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ITIH3 Is a Potential Biomarker for Early Detection of Gastric Cancer

Poh Kuan Chong^{*,†}, Huiyin Lee[†], Jianbiao Zhou[†], Shaw-Cheng Liu[†], Marie Chiew Shia Loh[†], Ting Ting Wang[†], Siew Pang Chan[‡], Duane T. Smoot[§], Hassan Ashktorab[§], Jimmy Bok Yan So^{||}, Khong Hee Lim[⊥], Khay Guan Yeoh[#], and Yoon Pin Lim^{*,†,▽,◊}

[†]Cancer Science Institute of Singapore, National University of Singapore, Singapore 117456

[‡]Singapore Institute of Management University, Singapore 599491,

[§]Department of Medicine and Cancer Center, Howard University, Washington, DC 20060

^{||}Department of Surgery, National University Hospital, Singapore 119074

[⊥]Department of Surgery, Tan Tock Seng Hospital, Singapore 308 433

[#]Department of Medicine, Yong Loo Lin School of Medicine, National University of Singapore, Singapore 6516 6914

^ΔDepartment of Biological Sciences, Faculty of Science, National University of Singapore, Singapore 117546

[◊]Bioinformatics Institute, Agency for Science, Technology and Research, Singapore 138668

Abstract

Gastric cancer has one of the highest morbidities and mortalities worldwide. Early detection is key measure to improve the outcome of gastric cancer patients. In our efforts to identify potential markers for gastric cancer detection, we coupled xenotransplantation mouse model with a plasma proteomic approach. MKN45 gastric cancer cells were subcutaneously injected into nude mice and plasma samples from mice bearing different sizes of tumors were collected and subjected to iTRAQ and mass spectrometry analysis. ITIH3 protein was found to be more highly expressed in plasma of tumor bearing mice compared to control. Subsequent screening of ITIH3 expression in 167 clinical plasma samples, including 83 cancer-free subjects and 84 gastric cancer patients, revealed higher ITIH3 level in the plasma of gastric cancer patients. A receiver operating characteristics (ROC) curve estimated a maximal sensitivity of 96% at 66% specificity for ITIH3 in gastric cancer detection. In addition, plasma from early stage gastric cancer patient has significantly ($p < 0.001$) higher level of ITIH3 compared to that from noncancer subject. Our data suggest that ITIH3 may be a useful biomarker for early detection of gastric cancer.

Keywords

ITIH3; gastric cancer; plasma; proteomics; mouse xenograft

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* To whom all correspondence should be addressed. Chong Poh Kuan (csicpk@nus.edu.sg); Lim Yoon Pin (csilyp@nus.edu.sg); Cancer Science Institute of Singapore, Centre for Life Sciences, 28 Medical Drive, #02-14C, Singapore 117456. Tel: (65) 65168435. Fax: (65) 68739664.

Supporting Information Available: Supplemental tables (excel files). This material is available free of charge via the Internet at <http://pubs.acs.org>.

Introduction

Gastric cancer is the second leading cause of cancer-related deaths worldwide and early detection is the key in its management. Endoscopy is widely used for screening; however, this methodology involves invasive procedure and its cost effectiveness remains an issue. One of the main goals in molecular biomarker discovery today is to develop a non/minimally invasive method for cancer screening. In clinical diagnosis, body fluids, especially plasma, serum, and urine, are frequently sampled from patients since they are easily obtained without complicated procedures. Hence, they represent ideal substrates for diagnostic purposes. However, biomarker discovery in body fluid is not without challenges. The wide dynamic range of more than 9 orders of magnitude in protein concentration coupled with 20 abundant proteins accounting for 99% of total protein mass has hindered the detection of low-abundance proteins.¹

Mouse xenograft model is an alternative to analysis of clinical samples for biomarker discovery. It presents less genetic and biological variations compared to human model at the same time allowing environmental factor to be tightly controlled.² Another advantage is that xenograft model tends to have bigger tumor/blood ratio than humans, thereby enabling easier detection of cancer-induced changes. The feasibility of using xenograft model for biomarker discovery had been demonstrated by various serum biomarker studies including that for breast,³ pancreatic,⁴ stomach,⁵ and prostate.⁶ For example, identification of nm23/nucleoside-diphosphate kinase and human glycolytic enzyme in prostate cancer mouse xenograft has been achieved.⁶ Another study on nasopharyngeal carcinoma identified peroxiredoxin 2 up-regulation in the plasma of tumor-bearing compared to control mice and subsequent ELISA screening on clinical samples showed the similar observation.⁷

This study utilized the Isobaric Tags for Relative and Absolute Quantification (iTRAQ) approach to profile the levels of proteins in the plasma from control mice and mice bearing different sizes of MKN45 gastric cancer cell line-derived tumors. The aim of this study is to identify potential protein candidates in mice and further validate the selected candidate in human samples.

