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Cell Secretome Analysis Using Hollow Fiber Culture System Leads to the Discovery of CLIC1 Protein as a Novel Plasma Marker for Nasopharyngeal Carcinoma

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Nasopharyngeal carcinoma (NPC) is one of the most common malignant tumors in Southeast Asia. Unfortunately, most NPC victims have had metastasis when first diagnosed due to its deep location and vague symptoms. To date, discovery of sensitive and specific biomarkers for improving detection of NPC remains a challenge. Our previous study established a strategy for cell secretome analysis using a hollow fiber culture (HFC) system combined with liquid chromatography mass spectrometry. Herein, the above platform was used to collect NPC secretome for the discovery of relevant clinical biomarkers. Among 66 identified proteins, chloride intracellular channel 1 (CLIC1) was sieved out for intended use as a potential NPC biomarker candidate. Approximately 75% of NPC tissue specimens showed positive CLIC1 staining by IHC. The plasma levels of CLIC1 in NPC patients ($N = 70$), as presented by sandwich ELISA, were significantly higher than those in the healthy controls ($N = 74$) (mean $\pm$ SD, 16.38 $\pm$ 26.53 vs 2.39 $\pm$ 5.32 μg/mL; $p = 0.00005$). Using a cutoff point of 2.58 μg/mL, CLIC1 successfully discriminated NPC from the benign healthy control group with a sensitivity of 63% and a specificity of 77%. The area under the receiver operating characteristic curve was determined to be 0.74 (95% CI, 0.652−0.818). The statistical analysis of CLIC1 level in plasma shows that CLIC1 could be applied as a marker for early detection of NPC. This is the first report for the detection of CLIC1 as a plasma marker. Our results indicate that the analytical platform could provide a feasible strategy to profile tumor cell secretome for identifying cancer biomarkers, and CLIC1 may be a novel plasma tumor marker for NPC.

Keywords: CLIC1 • Nasopharyngeal carcinoma • Secretome • Tumor markers

Introduction

Nasopharyngeal carcinoma (NPC) is one of the most common cancers in southern China and Southeast Asia. The incidence rate of NPC among the Chinese, particularly the population of southern China, is approximately 100-fold higher than that of other countries.[1] NPC is commonly diagnosed late due to its deep location and vague symptoms.[2] Metastasis is found in 7% of patients at initial diagnosis and 20% or more develop metastasis after treatment.[3,4] Detection of the DNA and/or viral capsid antigen of NPC-associated Epstein−Barr virus (EBV) is the method currently used for serological diagnosis of NPC; however, these serological markers are lacking in sensitivity and specificity.[5−10] Therefore, the identification of novel biomarkers for NPC is critical for early detection and management of this disease.

The proteins secreted from cells are referred as secretome. In cancer progression, some particularly interesting proteins released from the tumor cells can enter into blood circulation. These proteins could be detected in serum or plasma and used as cancer screening markers. For instance, CA-125, a plasma marker, is currently the most widely used tumor marker for ovarian epithelial cancer.[11,12] Proteomics technology is a powerful tool for large-scale study of proteins. However,

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identification of these proteins from serum or plasma using proteomics analysis has inherent limitations. Serum and plasma proteomes predominantly consist of some high-abundance proteins, including albumin and immunoglobulins, which comprise over 90% of the proteome and could cause difficulties in identification and characterization of the low-abundance proteins.[13] Considering the complex nature of serum or plasma, examination of proteins released from cultured cells, as derived from specific cancer types, may represent an attractive strategy for marker discovery. The analysis of secretome obtained from cultured cells could be used to further design investigative methods for the discovery of diagnostic and prognostic markers in plasma and serum.

Previously, we have analyzed cancer cell secretome using a hollow fiber culture (HFC) system combined with liquid chromatography mass spectrometry.[14] The HFC system provides high-density 3D cell cultures that mimic solid tumors *in vivo*, and in addition, collects cell secretome with improved efficiency, which is critical when the concentrations of the secreted proteins are low. Currently, the above platform is being used to discover novel clinical biomarkers for NPC. Proteomics analysis was performed to identify the NPC cell secretome using SDS-PAGE, followed by nanoliquid chromatography tandem mass spectrometry (nano-HPLC-MS/MS). From bioinformatics analysis of 66 identified proteins, chloride intracellular channel 1 (CLIC1) appears to be a potential marker candidate and is further confirmed as a plasma marker in patients.

