


RESEARCH ARTICLE

NAD(P)-dependent steroid dehydrogenase-like protein and neutral cholesterol ester hydrolase 1 serve as novel markers for early detection of gastric cancer identified using quantitative proteomics

Yang Xiao¹ | Jiebin Xie² | Lan Liu¹ | Wentao Huang¹ | Qiang Han¹ |
Jiayi Qin¹ | Shunying Liu¹ | Zhen Jiang¹ 

¹Department of Biochemistry, School of Preclinical Medicine, North Sichuan Medical College, Nanchong, China

²Department of Gastrointestinal Surgery, Affiliated Hospital of North Sichuan Medical College, Nanchong, China

Correspondence

Zhen Jiang, Department of Biochemistry, School of Preclinical Medicine, North Sichuan Medical College, Nanchong, Sichuan Province 637100, China.
Email: jiangzhenbiochem@126.com

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Abstract

Background: Gastric cancer (GC) is the third most common cause of cancer deaths worldwide. In the present study, we aimed to identify novel GC biomarkers by integrating isobaric tags of relative and absolute quantitation (iTRAQ) for aberrantly expressed proteins in GC patients.

Methods: Using stable isotope tags, we labeled an initial discovery group comprising four paired gastric cancer and adjacent gastric tissue samples, and subjected them to LC-ESI-MS/MS. We used a validation set comprising 129 paired gastric cancer and adjacent gastric tissues from patients and benign healthy controls to validate the candidate targets.

Results: We identified two proteins, NAD(P)-dependent steroid dehydrogenase-like (NSDHL) and neutral cholesterol ester hydrolase 1 (NCEH1), that were significantly overexpressed in GC tissues. The sensitivity and specificity of NSDHL were 80.6% and 74.4%, respectively, in GC compared with a sensitivity of 25.6% in adjacent tissues and 24% in benign healthy controls. The area under the ROC curve (AUC) for NSDHL was 0.810 for GC detection. Overexpression of NSDHL in GC was significantly correlated with local tumor invasion. The sensitivity and specificity of NCEH1 were 77.5% and 73.6%, respectively, in GC compared with a sensitivity of 26.4% in adjacent tissues and 20% in benign controls. The AUC for NSDHL was 0.792. Overexpression of NCEH1 was significantly associated with tumor histological classification and local invasion. Moreover, a combined analysis of NSDHL and NCEH1 achieved a sensitivity and specificity of 85.7% and 83%, respectively, and the AUC was 0.872. The combined analysis of NSDHL and NCEH1 was significantly correlated with histological grade and TNM II-IV staging.

Conclusions: iTRAQ-labeled quantitative proteomics represents a powerful method to identify novel cancer biomarkers. The present study identified NSDHL and NCEH1

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as useful biomarkers for screening, diagnosis, and prognosis of patients with gastric cancer.

KEYWORDS

Cholesterol metabolism, Gastric cancer, iTRAQ, NCEH1, NSDHL

1 | INTRODUCTION

Gastric cancer (GC) is the third most common cause of cancer deaths worldwide. Because of a lack of early-stage gastric cancer diagnostic and therapeutic technologies and targets, the majority of gastric cancer patients exhibit poor survival rates.^{1–3} Although research in this area has steadily progress over the last several decades, the currently available gastric cancer diagnostic biomarkers, such as carbohydrate antigen 19.9 (CA 19.9), carcinoembryonic antigen (CEA), and pepsinogen I/II, have limited diagnostic sensitivity and specificity.^{4–6} Therefore, it is important to identify novel diagnostic biomarkers that are responsible for gastric carcinoma etiology and progression. This would be beneficial for early-stage detection of gastric cancer and for the development of effective therapeutic strategies.