Materials and Methods

MKN45 Xenograft in Nude Mice

All animal work was done with IACUC approval and complies with the Agri-Food and Veterinary Authority of Singapore (AVA) guidelines. BALB/C-nu strain female nude mice (4–6 weeks) were purchased from Biological Resources Centre (BRC), Agency for Science, Technology and Research (A*STAR). These mice were caged and maintained using laminar air-flow rack.

Human gastric cancer cell line MKN45, a poorly differentiated adenocarcinoma, was purchased from Japanese Riken Cell Bank (Tsukuba, Japan). Others cell lines including MKN28, N87-HCl, SCH, KATO3, SNU1, SNU16, IM95, NUGC3, NUGC4, SNU719, MKN74, N87, OCUM1, SNU84, HGC27, HS746T, TMK1, AZ521, and SNU620 were purchased from either American Type Culture Collection (Manassas, VA) or Japanese Riken Cell Bank (Tsukuba, Japan), whereas HFE-145 normal gastric epithelial cell was a courtesy from Dr. Hassan Ashktorab (Howard University Cancer Center, Washington, DC). These cells were cultured in controlled humidified atmosphere containing 5% CO₂ at 37 °C using RPMI 1640 media supplemented with 10% fetal calf serum.

Trypsinized and pelleted MKN45 cells were washed and resuspended in PBS buffer. A total of 5×10^6 cells were transplanted by subcutaneous inoculation into the left flank of 15 mice

(i.e., 5 mice per group). The MKN45 mice xenografts were sacrificed once the tumor size reached approximately: (a) 1–2 mm for low tumor burden group, (b) 7.5 mm for mid tumor burden group, and (c) 15 mm for high tumor burden group. Five control mice were injected with Phosphate Buffered Saline (PBS) buffer and were sacrificed at the middle time point of the study, about 4 weeks after the injection. Blood from each group was collected into K₂EDTA Microtainer tube (BD) via cardiac puncture using 23G butterfly needle. Following centrifugation at 1100g at 4 °C for 10 min to separate plasma from the red blood cells, protein inhibitors were added to the plasma sample. Separate pools of individual mice were stored at –80 °C.

Sample Preparation, iTRAQ Labeling, and LC-MS/MS Analysis

The same volume (50 µL) of plasma samples from each mouse was pooled according to the groups they are in (i.e., control, low, mid, and high tumor burden groups). The pooled samples were depleted using Removal System (MARS Ms-3) affinity column (Agilent Technologies, CA). Depleted plasma samples were then concentrated and washed 3 times with 50 mM TEAB buffer pH 8 on 5 kDa cutoff centrifugal filter units (Millipore), prior to BCA assay.

Protein samples were then reduced, alkylated, digested, and labeled with iTRAQ reagents according to the recommended protocol (Applied Biosystems, Framingham, MA). The samples were labeled as follows: 114, control; 115, low tumor burden; 116, mid tumor burden; and 117, high tumor burden. Triplicate replicate set of iTRAQ experiments were carried out to increase the reliability of the results. Dried labeled peptide mixture was fractionated using a PolySULFOETHYL A Column (PolyLC, Columbia, MD) 5 µm of 200 mm length × 4.6 mm i.d., 200 Å pore size, on an AKTA Purifier FPLC unit (GE Healthcare, U.K.) using a 60 min gradient. A total of 30 fractions were pooled and cleaned-up using a C18 Discovery DSC-18 SPE column (100 mg capacity, Supelco, Sigma-Aldrich).