Materials and Methods

Patient Population and Clinical Specimens. Plasma samples were collected from 74 healthy controls (41 men and 33 women, ranging from 12 to 75 years of age, mean age 46.0 ± 13.3 years), 70 NPC patients (51 men and 19 women, ranging from 16 to 84 years of age, mean age 48.7 ± 14.1 years), 43 lung cancer patients (27 men and 16 women, ranging from 32 to 88 years of age, mean age 64.7 ± 15.7 years), and 45 colorectal carcinoma (CRC) patients (30 men and 15 women, ranging from 34 to 75 years of age, mean age 56.8 ± 14.9 years). The clinicopathological data of these patients are shown in Supplementary Table S1. All the blood samples were collected at Chang Gung Memorial Hospital (Lin-Kou, Taiwan, Republic of China) from 2003 to 2007. The study protocol was approved by the Medical Ethics and Human Clinical Trial Committee at Chang Gung Memorial Hospital. All patients entered into the study were informed of the testing situation when signing a consent form.

Blood samples were collected from the patients preoperatively following a standardized protocol. Plasma samples were prepared by collecting blood in EDTA tubes, and centrifuging at 2000*g* for 10 min at room temperature to pellet the cells. After centrifugation, samples were divided into 1.0-mL aliquots in sterile cryotubes and immediately frozen at −80 °C for storage until they would be used for the ELISA assays. The samples had undergone only one freeze/thaw cycle before the measurements were conducted.

Tumor specimens for the immunohistochemical analysis were obtained from 40 NPC patients diagnosed at the Chang Gung Memorial Hospital (Tao-Yuan, Taiwan) from 1996 to 2005. The clinicopathological data for these patients are shown in Supplementary Table S2.

Harvest of Conditioned Medium from NPC Cancer Cell Line Using the Hollow Fiber Culture (HFC) System. NPC-TW04 cell line was kindly provided by Dr. C.-T. Lin (Institute of Pathology, College of Medicine, National Taiwan University, Taipei, Taiwan).[15] Complete details for the procedures for the collection of conditioned medium are as previously described.[14] Briefly, the cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum and 1% antibiotics in a humidified incubator at 37 °C under 5% CO_2. The volume of serum medium was slowly reduced and replaced with serum-free medium consisting of RPMI 1640 with 10% SynQ (CCS Cell Culture Service, Hamburg, Germany) and 1% antibiotics. The cells were transferred to the HFC system following 2−3 passages after the cells were completely adapted to serum-free medium. Secretome samples in conditioned media from the extracapillary space (ECS) of HFC system were collected every 24 h, and the CM proteins of the first 2 days were combined for further analysis. Glucose and lactate concentrations were measured to monitor cell growth in the HFC system. The conditioned media harvested from ECS of the HFC system were concentrated using Amicon Ultra-15 tubes (molecular weight cutoff 3 kDa; Millipore, Billerica, MA). The protein concentrations of the secretome samples were determined using the Bradford assay (Bio-Rad, Hercules, CA).

Mass Spectrometric Analysis of Gel-Fractionated Proteins. Five micrograms of proteins obtained from conditioned medium in each lane was resolved on 12% SDS-PAGE visualized by silver staining. All visualizing bands were excised and digested in-gel with trypsin. The gel pieces were destained in a mixture of 20 μL of 30 mM potassium ferricyanide and 20 μL of 100 mM sodium thiosulfate for 10 min, and, subsequently, washed three times with 25 mM ammonium bicarbonate and 50% (v/v) acetonitrile (ACN)/25 mM ammonium bicarbonate. The gel pieces were reduced with 10 mM dithiothreitol (DTT) at 56 °C for 1 h and then alkylated with 55 mM iodoacetamide at room temperature for 45 min. The trypsin digestion was achieved by the addition of 10 μL of 0.01 μg/μL modified trypsin to the gel pieces and incubated overnight at 37 °C. The resulting peptides were extracted from the gel pieces with 50% (v/v) ACN/5% (v/v) formic acid and followed by nano-HPLC-MS/MS analysis to identify SDS-PAGE separated proteins. The tryptic digests were fractionated on a nano-HPLC system (LC Packings, Amsterdam, Netherlands) coupled with an ion trap mass spectrometer (LCQ DECA XP Plus, ThermoFinnigan, San Jose, CA) equipped with electrospray source. The chromatographic separation was achieved using a C18 microcapillary column (75 μm i.d. × 15 cm) at a flow rate of 0.02 μL/min with the mobile phase as 5% ACN/0.1% (v/v) formic acid (buffer A) and 80% ACN/0.1% (v/v) formic acid (buffer B). Chromatographic elution was performed using a 40 min gradient from 0 to 60% buffer B. The peptides eluted from the microcapillary column were electrosprayed into the ESI-MS/MS by application of distal 1.3 kV spraying voltage. Each cycle of one full scan mass spectrum (m/z 450−2000) was followed by three data dependent MS/MS acquisition.