Proteomics represents a powerful tool for the identification of novel biomarkers in malignant tumors. Compared with 2-D electrophoresis, mass spectrometry is a superior technique for detecting the relative abundance of proteins in biological samples. A great number of methods have been developed that support relative quantification measurements. Stable isotope labeling of proteins in samples prior to analysis is one of the most popular methods for relative quantification by mass spectrometry.⁷ Isobaric tags for relative and absolute quantification (iTRAQ) is now the most widely used method for high-throughput protein quantification, and it is capable of simultaneously quantitating four to eight different biological samples.^{8,9} Thus, iTRAQ provides an unbiased method to identify novel potential diagnostic and therapeutic targets for early cancer stage detection.

In this study, we used iTRAQ-labeled quantitative proteomics as a gold standard technique to identify novel biomarkers in gastric cancer compared with adjacent tissues. We identified NAD(P)-dependent steroid dehydrogenase-like (NSDHL) and neutral cholesterol ester hydrolase 1 (NCEH1) as potential novel biomarkers, which were further validated in a separate series of surgical gastric cancer samples for early-stage gastric cancer screening.

2 | MATERIALS AND METHODS

2.1 | Clinical specimens

The specimens comprised fresh stomach cancer tissues surgically removed from gastric cancer patients along with surrounding adjacent tissues, which were 5 cm from the edge of the tumor and had

no obvious tumor cell content as determined by an experienced pathologist. All 129 patients with gastric cancer and 25 healthy control subjects with benign gastritis from January 2010 to December 2018 were included, and no patient underwent radiotherapy or chemotherapy prior to surgery. The study was approved by the Human Research Ethics Committee of North Sichuan Medical College.

2.2 | Sample preparation and iTRAQ labeling

Frozen tissue samples representing four pairs of samples in the discovery group were quickly weighed, homogenized in lysis buffer, and centrifuged. The supernatant containing the proteins was collected and stored at -80°C . iTRAQ 8-plex experiments were performed on the tissue extracts. Each iTRAQ channel contained 50 μg of protein per sample. The proteins were digested with trypsin (10% w/w, sequencing grade modified trypsin, Sigma). The iTRAQ 8-plex labeling was performed according to the manufacturers' instructions (iTRAQ labeled for 2.5 hours). iTRAQ labels 113 and 114 were used to label the first pair of gastric cancer and adjacent normal gastric tissue, respectively. Labels 115/116, 117/118, and 119/121 were used for the remaining three tissue pairs, respectively. After iTRAQ labeling, the samples were pooled, desalted on a 500-mg Sep-Pak C18 column (Millipore), dried in a Speed Vac Concentrator (Thermo Scientific), and subjected to peptide fractionation.

2.3 | Strong cation exchange chromatography

The dried iTRAQ-labeled sample was diluted 10-fold in a loading buffer. Then, the samples were reconstituted in 4 mL of buffer A (25 mM NaH_2PO_4 in 25% ACN, pH 2.7) and loaded onto a 5- μm particle size, 4.6 \times 250 mm Ultremex SCX Column (Phenomenex). The column was eluted with a gradient of buffer A for 10 min, 5%–35% buffer B (25 mM NaH_2PO_4 , 1 M KCl, 25% ACN, pH 2.7) for 11 min, and 35%–80% buffer B for 1 min. The peptides were pooled into 20 fractions, and each fraction was collected at 1-min intervals. Finally, samples were dried under vacuum and stored at -20°C until the MS analysis.

2.4 | LC-ESI-MS/MS analysis

The LC setup was equipped with a linear ion trap mass spectrometer, the LTQ-Orbitrap Discovery (Thermo Fisher, San Jose, CA, USA). A

data-dependent acquisition protocol, in which one MS scan was followed by three MS/MS scans for the eight most abundant precursor ions in the MS survey scan, was applied. The sample volume was 10 μ l per injection. A blank was introduced after each sample to clear the system. A 1.8 kV electrospray voltage was applied. The dynamic exclusion settings were 2 s for repeat counts, 30 s for repeat duration, and 120 s for exclusion duration. The automatic gain control to avoid overfilling of the ion trap and the PQD spectra was generated by 5×10^4 ions accumulated in the ion trap. The m/z scan range was 350–2,000 Da.

