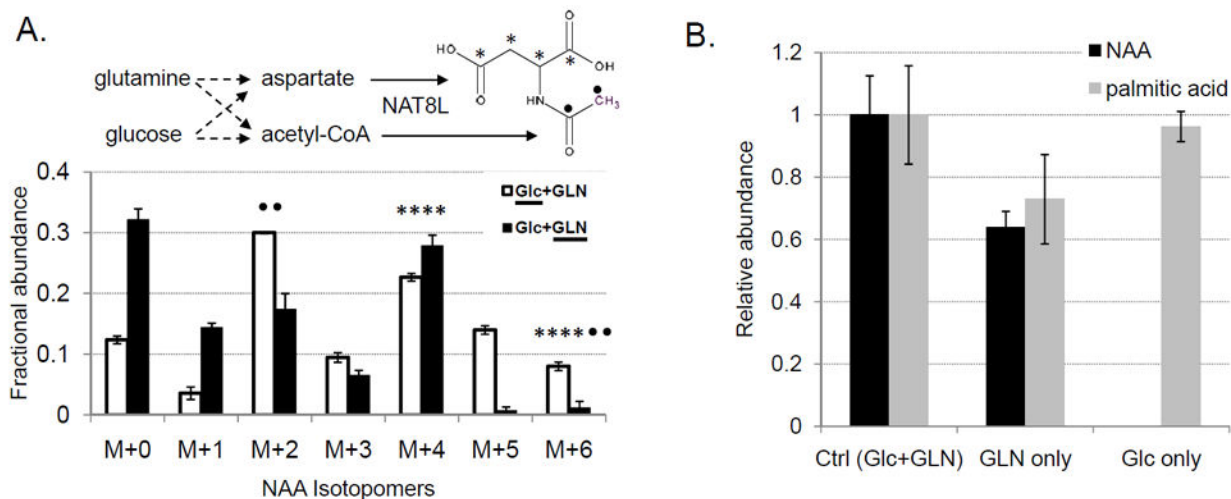
Figure 5. Glutamine dependence of NAA production in lung cancer cells

(A) Isotope labeling patterns of NAA in H1299 cells grown in medium containing [U-^{13}C] glucose and unlabeled glutamine (<u>Glc</u>+GLN), or unlabeled glucose and [U-^{13}C] glutamine (Glc+<u>GLN</u>). ^{13}C-labeled aspartate and acetyl-CoA in NAA are marked with asterisks and closed circles, respectively. M+0 indicates unlabeled isotopomer of NAA (m/z=304) while M+X indicates NAA labeled with X carbons from ^{13}C-labeled precursors. Error bars indicate standard errors of normalized metabolite levels (N=3).

(B) Levels of NAA and palmitic acid in H1299 cells under control, glutamine (GLN)-only, or glucose (Glc)-only conditions. Error bars indicate standard errors (N=3).
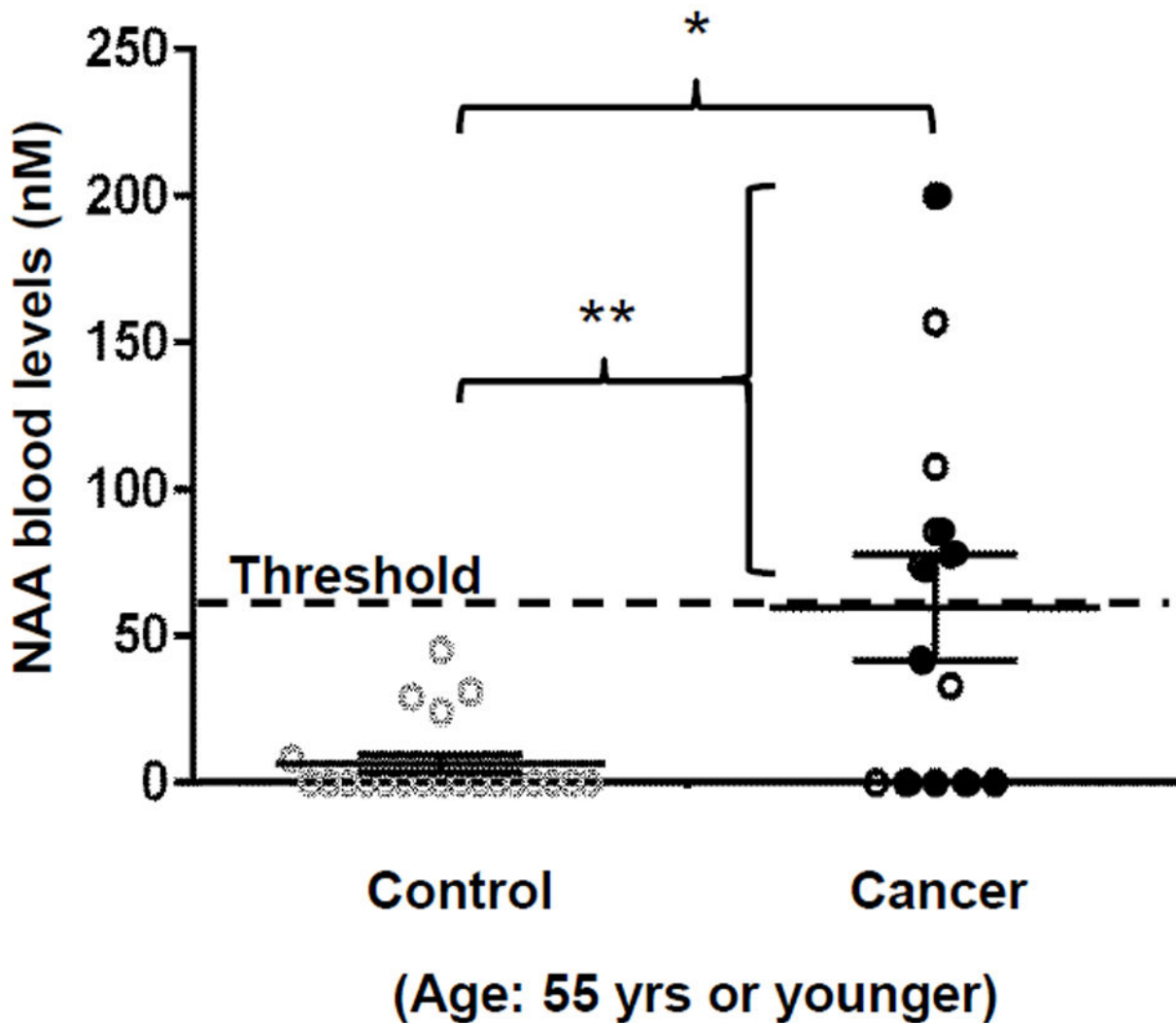
Figure 6. Preliminary evaluation of NAA as a circulating biomarker for lung cancer
NAA blood levels in healthy controls ("Control") vs. NSCLC patients ("Cancer") at 55 years of age or younger (** = $p<0.01$, * = $p<0.05$). Filled circles indicate nine NSCLC patients diagnosed as stage 1A. Horizontal lines and error bars indicate means and standard errors of NAA blood levels.