Protein Identification and Database Search. The resulting MS/MS spectra of monoisotopic peptide masses were assigned and used for database searches performed with the Mascot search engine (version 2.1, Matrix Science, London, U.K.) against Swiss-Prot. The search parameters were restricted to the human taxonomy (134 728 sequences), allowing two missing tryptic cleavages, 1 Da peptide mass tolerance for both MS and MS/MS, and variable modifications of carbamidomethyl, deamidation, and oxidation. The Scaffold (version Scaffold-01_05_19, Proteome Software, Inc., Portland, OR) was used to improve protein identification. The acceptance criteria were

set as greater than 90.0% probability, as specified by the ProteinProphet algorithm,[16] and contained at least two identified peptides. The amino acid sequences of identified proteins were further analyzed by various software, SignalP 3.0 for the prediction of the presence of secretory signal peptide sequences,[17,18] SecretomeP 2.0 for divining the nonsignal peptide triggered protein secretion,[19] and TMHMM for the prediction of transmembrane helices in proteins.[20] The molecular functions of the proteins were classified by ProteinCenter software (Proxeon, Denmark). The gene expression profile of NPC-TW04-related data set GSM44776 obtained from the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/projects/geo/) was used as the basis for the selection of NPC biomarker candidates.

Western Blot Analysis. The anti-CLIC1 antibody produced in rabbits, as previously described,[21] was kindly provided by Dr. K. H. Lin (Department of Biochemistry, Chang Gung University, Taoyuan, Taiwan, Republic of China). Anti-alpha actin antibody (Chemicon) was used to evaluate cell leakage in conditioned medium, collected from ECS of the HFC system. Twenty micrograms of secreted proteins from conditioned media and cell extract was separated on 12% SDS-PAGE and transferred to PVDF membranes (Millipore). The membrane was blocked in a 5% nonfat milk solution for 1 h at room temperature. Afterward, the membranes were probed using anti-alpha actin and anti-CLIC1 antibody at 1:10 000 and 1:2000 dilutions, respectively, for 2 h at room temperature. Alpha-actin and CLIC1 were detected with alkaline phosphatase-conjugated goat anti-mouse and anti-rabbit IgG antibodies (Santa Cruz Biotechnology) at 1:5000 dilution for 50 min at room temperature and visualized with enhanced chemiluminescence detection.

Immunohistochemistry. Immunohistochemical staining was performed according to the procedures previously described.[22] Briefly, The tissue sections were dehydrated with ethanol, then heated in 0.01 M of citrate buffer (pH 6.0) for 45 min. Endogenous peroxidase activities were then inactivated in 3% hydrogen peroxide for 10 min at room temperature. The sections were further blocked with 3% normal goat serum in 0.2 M PBS (pH 7.4) followed by incubation with primary CLIC1 antibody for 2 h at room temperature. The primary antibody was detected using the Envision-kit (DAKO, Carpinteria, CA) by incubating the secondary anti-rabbit antibody-coated polymer peroxidase complexes (DAKO) for 30 min at room temperature, followed by treatment with substrate/chromogen (DAKO), and incubated for 1 min at room temperature. Slides were counterstained with hematoxylin for long-term storage. The simplified H score system was used to evaluate the expression of CLIC1,[23] which was based on the percentage of immunopositive stained cells: 0, 0−9% cells; 1, 10−49% cells; 2, 50−89% cells, and 3, ≥90%, and the intensity of the cell staining (3 (strong), 2 (moderate), 1 (weak), or 0 (no cell staining)). The two scores were multiplied, and divided by three to give a final score. A final score greater than 1 was defined as a positive staining.