2.5 | Database search and bioinformatics

The MASCOT search engine (Matrix Science, London, UK; version 2.20) was used to compare MS/MS spectra with that of the International Protein Index (IPI) human database (<https://www.ebi.ac.uk/IPI>). A threshold of 10 ppm for intact peptide tolerance masses and that of 0.05 Da for PQD fragment ions were set for protein identification. For quantification of the identified proteins, we defined the threshold for a significant change as a 1.2-fold increase or decrease in expression. We also searched the spectra against a decoy database and estimated that the false-discovery rate (FDR) of our identified peptides was 1.2%. Gene ontology analysis was performed by Blast2GO software, and pathways associated with the candidate proteins were identified using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database.

2.6 | Immunohistochemical staining

Immunohistochemical (IHC) staining was performed as previously described.¹⁰ Briefly, 5- μ m-thick consecutive sections of formalin-fixed,

paraffin-embedded specimens from gastric cancer patients were stained with antibodies using the Boster immunohistochemical kit (Boster Biology). IHC staining was performed using specific antibodies against NSDHL (Santa Cruz; diluted 1:50–100) and NCEH1 (Abcam, MA, USA; diluted 1:50–100). Two pathologists independently evaluated the intensities and percentages of the stained sections. The staining intensity was scored as 0 for a negative stain and 1, 2, or 3 to indicate weak, moderate, or strong staining, respectively. The percentages of positive tumor cells were scored as 0 (<5%), 1 (6%–25%), 2 (26%–50%), 3 (51%–75%), or 4 (76%–100%). The IHC scores were calculated by multiplying the percentage of positively stained cells by their corresponding intensities to obtain a final protein expression score. Slides with final scores ≥ 3 were considered positive, and those with scores <3 were considered negative.

2.7 | Statistical analysis

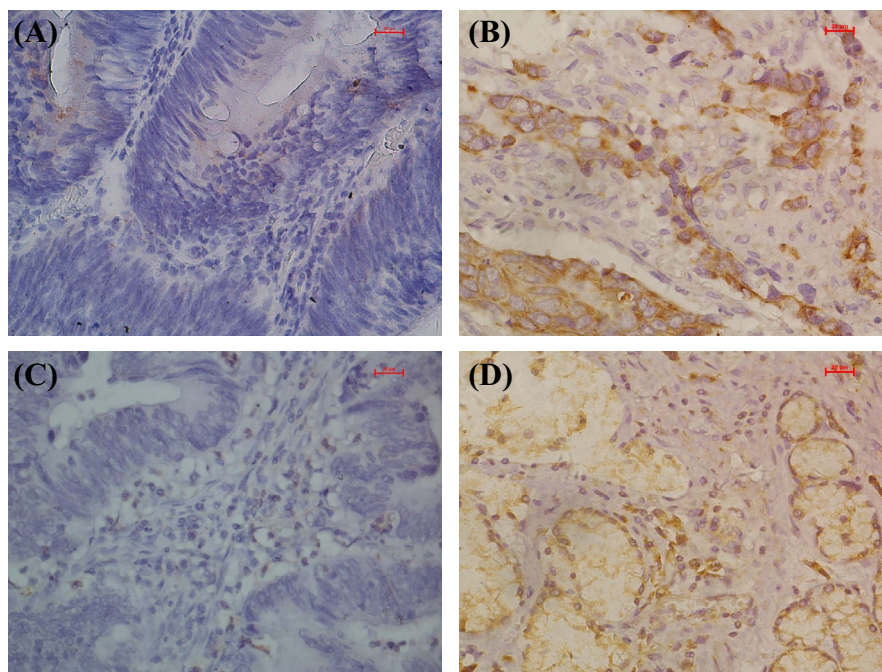
The differences between experimental groups were analyzed with a chi-square test. All analyses including chi-square tests and area under the ROC curves (AUCs) were performed using SPSS 23.0 (SPSS, Chicago, IL, USA) software for Windows. $p \leq .05$ (two-sided) was considered significant.