Those fractions were then analyzed using Ultimate 3000 nanoflow HPLC (Dionex, Surrey, U.K.) coupled online to a quadruple time-of-flight mass spectrometer (QStar XL, Applied Biosystems). Samples were resuspended in 0.1% formic acid and 2% acetonitrile in water (Buffer A), prior loading to a 5 cm × 300 µm i.d. LC-Packing C18 100 Å PepMap100 trap cartridge for desalting at 20 µL/min for 5 min. Then, the trap was switched online with a 15 cm × 75 µm i.d. LC-Packing C18 100 Å PepMap100 analytical column. A 120 min or 85 min gradient was used, ramping from 5% to 100% Buffer B (0.1% formic acid in 98% acetonitrile) in 2 linear gradient steps to elute peptides. Eluent from the reverse phase nLC was directly subjected to positive ion nanoflow electrospray analysis in an information dependent acquisition mode (IDA), with a ToF MS survey scan was acquired (*m/z* 300–1800), with the 3 most intense multiple charged ions (counts >20) sequentially subjected to MS/MS analysis. The time of summation of MS/MS events was set to be 2 s in the mass range of *m/z* 100–1600.

Protein identification and quantification for iTRAQ samples were carried out using ProteinPilot software (version 2.0; Applied Biosystems, MDS-Sciex). The search was performed against IPI mouse database (version 3.56, date of release: March 2009, 56 073 sequences). The search was performed using Paragon Algorithm, which is discussed in detail elsewhere.⁸ Only those proteins identified with at least 95% confidence were taken into account. All results were then exported into Excel for manual data interpretation.

Clinical Plasma Samples

Since January 2006, patients with newly diagnosed gastric cancer at the National University Hospital and Tan Tock Seng Hospital, Singapore, have been prospectively enrolled with

informed consent in a research study (Gastric Cancer Biomarker Discovery II, GASCAD II) and blood samples were obtained together with clinical and pathologic annotation. Blood collection was obtained before surgery or chemotherapy. Staging information was determined histo-pathologically and in combination with all clinical information. There was no evidence of other malignancies. American Joint Committee on Cancer (AJCC) (6th edition) on gastric cancer staging system and Lauren's classification of gastric cancer were used. Non-cancer controls were obtained from a clinical study in which subjects had undergone screening with upper gastrointestinal endoscopy which determined they were free of gastric cancer. The same blood collection protocols were used in all patients. The study was approved by the respective institutional review boards and all patients gave written informed consent.

Western Blotting and Immunohistochemistry

To validate iTRAQ result, the same pooled mouse plasma samples used for iTRAQ analysis were subjected to immunoblotting against ITIH3 (Santa Cruz), as previously described.^{9,10} Triplicates blots were carried out for each sample to ensure robustness of data generated. For human plasma validation, a total of 167 samples, comprising 83 normal and 84 gastric cancer samples, were screened for ITIH3 expression. The clinical data for these samples were tabulated in Supplementary Table 1. Plasma protein from each sample was loaded into 1D-SDS PAGE. Gel strips spanning the desired molecular weight range were cut out from various 1D gels, laying onto the same PVDF membrane, and Western blotted using ITIH3 and secondary antibodies at 1:1000 and 1:15 000, respectively. The densitometry of these bands was analyzed and estimated using Image Scanner and ImageQuantTL software v2003.03 (General Electric Healthcare).

For immunohistochemistry, matched malignant and adjacent normal gastric tissues and the histopathology reported were requested from the Tissue Repositories of National Cancer Centre of Singapore (NCCS) and National University Hospital (NUH) following approvals from Institutional Review Board from NCCS, NUH and National University of Singapore. Immunohistochemistry was carried out as previously described,^{11,12} with primary antibody (ITIH3) at 1:500 dilutions. All interpretations of H&E sections and analysis/ scoring of IHC data were all done by the same certified pathologist to maintain consistency.

Statistical Analysis

To investigate statistically differences of the protein expression level obtained via immunoblot in normal plasma compared to cancer plasma, 2-sample Student's *t* test analysis was performed at 5% significance using SPSS16.0 software for Windows. All other statistical analyses including Fisher's exact test, Kruskal-Wallis test, and likelihood ratio test described in subsequent result section were also performed using the aforementioned statistical software. Receiver operating characteristics (ROC) curve was also generated to estimate the protein sensitivity and specificity using Stata 10.0 software package (Stata Corp, TX).

Results

Identification and Relative Quantification of Mouse Plasma Proteins

All experimental mice developed tumors after 5 days of inoculation. The volumes of tumors for the low-, mid-, and high-tumor burden groups of mice ranged from 2.1–2.5, 127.4–210.2, and 726.1–1077.5 mm³, respectively (Figure 1a). These various sampling time points allow closer monitoring of tumor progression via protein profiling.