Sandwich ELISA. White polystyrene 96-well microtiter plates (Corning Corp., Corning, NY) were coated with mouse anti-CLIC1 (Abnova Corp., Taipei, Taiwan) antibodies by incubation at 4000 ng/mL in PBS (50 μL in each well) for 1 h at 37 °C. After removing the antibody residue mixture, the plates were blocked by the addition of 200 μL per well of bovine serum albumin (BSA) (Sigma) (1 mg/mL in PBS) overnight at 4 °C. Five microliters of plasma samples from 70 NPC patients, 74 healthy controls, 43 lung cancer patients, and 45 colorectal carcinoma patients was added to 50 μL of PBS containing 1% BSA, and incubated for 1 h at 37 °C. A recombinant CLIC1 protein (Abnova Corp., Taipei, Taiwan) was used as a standard. Subsequently, the rabbit anti-CLIC1 antibody (1:200 dilution in PBS containing 1% BSA) was then applied and incubated for an additional 1 h at 37 °C. The alkaline phosphatase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA) (50 μL, diluted 2000-fold in PBS containing 1% BSA) was added and incubated for 1 h at 37 °C. The substrate 4-methylumbelliferyl phosphate (Molecular Probes, Eugene, OR) was diluted to 100 μM with an alkaline phosphatase buffer (6.05 g of Tris, 8.75 g of NaCl, and 0.05 g of $MgCl_2$ in 1000 mL of H_2O), and 100 μL was added to each well. The fluorescence was measured with a spectrofluorometer (Hidex, Turku, Finland) with excitation and emission wavelength set at 355 and 460 nm, respectively.

Statistical Analysis. This study used SPSS software (SPSS 12.0, Chicago, IL) and two-tailed tests, and a statistical significant p-value of less than 0.05 was applied in all tests. The Mann−Whitney U test and t test were used for ELISA group analysis. The receiver operating characteristic (ROC) curve was used to discriminate expression levels of CLIC1 in the plasma of the NPC patients and that of the healthy controls by plotting sensitivity versus (1 − specificity), and considering each observed value as a possible cutoff value. The area under the ROC curve (AUC) was calculated as an indicator for the likelihood of the NPC markers.[24,25]

Results and Discussion

Profiling of NPC Cell Secretome Collected from the HFC System. The proteins secreted from tumor cells *in vivo* are believed to enter the blood circulation.[26,27] These cell-type specific or tumor specific secreted proteins detected in serum or plasma have the potential to be used for the monitoring or screening of particular types of diseases. Our previous study established a platform using HFC system combined with liquid chromatography mass spectrometry for cell secretome analysis.[14] We have adopted this strategy to facilitate NPC biomarker discovery and the flowchart of the experimental design is illustrated in Figure 1. The NPC-TW04 cells adapted in serum-free medium were cultured in HFC system for conditioned medium (CM) collection. The secretome in CM was separated using a 12% SDS-PAGE, followed by visualization with silver staining (Figure 2A). Actin is one of the most highly conserved intracellular proteins. The presence of actin in the CM was used to accurately indicate that intracellular protein was released as a result of cellular lysis. The relative distribution of actin in CM and cell extract (CE) was examined by Western blot. As shown in Figure 2B, actin was distinctly revealed in the CE, but was not perceptible in the CM. The average cell number in HFC during these 2 days was 10^8, from which, ~170 μg of CM protein was obtained. The content of a single cell generally contains about 1 ng of protein, and actin protein accounts for 20% of the total proteome. Approximately 20 000 cells, the equivalent of 20 μg of CE, have lysed in CE. If 10^8 cells produced ~170 μg of CM protein in the HFC system, then a simple ratio would reveal that 20 μg of CM protein would be produced by 1.18×10^7 cells. If 1.18×10^7 cells represent 100% of the cells, then 20 000 cells yield 0.17%. From this, we calculate the cell death rate to be under 0.17% during the first 2 days in the HFC