3 | RESULTS

3.1 | NSDHL and NCEH1 are overexpressed in gastric cancer tissues

In this study, we used four technical replicates to demonstrate the reproducibility of the experimental results and to perform

FIGURE 1 Immunohistochemical analysis of the expression of NSDHL and NCEH1 in gastric cancer tissues. NSDHL was primarily located in the cytoplasm (A, adjacent tissues; B, gastric cancer tissues), and NCEH1 was mainly located in the cytoplasm (C, adjacent tissues; D, gastric cancer tissues;)



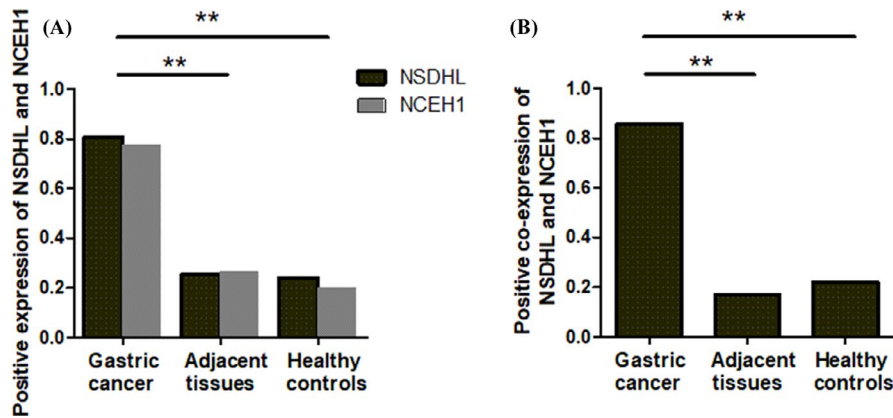


FIGURE 2 Expression of NSDHL (A) and NCEH1 (A) and both of them (B) in gastric cancer tissues, adjacent tissues, and benign healthy controls

TABLE 1 Validation of NSDHL and NCEH1 expression in gastric cancer tissues, adjacent normal tissues, and benign healthy controls

	Adjacent normal tissues	Cancer tissues	Healthy controls	<i>p</i> -values [*]
NSDHL(-)	96	25	19	
NSDHL(+)	33	104	6	<.001
NCEH1(-)	95	29	20	
NCEH1(+)	34	100	5	<.001
NSDHL and NCEH1(-)	78	15	18	
NSDHL and NCEH1(+)	16	90	5	<.001

*The *p*-values were determined by the chi-square test.