To profile the protein expression levels in MKN45 mouse xenograft model, quantitative iTRAQ approach was employed. Triplicate iTRAQ replicates were carried out to ensure result reproducibility and reliability. Only common proteins identified across the iTRAQ triplicates were considered further. Similarly with previous studies, proteins were considered up- or down-regulated when their ratios were >1.3 or <0.77 . This cutoff is widely accepted and adapted.^{11,13,14} Table 1 shows 31 proteins whose expression levels differed between at least 1 tumor loads compared to control. The complete list of the proteins identified is tabulated in Supplementary List 1.

The molecular function classification of these 31 common proteins was achieved using the Panther Classification (<http://www.pantherdb.org>).¹⁵ A total of 40 protein assignments were obtained and sorted into 13 molecular classifications, since some of these proteins have multiple classifications assigned (Figure 1b). A majority of the common proteins were grouped under transport/carrier protein followed by defense/immunity protein and select regulatory molecule, typical of proteins found in the plasma.

Novel Candidates for Gastric Cancer Detection

As seen from Table 1, Rbp4 was the most interesting candidate since it is highly overexpressed in all 3 groups of tumor load. However, when compared to our human gastric cancer plasma data set (unpublished data), this protein was found to be underexpressed in gastric cancer patients compared to cancer free subjects. Because of the non-congruence of the data between the human and mouse plasma data set, Rbp4 was dropped from this study. Instead, to add rigor to the study, we focused on 9 of the 31 candidates that have similar iTRAQ trend with the human gastric cancer plasma data set (as highlighted in Table 1).

Since our objective is to identify new and better markers than current ones, intensive literature search against Pubmed was carried out to identify which of the 9 proteins have not been previously associated with gastric cancer plasma detection. Inter-alpha-trypsin inhibitor heavy chain H3 (ITIH3) emerged as the only candidate. On the basis of the iTRAQ result, ITIH3 was found to be more highly expressed in high tumor load mice compared to control (Table 1).

To validate the expression level of ITIH3 indicated by iTRAQ analysis, the same pooled samples used for iTRAQ analysis were probed for ITIH3 expression level via immunoblotting. The results obtained via immunoblotting is shown in Figure 2a and densitometry of the immunoblotting data showed overexpression of ITIH3 in the plasma of high tumor load mice compared to normal mice by 3.1-fold (Figure 2b). These experiments were conducted in triplicates and equal loadings in these experiments were also inspected (Figure 2c). Attaining consistent observations between two independent approaches (iTRAQ and immunoblotting) authenticated our findings.

ITIH3 Expression in Human Plasma

Next, we proceeded to investigate ITIH3 expression level in human plasma samples to determine whether it might be a viable biomarker candidate for gastric cancer detection. A total of 167 plasma samples, comprising 83 from cancer-free subjects and 84 from gastric cancer patients, were subjected to immunoblotting with ITIH3 antibody. Triplicate blots were carried out and the average densitometry reading of ITIH3 expression for each sample was tabulated according to the samples type (normal or cancer). The average densitometry reading for each patient was plotted in a bar chart according to the samples' nature (Figure 3A). The average densitometry of ITIH3 expression level for cancer group (183 933) was found to be 1.9-fold higher compared to the normal control group (97 393). A 2-sample *t* test was carried out, revealing a *p*-value of less than 0.001 (Figure 3B), indicating that

expression level of ITIH3 can be used to discriminate the gastric cancer group from normal control. A receiver operating characteristics (ROC) curve was generated based on these results to estimate ITIH3 sensitivity and specificity in gastric cancer detection. As shown in Figure 3C, at an average densitometry reading cutoff of 111 305 units, the area under the ROC curve was estimated to be 0.864, giving ITIH3 a specificity and sensitivity of 66% and 96%, respectively, at 95% confidence for gastric cancer detection.

As early detection of gastric is an important goal, we investigated whether ITIH3 is capable of differentiating early stage gastric cancer patients from non-cancer subjects. To this end, we segregated the gastric cancer patients according to their disease stages, i.e., early stage (I–II) and advanced stage (III–IV) based on American Joint Committee on Cancer (AJCC) staging system. Among the 84 gastric cancer samples screened, 36 were from early and 48 from late stage gastric cancer. The box-plot in Figure 3D generated from ANOVA statistical analysis revealed that the difference in ITIH3 expression level was significant (p -value <0.001) between normal subjects and early stage gastric cancer patients.