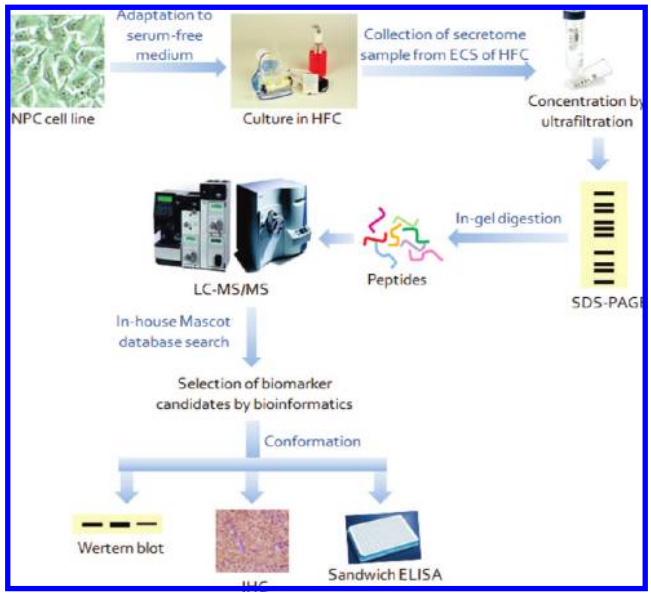

Figure 1. Outline of experimental workflow for NPC-TW04 cell secretome analysis, and potential biomarker selection.

system. These results verify that the release of proteins into the CM was not caused by cell lysis.

The 62 distinctive bands were individually excised for following tryptic digestion and LC-MS/MS analysis as described in Materials and Methods. The tandem mass spectra were searched against the Swiss-Port database using Mascot software for protein identification which had their probability further improved by the program Scaffold. Protein identification was accepted to be entered into the final list only if the identification established a probability greater than 90.0% based on the ProteinProphet algorithm and with at least two identified peptides. A total of 66 proteins identified fulfill the above requirement and are listed in Supplementary Table S3. Their peptide sequences and spectra are shown in Supplementary Table S4 and Supplementary Figure S1.

The NPC-TW04 cell secretome was previously described by Wu and co-workers.[22] The conditioned medium was harvested from the cells incubated in a serum-free medium using a conventional dish culture for 24 h for the collection of secreted proteins. A total of 32 proteins from the conditioned medium were identified using SDS-PAGE combined with MALDI-TOF MS. With the incorporation of different platforms and strategys, we have identified over 50% of those proteins reported by Wu et al. and the proteins were annotated in the Supplementary Table S3.

Distribution and Ontology Analysis of the Identified Proteins. To predict the secretion pathways, 66 identified proteins were further analyzed using SignalP 3.0, SecretomeP 2.0 and TMHMM to predict the possibility of protein secretion through classic secretion pathways or nonclassic secretion pathways and the presence of transmembrane domains in the protein sequence. According to the prediction, 56.1% (37/66) of the identified proteins could be released into CM of NPC-TW04 cells (Supplementary Table S3). Previously, the proteome of exosome, one of the protein secretion pathways, has been profiled.[28−30] An additional 15.2% (10/66) of identified proteins from NPC secretome were also found in exosome secretome as annotated in Supplementary Table S3. Collectively, 71.2% (47/66) of the identified proteins could be secreted from NPC cells via the various mechanisms.