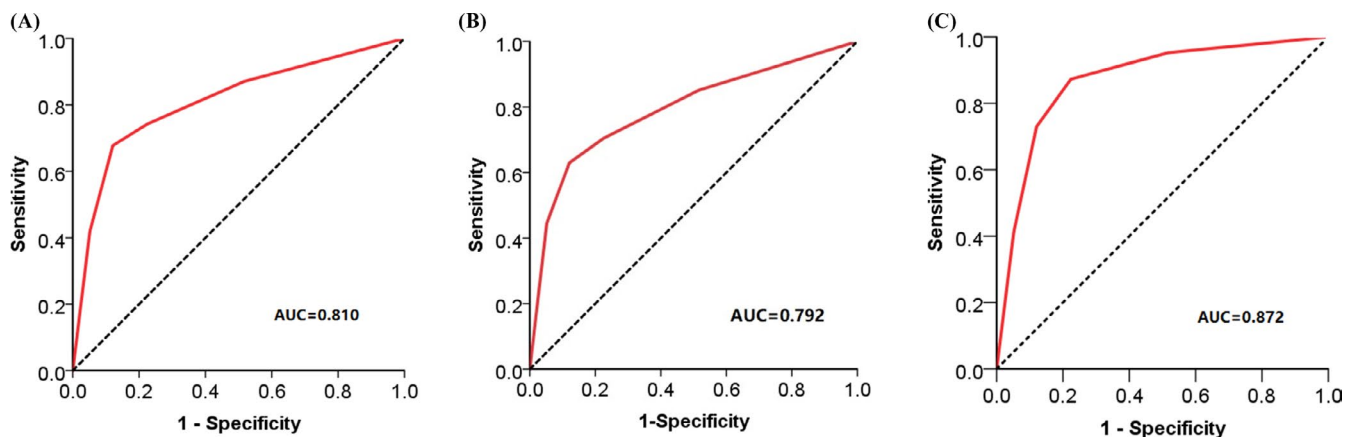


FIGURE 3 ROC curves for NSDHL and NCEH1. (A), The AUCs were 0.810 for NSDHL. (B), The AUC were 0.792 for NCEH1. (C), The AUC were 0.872 for the combination of NSDHL and NCEH1. *p* < .001

complementation. A total of 431 proteins were characterized as differentially expressed in gastric cancer tissues compared with adjacent tissues.¹¹ Functional clustering of the differentially expressed proteins revealed that the majority represented metabolic proteins. We found that two key enzymes, NSDHL and NCEH1, which are associated with cholesterol metabolism, were significantly overexpressed in gastric cancer tissues. NSDHL was upregulated 1.476-fold, and NCEH1 was upregulated 1.303-fold in gastric cancer tissues.

3.2 | Validation of NSDHL and NCEH1 expression in gastric cancer tissues by immunohistochemistry

The expression of NSDHL (Figure 1A, B) and NCEH1 (Figure 1C, D) was further validated by immunohistochemistry. NSDHL was overexpressed in 80.6% of GC tissues compared with 25.6% of surrounding normal tissues (Figure 2A). The localization of NSDHL was mainly in the cytoplasm. NCEH1 was overexpressed in 77.5% of GC tissues compared with 26.4% in adjacent normal tissues (Figure 2A, Table 1).

TABLE 2 Correlation of NSDHL expression with clinicopathological features in gastric cancer

Clinicopathological features	NSDHL (-)	NSDHL (+)	Case	<i>p</i> -values [*]
Histology classification				
Adenocarcinoma	20 (18.7%)	87 (81.3%)	107	.663
Signet-ring cell carcinoma	5 (22.7%)	17 (77.3%)	22	
pT				
T1 + T2	17 (28.8%)	42 (71.2%)	59	.013
T3 + T4	8 (11.4%)	62 (88.6%)	70	
pN				
pN0	13 (23.2%)	43 (76.8%)	56	.402
pN1 + pN2	8 (20.5%)	31 (79.5%)	39	
pN3a + pN3b	4 (11.8%)	30 (88.2%)	34	
pM				
pM0	25 (20.3%)	98 (79.7%)	123	-
pM1	0	6 (-)	6	

*The *p*-values were determined by the chi-square test.

TABLE 3 Correlation of NCEH1 expression with clinicopathological features in gastric cancer

Clinicopathological features	NCEH1 (-)	NCEH1 (+)	Case	<i>p</i> -values [*]
Histology classification				
Adenocarcinoma	20 (18.7%)	87 (81.3%)	107	.023
Signet-ring cell carcinoma	9 (40.9%)	13 (59.1%)	22	
pT				
T1 + T2	20 (33.9%)	39 (66.1%)	59	.004
T3 + T4	9 (12.9%)	61 (87.1%)	70	
pN				
pN0	15 (26.8%)	41 (73.2%)	56	.217
pN1 + pN2	10 (25.6%)	29 (74.4%)	39	
pN3a + pN3b	4 (11.8%)	30 (88.2%)	34	
pM				
pM0	28 (22.8%)	95 (77.2%)	123	-
pM1	1 (16.7%)	5 (83.3%)	6	

*The *p*-values were determined by the chi-square test.