Helicobacter pylori (HP) infection and gastritis are frequently associated with gastric cancer. To ensure that ITIH3 is not a marker for these events, we asked whether there is any statistically significant correlation between ITIH3 expression level and HP or gastritis status. To investigate the former, 2-sample t test statistical analysis was performed. No significant difference in the mean level of ITIH3 expression between HP-positive and HP-negative subjects were obtained (p -value = 0.187) (Supplementary Table 3). Similarly, gastritis status has no impact on ITIH3 expression, in which Fisher's exact test showed a p -value of 1.00. From a clinical and biological point of view, diffuse and intestinal types of gastric cancer have different behaviors. Thus, we asked whether ITIH3 expression in the plasma might be different between the cancer subtypes by performing the Kruskal-Wallis test. Results showed no significant correlation (p -value = 0.226) between ITIH3 expression with the various histology types. Thus, the statistical analyses indicate that plasma ITIH3 expression was independent of the HP infection, gastritis inflammation, and the histology type of gastric cancer.

To test the role of ITIH3 as a biomarker, a small-scale blinded study was performed using the cutoff value obtained from the ROC plot to predict whether the plasma sample tested was from cancer or non-cancer subject. For this purpose, 39 newly collected plasma samples (19 from normal and 20 from gastric cancer) were analyzed using the immunoblotting approach. Samples with ITIH3 average densitometry reading of less than 111 305 units were considered as normal, whereas higher than 111 305 units were considered as cancer. Using these criteria, 18 out of 20 gastric cancer plasma samples were correctly predicted, giving a sensitivity of 90% (Supplementary Table 4). On the other hand, among the 19 normal samples screened, only 9 of them were correctly predicted, giving ITIH3 specificity of 47% (Supplementary Table 4). The performance of ITIH3 in the blind test study is comparable that revealed by the ROC curve.

ITIH3 Expression in Gastric Cancer Cells

Next, we were interested to know the expression level of ITIH3 in gastric cancer tissues since this has never been reported before. As a pilot study, 19 matched pairs of normal and gastric cancer tissues were subject to immunohistochemistry. The raw IHC results for the 19 match cases are provided in Supplementary Table 5. Forty-two percent of the cases (8/19) showed no difference in ITIH3 level between tumor and normal tissue; 21% (4/19) showed higher ITIH3 expression and 26% (5/19) showed lower ITIH3 expression in the tumors when compared to their matched adjacent normal tissue. In cases where normal tissues stained positive for ITIH3, we noticed that only discrete individual cells expressed ITIH3 as shown in Figure 4A. This reflects that only specific cells in the crypts expressed ITIH3. In

contrast, ITIH3 expression in cancer cells of tumor sections was usually very widespread (Figure 4B). Likelihood ratio test carried out showed no significant difference (p -value = 0.236) between ITIH3 expression and the various histological types of gastric cancer (Supplementary Table 3).

We also examined the expression of ITIH3 in a panel of 19 gastric cancer cell lines. As shown in Figure 4C, only 1 of the gastric cell lines (MKN45) showed overexpression of ITIH3 by 1.5-fold. Another one (SNU719) showed 1.3-fold overexpression while 4 others (MKN74, SNU1, NUGC3 and HGC27) showed only 1.2-fold. The IHC and cell line data are largely consistent, and collectively, they implied that the up-regulation of ITIH3 in the plasma is unlikely to be result of up-regulation of ITIH3 in cancer cells. Instead, these data supported the notion that the increase in ITIH3 plasma level could be a result of host response to the tumor. It further suggests that plasma rather than tissues is a better substrate for profiling ITIH3 during diagnostic examination.

Discussion

Xenograft model facilitates target candidate identification for validation in human clinical samples, as exemplified by the discovery of the higher expression of ITIH3 protein in the plasma of gastric tumor-bearing mice and gastric cancer patients compared to normal condition. To date, this is the first study that showed elevated ITIH3 expression in the plasma of gastric cancer patients. Interestingly, ITIH3 has also been recently reported to be up-regulated in the sera of lung cancer of c-myc transgenic mice¹⁶ and lung cancer patients.¹⁷