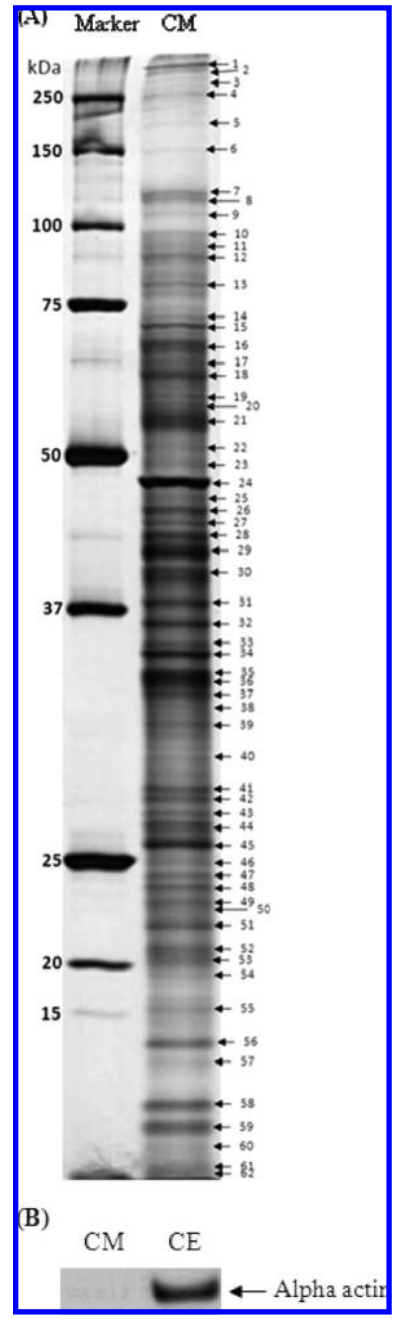

Figure 2. (A) SDS-PAGE analysis of NPC-TW04 cells conditioned medium harvested from the HFC system. The 62 visualized protein bands were marked, numbered, and individually excised for tryptic digestion and LC-MS/MS analysis. Lane Secretome denotes conditioned medium from HFC system. Lane Marker, molecular weight markers. (B) Proteins (20 μg) from the conditioned medium (CM) and cell extracts (CE) underwent Western blot analysis using an anti-alpha actin antibody.

The molecular functions of the identified proteins were classified by ProteinCenter software as shown in Figure 3. Among the 66 identified proteins, 81.8% (54/66) and 71.2% (47/66) proteins possessed molecular functions related to catalytic activity and protein binding, respectively. The proteins classified with catalytic activity possess specific binding sites for substrates and, thus, cause the catalysis for a biochemical reaction. For instance, members of the cathepsin family, including serine, cysteine or aspartate protease, are often found in lysosomes and become activated at low pH. They play

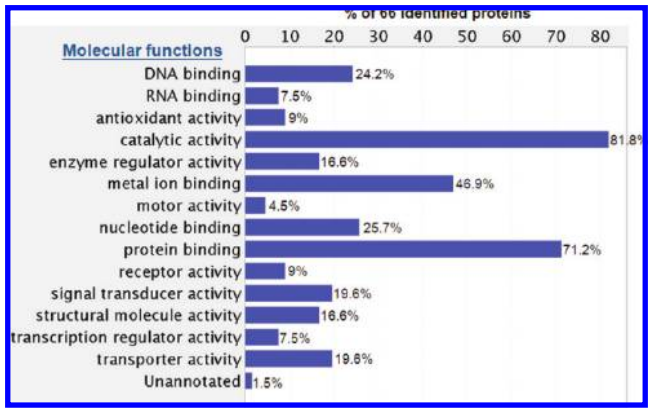

Figure 3. Classification of 66 identified proteins by the molecular functions.

important roles in cancer progression. Many members of the cathepsin family are identified by the strategy applied in the current study. Cathepsin A is a tumor-associated enzyme, and it may function together with other proteinases in extracellular proteolysis. Following its secretion from tumor or tumor-associated cells, it may participate in malignant transformation and metastasis.[31] Cathepsin B expression is increased in many human cancers at mRNA, protein expression and activity levels. Cathepsin B is also secreted from tumor cells, a mechanism likely to be facilitated by lysosomal exocytosis or extracellular processing of surface activators. Increased expression of cathepsin B suggests that this enzyme might have pro-apoptotic features, and has been linked with tumor invasion and metastasis.[32] During tumor progression, cathepsin D can directly or indirectly stimulate cancer cells proliferation and tumor angiogenesis, as well as independently of its proteolytic activity, might provide some protection against tumor apoptosis by proteolysis.[33] Both galectin-3-binding protein and E-cadherin among the 47 identified proteins exhibit the protein binding activity, which indicates that these proteins can selectively interact with other proteins or protein complexes. Galectin-3-binding protein is a secreted glycoprotein, known to enhance both cell−cell and cell−extracellular matrix adhesion through its ability to bind collagens, fibronectin, and nidogen.[34,35] However, the exact functions are not well-understood. E-cadherin is a transmembrane glycoprotein, calcium-dependent cell−cell adhesion molecule, as well as the principal component of adherence junctions in epithelial cells. Intercellular adhesion through E-cadherin is controlled by its association with the catenin family proteins. These associations contribute to the regulation of diverse cellular processes, such as cell polarization, aggregation, and migration.[36] During the process of cancer development, down-regulation of E-cadherin played a crucial role in the progression of a well-differentiated adenoma to an invasive carcinoma.[37] E-cadherin is therefore considered to act as a tumor suppressor.[38]