The NCEH1 was mainly localized to the cytoplasm. Furthermore, a combined analysis revealed that NSDHL and NCEH1 were over-expressed in 85.7% of gastric cancer tissue compared with those in 17% of adjacent gastric tissues and 22% of healthy controls (Figure 2B). The AUCs were 0.810 for NSDHL, 0.792 for NCEH1, and 0.872 for the combination of NSDHL and NCEH1 (Figure 3A, B, and C, respectively). These results suggest that the diagnostic value for gastric cancer was significantly increased by the combined analysis of NSDHL and NCEH1.

3.3 | Clinicopathological features between NSDHL-positive and NSDHL-negative patient groups

Next, we performed a chi-square test to analyze the relationship between NSDHL-positive and NSDHL-negative patient samples

with clinicopathological factors including histological type, T staging, lymph node involvement, and distant metastasis. As shown in Table 2, NSDHL expression was significantly upregulated in the T3 and T4 group compared with the T1 and T2 group ($p = .013$).

3.4 | The correlation of NCEH1 expression with clinicopathological features

To explore the clinical association of NCEH1 expression with clinicopathological features, we found that NCEH1 was highly expressed in gastric adenocarcinoma compared with that in signet-ring cell carcinoma (Table 3, $p = .023$). Furthermore, patients with T3 and T4 TNM stage disease exhibited a higher expression of NCEH1 compared with patients in stage T1 or T2 (Table 3, $p = .004$).

Clinicopathological features	NSDHL and NCEH1 (-)	NSDHL and NCEH1 (+)	Case	<i>p</i> -values*
Age (year)				
≥60	10 (14.9%)	57 (85.1%)	67	.804
≤59	5 (13.2%)	33 (86.8%)	38	
Sex				
Male	11 (14.9%)	63 (85.1%)	74	.793
Female	4 (12.9%)	27 (87.1%)	31	
Histology classification				
Adenocarcinoma	10 (11.5%)	77 (88.5%)	87	.072
Signet-ring cell carcinoma	5 (27.8%)	13 (72.2%)	18	
Histological grade				
I	7 (9.7%)	65 (90.3%)	72	.048
II and III	8 (24.2%)	25 (75.8%)	33	
Lymph node metastasis				
Positive	7 (11.5%)	54 (88.5%)	61	.333
Negative	8 (18.2%)	36 (81.8%)	44	
TNM Staging				
I	10 (25.6%)	29 (74.4%)	39	.011
II-IV	5 (7.6%)	61 (92.4%)	66	

*The *p*-values were determined by the chi-square test.

TABLE 4 Correlation of NSDHL and NCEH1 expression with clinicopathological features in gastric cancer

3.5 | Clinical association of NSDHL and NCEH1 expression with pathological features in gastric cancer

As shown in Table 4, to further explore the combined diagnostic values of NSDHL and NCEH1 in gastric cancer, we found that NSDHL and NCEH1 expression was significantly associated with histological grade and TNM staging. Combined NSDHL and NCEH1 expression was significantly higher in patients with histological grade I compared with that in patients with histological grade II and III ($p = .048$). Compared with TNM stage I, gastric cancer patients with TNM stage II to IV presented with higher NSDHL and NCEH1 expression (Table 4).

4 | DISCUSSION

Although there have been improvements in the treatment of gastric cancer in recent years, prognosis remains poor primarily because the disease is often diagnosed at advanced stages. Identification of reliable biomarkers is an essential for early detection and prognosis of gastric cancer. We are the first to discover that NSDHL and NCEH1 are significantly overexpressed in gastric cancer tissues compared with adjacent gastric tissues and healthy controls. Notably, the aberrantly high expression of NSDHL and NCEH1 in gastric cancer patients was significantly associated with clinicopathological histology classification, local invasion, histological grade, and TNM staging.