ITIH3 is one of the five heavy chains (ITIH1, ITIH2, ITIH3, ITIH4, and ITIH5) belonging to the family of inter-alpha-trypsin inhibitor, comprising a family of protease inhibitors that could be found in extracellular matrix of various organ including in blood circulation. One of the well-known functions of these heavy chains is the ability to link covalently to hyaluronic acid (HA), a major component of extracellular matrix.¹⁸ A study proposed that ITIH acts as an important factor to stabilize the extracellular matrix.¹⁹ There are evidence showing ITIH's antagonistic relationship with tumor invasion and metastasis.^{20,21} Studies so far have ascribed a tumor suppressor role for ITIH, which largely exerts a negative effect on cancer progression. For example, Paris *et al.* reported that ITIH1 and ITIH3 expression significantly reduced the number of lung metastases in mouse xenograft and increased cell attachment *in vitro*.²² Another study demonstrated that down-regulation of ITIH5 expression is a prognostic marker for tumor invasion and metastasis.^{23,24} The reported loss of ITIH genes including ITIH3 expression level in the tissues of multiple solid cancers including that of the breast, stomach, lung, ovary, and colon²⁵ seem to contradict the observed up-regulation of ITIH3 in the plasma of lung cancer^{16,17} and, in this study, gastric cancer subjects. We do not understand this apparent discrepancy but several potential reasons exist. First, the down-regulation of ITIH3 reported was based on mRNA profiling, which may not necessarily correlate with protein levels. Second, the expression profiling was performed on tissues and not in body fluids like blood. It is possible that the levels of ITIH3 in the intracellular and extracellular compartments are different. Third, the ITIH3 that was present at higher level in the plasma of gastric cancer patient did not derive from tumor/cancer cells but was instead a consequence of the host response to the tumor. The detect-ability of ITIH3 in early stage cancer, the IHC and *in vitro* cell line data all supported this notion. However, further investigations would be required to prove this hypothesis.

Currently, there is no highly sensitive marker used for gastric cancer detection. Conventional markers including CEA, CA19-9, and CA72-4 are not effective in detecting gastric cancer. The sensitivity of these markers in gastric cancer detection ranged from

16%–63% as summarized in a review.²⁶ Studies had also evaluated M2-pyruvate kinase (M2-PK), a tumor-associated metabolic marker for gastric cancer detection, giving the range of sensitivity and specificity of 57–67% and 89–95%, respectively.^{27,28} A study on gastric cancer detection using serum pepsinogen measurement had reported a sensitivity of 77% with a specificity of 73%.²⁹ A mass screening in Japan also revealed large proportion of early stage gastric cancer was detected, that is, 80% of all detected cancers, using serum pepsinogen screening.³⁰ Biomarkers for early stage gastric cancer detection remain limited. On the basis of the results of our study, the high sensitivity of ITIH3 from 90–96% and specificity of 47–66% may be exploited for gastric cancer detection. Most interestingly is the potential of ITIH3 for early stage gastric cancer detection and cancer screening.

Because of the heterogeneity of cancer, it is not surprising that combination of biomarkers may be required to achieve better specificity and sensitivity than individual markers. In ovarian cancer, a study had reported the use of 11 analytes including CA125 marker which successfully distinguished ovarian cancer from benign condition with a specificity and sensitivity of approximately 90%.³¹ Another study reported higher specificity and sensitivity of 89% and 96% for pancreatic cancer detection by using CA19–9 concurrently with tumor marker M2-PK compared to CA19–9 alone.³² M2-PK has also been used in conjunction with CA72–4 for gastric cancer detection. Compared to the use of M2-PK alone (sensitivity of 67%) or CA72–4 alone (sensitivity of 41%), combinatorial use of both markers resulted in a higher sensitivity of 82%, although the specificity remained at 89%.²⁸ ITIH3 has high sensitivity but relatively lower specificity. Exploring markers that could synergize with ITIH3 should improve early detection of gastric cancer that will save lives.

In conclusion, the study supported the role of ITIH3 as a biomarker for gastric cancer. Future larger-scale studies would certainly clarify the clinical utility of ITIH3 for gastric cancer management.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(a) An overview of the workflow employed in this study. Three iTRAQ replicates were carried out to ensure the consistency and reliability in results. (b) The protein molecular function classification of the 31 common proteins identified with similar iTRAQ trend across 3 iTRAQ replicates. The classification was performed using Panther classification systems (www.pantherdb.org). A total of 40 assignments were obtained and sorted into 13 molecular classifications, despite the fact that there were only 31 proteins, as some of these proteins were assigned with more than 1 classification. The number showed in each category represented the number of protein assignment in a particular classification. The details for each classification are the following: Defense/immunity protein (Mbl2, Saa4, Apcs, C9);