Cholesterol is a major component of cell membranes and a precursor for steroid hormones. The biosynthesis and catabolism

reactions of cholesterol ester are central cholesterol metabolic pathways in animal cells. Comprising more than 20 reactions, cholesterol biosynthesis is strictly regulated within cells. Dysregulation or imbalance of cholesterol metabolic pathways contributes to many human diseases such as atherosclerosis, neuropathy, and cancer progression.^{12,13} Several recent studies have demonstrated that dysregulated cholesterol metabolism is a salient feature in the progression of cancer.^{14,15} In the present study, we found that NSDHL and NCEH1 were significantly overexpressed in patients with gastric cancer. These results suggest that patients with gastric cancer may experience dysregulated cholesterol metabolism because NSDHL and NCEH1 are key enzymes that contribute to cholesterol metabolism.

NAD(P)-dependent steroid dehydrogenase-like is an essential enzyme in the penultimate step of cholesterol biosynthesis. It catalyzes NAD⁺-dependent oxidative decarboxylation of the C4 methyl groups of 4 α -carboxysterols. Previous studies have demonstrated that a loss-of-function mutation in the human NSDHL gene results in congenital hemidysplasia with ichthyosiform nevus and limb defect (CHILD) syndrome. This is an X-linked human embryonic developmental disorder that is lethal in males and causes limb reduction in females.¹⁶ Recently, several studies have revealed that NSDHL may also serve as an oncogene in cancer progression.¹⁷⁻¹⁹

Xue et al found that NSDHL was overexpressed in 67NR, 168FARN, and 4T1 murine breast cancer cell lines and translocated to the plasma membrane from the intracellular compartment. The study suggested that the altered localization and differential expression of NSDHL may contribute to breast cancer cell metastasis and serve as a novel target for anti-metastasis therapy.¹⁷ Mechanistic

studies revealed that NSDHL is a key regulator of growth and pro-oncogene signaling in cancer cells. Gabitova et al demonstrated that NSDHL deficiency resulted in the accumulation of sterol metabolites, which inhibit tumor growth.¹⁸ In addition, the loss of NSDHL gene expression sensitized the response of cancer cells to EGFR-targeting inhibitors.¹⁹ To develop a targeted compound, Kim et al analyzed the protein structure of NSDHL and identified a novel molecule (compound 9), which could inhibit NSDHL and its biological function in cells. The results revealed that compound 9 decreased EGFR expression and signal transduction. Moreover, when combined with an EGFR inhibitor, compound 9 enhanced its antitumor effect.¹⁴

Consistent with the above studies, we found that NSDHL was overexpressed by 80.6% in gastric cancer tissues and more than 25.6% in surrounding normal gastric tissues. In addition, NSDHL expression in gastric cancer patients with T3 and T4 stage disease was significantly higher than that in patients with T1 and T2 stage (Table 2). The results suggest that NSDHL has the potential to regulate the local invasion of gastric cancer cells. NSDHL exhibited a sensitivity of 80.6% and a specificity of 74.4% in the detection of patients with gastric cancer. To further improve the sensitivity and specificity, we analyzed the combined expression of NSDHL and NCEH1 in gastric cancer.

Neutral cholesterol ester hydrolase 1 (NCEH1), also known as arylacetamide deacetylase-like 1 (AADACL1) and KIAA1363, is a serine hydrolase, which has diverse biological activities and cellular functions.^{20–24} NCEH1 hydrolyzes cholesterol esters to free cholesterol, which contributes to the development of atherosclerosis. NCEH1 also hydrolyzes 2-acetyl monoalkylglycerol, a metabolic intermediate in the ether lipid pathway to form alkyl-LPA. Moreover, NCEH1 may also hydrolyze exogenous organophosphorus compounds during organ detoxification.²⁵ NCEH1 is overexpressed in a variety of aggressive human cancer cell lines and primary tumors.^{20–23} Although NCEH1 regulates the levels of neutral ether lipids and cholesterol ester metabolism, the manner in which these metabolites regulate the progression of cancer remains unclear.

Recent studies have demonstrated that after a single and repeated treatment of chlorpyrifos and chlorpyrifos-oxon, NCEH1 is elevated in MCF-7 and MDA-MA-231 cells and induces proliferation. The results indicated that NCEH1 may participate with chlorpyrifos as factors in the induction of breast cancer.²⁶ Chang et al reported that NCEH1 was highly expressed in prostate cancer cells. Using covalent PET probe imaging, they observed that NCEH1 was primarily localized to the outer edges of xenograft tissues, with less localization within the tumor. These results indicate that NCEH1 may contribute to the aggressiveness and metastasis of breast cancer cells.²¹ By hydrolyzing the metabolic intermediate, 2-acetylmonoalkylglycerol, NCEH1 may regulate the ether lipid signaling network that links platelet activating factor and lysophosphatidic acid metabolism. To develop a novel inhibitor of NCEH1, they identified JW480, an O-aryl carbamate, which acts as a potent and selective inhibitor of NCEH1. JW480 reduced monoalkylglycerol ether and inhibited prostate cancer cell migration, invasion, and growth *in vivo*.²⁰

In addition to Chang's studies, other researchers have designed and developed compounds to detect and inhibit NCEH1 activity. Fan et al designed the first "off-on" NIR probe, NB-AX, which exhibits a high selectivity for NCEH1. This probe can rapidly distinguish breast cancer cells from normal cells with a detection limit of 0.58 $\mu\text{g}/\text{ml}$.²⁷ To develop novel inhibitors for NCEH1 in cancer, Shreder et al demonstrated that AX13057 inhibits NCEH1 activity in SK-OV-3 xenograft-bearing mice based on protein profiling.²⁸

In the present study, we found that NCEH1 was highly expressed in 77.5% of gastric cancer tissues and was higher compared with the 26.4% expression observed in adjacent gastric tissues. Further analysis indicated that the high expression of NCEH1 was significantly associated with histological type and local invasion of T3 and T4 stage disease. Traditional biomarkers, such as CA 19.9, exhibit a sensitivity of 20%–56%. However, our study revealed that NCEH1 had a sensitivity of 77.5% and a specificity of 73.6% for the detection of gastric cancer. Our results suggest that NCEH1 may serve as a novel biomarker for gastric cancer detection and progression, particularly for patients with an adenocarcinoma histology type and T3 or T4 stage disease. To further improve the sensitivity and specificity of NCEH1, we combined NSDHL with NCEH1, another key enzyme in the cholesterol ester catabolism pathway, and analyzed their diagnostic potential in gastric cancer. We observed that the combination of NSDHL and NCEH1 exhibited a sensitivity of 85.7% and specificity of 83% in detecting gastric cancer. Moreover, the AUC value of 0.872 for combined NSDHL and NCEH1 was higher than either marker alone. In addition, the coexpression of NSDHL and NCEH1 was significantly associated with advanced TNM stage in gastric cancer patients. Our results suggest that the combined analysis of NSDHL and NCEH1 achieved a higher sensitivity and specificity for screening gastric cancer than that of a single marker and may be useful in the screening and follow-up assessment of gastric cancer patients.

In conclusion, we used iTRAQ-based quantitative proteomics to discover that NSDHL and NCEH1, key enzymes in cholesterol metabolism, were aberrantly expressed in gastric cancer. Dysregulated expression of NSDHL and NCEH1 in gastric cancer was significantly correlated with histological type, local invasion, and TNM staging. Moreover, our results indicate that the combined analysis of NSDHL and NCEH1 as coordinate markers was superior to a single marker in detecting gastric cancer. Therefore, our study indicates that NSDHL and NCEH1 may serve as novel biomarkers in the screening and follow-up of patients with gastric cancer.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data are available in the article.

ORCID

Zhen Jiang  <https://orcid.org/0000-0003-3006-3056>

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