Extracellular matrix (Fga, Lrg1, Lum, Fgb, Igfbp3, Fgfr); Hydrolase (Gpld1); Kinase (Egfr);
Lyase (Car2); Miscellaneous function (Orm2); Molecular function unclassified (Apoc4);
Receptor (Lifr, Egfr, Lrg1, Lum, Igfbp3); Select regulatory molecule (Itih3, Itih2, Serpina3k,
Ambp, Itih1, Serpina1d); Signaling molecule (Fga, Egfr, Fgb, Fgfr); Transfer/carrier protein
(Hbb-b2, Apoc1, Saa4, Apod, Hbb-b1, Rbp4, Hba-a2;Hba-a); Transferase (F13a1);
Transporter (Apoc1, Saa4)

Figure 2.

(a) Representative immunoblot of ITIH3 validation. (b) Bar chart shows the average densitometry ratio obtained from the triplicate immunoblots of ITIH3 protein. The trends observed in the immunoblotting were similar to the protein expression level obtained via iTRAQ approach in which ITIH3 was more highly expressed in high tumor load when compared against control. (c) The overall protein profile of the pooled plasma samples from control, low, mid, and high tumor load mice. The gel was stained with SYPRO Ruby to ensure equal loadings during immunoblotting analysis.

Figure 3.

(A) Triplicate immunoblots were carried out for each individual and their average densitometry reading were plotted in the bar chart according to their classification, i.e., normal and gastric cancer patients. The average densitometry reading for both the overall normal subject and gastric cancer group was also calculated and shown in the chart. The average expression level of ITIH3 in gastric cancer patients was found to be 1.9-fold higher compared to normal. (B) 2-sample *t* test analysis carried out using the average densitometry for each individual samples for the two categories. Significant difference (p -value < 0.001) in ITIH3 expression level was observed between normal versus cancer groups. (C) The receiver operating characteristics (ROC) curve using ITIH3 average densitometry reading for all individual plasma samples (167) screened in the study. ITIH3 sensitivity and specificity in detecting gastric cancer were estimated to be 96% and 66% at the cutoff point of the average densitometry reading of 111 305. (D) A box-plot showing the distribution of ITIH3 expression within normal, early and late stage gastric cancer. Analysis of variance (ANOVA) analysis performed showed that a significant difference (p -value < 0.001) in ITIH3 expression level was observed between normal versus early stage and late stage gastric cancer groups.

Figure 4.

(A) An illustration of ITIH3 expression level in normal tissue, in which ITIH3 expression was found to be randomly expressed in discrete individual cell across the tissue, as shown by the arrows in the figure. (B) An example of ITIH3 expression in tumor tissue, where ITIH3 expression was widespread in many cancer cells. (C) ITIH3 expression level in a panel of gastric cancer cell lines, compared to the normal gastric cell line, HFE145. The dotted line in the figure highlighted ITIH3 expression level in normal gastric cell line HFE145.

Table 1

Thirty-one Proteins That Showed Differences in Expression Levels between at Least 1 Tumor Loads Compared to Control^a

Gene symbol	proteinname	iTRAQ ratio of low tumor load: control			iTRAQ ratio of mid tumor load: control			iTRAQ ratio of high tumor load: control		
		iTRAQ replicate 1	iTRAQ replicate 2	iTRAQ replicate 3	iTRAQ replicate 1	iTRAQ replicate 2	iTRAQ replicate 3	iTRAQ replicate 1	iTRAQ replicate 2	iTRAQ replicate 3
Rbp4	Retinol-binding protein 4	2.40	2.78	2.59	3.75	4.66	4.11	3.09	3.71	3.67
ApoE*	Serum amyloid P-component	0.68	0.60	0.59	0.87	0.84	0.86	1.26	1.11	1.20
Serpina1d	Alpha-1-antitrypsin 1-4	0.79	0.69	0.75	0.67	0.64	0.65	0.84	0.69	0.74
Fgg*	Putative uncharacterized protein	0.83	0.75	0.74	0.66	0.56	0.61	1.36	1.20	1.18
Saa4	Serum amyloid A-4 protein	0.91	0.83	0.89	0.74	0.68	0.73	0.93	0.84	0.93
F13a1	Coagulation factor XIII A chain	0.98	0.94	0.88	0.64	0.67	0.59	1.17	1.16	1.13
Fgb	Fibrinogen beta chain	0.76	0.79	0.83	0.58	0.57	0.65	1.16	1.16	1.28
Fga	fibrinogen, alpha polypeptide isoform 1	0.80	0.73	0.78	0.60	0.54	0.64	1.29	1.14	1.27
Serpina3k	Serine protease inhibitor A3K	1.06	0.95	1.03	0.54	0.41	0.63	0.81	0.70	0.78
Egfr	Epidermal growth factor receptor	1.12	1.24	1.08	0.61	0.68	0.55	0.74	0.78	0.63
Car2	Carbonic anhydrase 2	1.13	1.02	1.07	1.48	1.46	1.61	1.03	0.87	1.09
Hba-a2;Hba-al	Hemoglobin subunit alpha	0.90	0.92	1.01	1.33	1.56	1.45	0.82	0.69	0.84
Hba-a2;Hba-al	hemoglobin alpha, adult chain 2	0.86	0.78	0.87	1.35	1.66	1.35	0.69	0.65	0.87
Hbb-b1	Hemoglobin subunit beta-1	0.93	1.42	0.90	1.50	2.39	1.46	0.68	0.96	0.65
Hbb-b2	Hemoglobin subunit beta-2	0.99	1.00	0.99	1.59	1.49	1.49	0.68	0.70	0.64
Lift	Isoform 2 of Leukemia inhibitory factor receptor	1.01	0.99	0.95	0.58	0.54	0.53	0.37	0.33	0.34
C9	complement component 9	0.96	0.95	0.86	0.58	0.53	0.57	0.61	0.55	0.57
Lum*	Lumican	1.01	0.95	1.03	0.96	0.82	0.90	0.74	0.67	0.68
Mbl2	Mannose-binding protein C	0.93	0.95	0.93	0.88	0.91	0.87	0.66	0.67	0.64
ApoE4*	Apolipoprotein C-IV	1.09	1.08	0.98	1.04	1.08	1.03	0.76	0.68	0.73
Gp1d1*	glycosylphosphatidylinositol specific phospholipase D1	1.10	1.09	1.12	1.08	1.06	1.12	0.69	0.54	0.57
ApoC1*	Apolipoprotein C-1	0.86	0.84	0.78	0.83	0.75	0.86	0.70	0.66	0.74
Igfals*	Insulin-like growth factor-binding proteininfactor-binding proteincomplex labile chain	1.23	1.01	1.24	0.91	0.75	0.89	0.76	0.59	0.69
2610016E04Rik	hypothetical protein LOC100039008	1.50	5.66	0.73	0.49	0.87	0.34	0.21	0.39	0.17

Gene symbol	proteinname	iTRAQ ratio of low tumor load: control			iTRAQ ratio of mid tumor load: control			iTRAQ ratio of high tumor load: control		
		iTRAQ replicate 1	iTRAQ replicate 2	iTRAQ replicate 3	iTRAQ replicate 1	iTRAQ replicate 2	iTRAQ replicate 3	iTRAQ replicate 1	iTRAQ replicate 2	iTRAQ replicate 3
Orm2	Alpha-1-acid glycoprotein 2	0.92	0.78	0.76	0.70	0.64	0.58	3.96	3.53	3.50
Ambp	Protein AMBP	0.80	1.82	0.87	1.37	2.54	0.75	2.85	4.69	1.72
Apod	Apolipoprotein D	0.88	0.86	0.83	1.08	1.08	1.11	1.45	1.52	1.72
Lrgl [†]	Leucine-rich alpha-2-glycoprotein 1	0.97	1.03	0.83	0.88	1.12	0.94	1.63	2.04	1.82
Itih1	Itih1 protein	1.14	1.09	1.07	1.15	1.20	1.13	1.65	1.65	1.82
Itih3*	Inter-alpha-trypsin inhibitor heavy chain H3	0.93	0.88	0.90	1.34	1.05	1.05	3.26	2.80	2.66
Itih2	Inter-alpha-trypsin inhibitor heavy chain H2	1.05	0.98	1.18	1.21	1.11	1.31	1.71	1.55	1.85

^aWithin the comparison between low, mid, or high tumor loads and control, a protein is considered to display expression difference (italicized) only when the ratios in all 3 iTRAQ replicates had the same trend and they satisfied the 1.3-fold cutoff. Other details such as *p*-value and error factor are tabulated in Supplementary Table 2. The 9 proteins that have the similar iTRAQ trends with human gastric cancer data set (unpublished data) were marked with (*).