Selection of Potential NPC Biomarker. To discriminate potential NPC markers among the 66 identified proteins, the proteins whose mRNA levels were over 2-fold in NPC-TW04 cell line compared with those in normal nasal epithelia were selected as primary candidates. The cDNA microarray data set GSM44776, which compared the gene expression levels of NPC-TW04 cells versus normal nasal mucosal epithelia and was obtained from Gene Expression Omnibus (GEO) database of National Center for Biotechnology Information (NCBI),[39] was used to distinguish the gene expression levels of 66 identified proteins in our study. With the use of 2-fold overexpression as a criteria, 14 proteins whose mRNA levels were recognized, including alpha-enolase, annexin A1, aspartate aminotransferase, chloride intracellular channel protein 1 (CLIC1), collagen alpha-1(VII) chain, DNA damage-binding protein 1, epithelial-cadherin, farnesyl pyrophosphate synthetase, glutathione synthetase, L-lactate dehydrogenase B chain, serpin B6, triosephosphate isomerase, transketolase, and transforming growth factor-beta-induced protein ig-h3 (Table 1).

The proteins alpha-enolase, DNA damage-binding protein 1, transforming growth factor-beta-induced protein ig-h3, transketolase, and triosephosphate isomerase were revealed in cell conditioned media in over 4 different types of cancers based on previous researches.[40−42] These proteins may be applied as general cancer screening markers, but not suitable for specific type of cancer. For instance, alpha-enolase (ENO1) has been considered as a diagnostic marker for variety types of cancers, including lung cancer, neuroblastoma, brain cancer, and oral cancer.[43−48]

The metabolism-related proteins, including aspartate aminotransferase (AST), farnesyl pyrophosphate synthetase, glutathione synthetase, and L-lactate dehydrogenase B chain, may cause the metabolic derangements in cancer cells. The altered metabolism of cancer cells is likely to imbue them with several proliferative, survival advantages, avoidance of apoptosis, and

Table 1. A Total of 14 Potential Tumor Marker Candidates for NPC

accession number	protein name	protein prob. (%)	unique peptide number	matched peptide number	gene name	molecular weight (amu)	cDNA microarray value
P06733	Alpha-enolase	100	8	17	ENO1	47152.2	2.56
P04083	Annexin A1	100	4	5	ANXA1	38697.9	2.13
P00505	Aspartate aminotransferase, mitochondrial	100	4	6	GOT2	47458.6	2.85
O00299	Chloride intracellular channel protein 1	98	2	2	CLIC1	26905.3	2.08
Q02388	Collagen alpha-1(VII) chain	95	2	3	COL7A1	295199.5	2.49
Q16531	DNA damage-binding protein 1	98	2	2	DDB1	126952.2	2.45
P12830	Epithelial-cadherin	100	2	2	CDH1	97439.6	2.05
P14324	Farnesyl pyrophosphate synthetase	100	2	5	FDPS	40515.6	3.13
P48637	Glutathione synthetase	100	5	8	GSS	52367.9	2.66
P07195	L-lactate dehydrogenase B chain	100	6	10	LDHB	36620.6	7.33
P35237	Serpin B6	98	2	2	SERPINB6	42572.7	2.21
Q15582	Transforming growth factor-beta-induced protein ig-h3	100	5	9	TGFBI	74664.9	2.49
P29401	Transketolase	100	6	16	TKT	67861.4	2.56
P60174	Triosephosphate isomerase	100	5	8	TPI1	26651.1	2.06

several vulnerabilities of cancer cells.[49] However, abnormal phenomena of metabolism are due to not only carcinogenesis, but also other factors, such as diet preference, cardiovascular disease, diabetes, and so forth. For example, low levels of AST are normally found in the blood, but when body tissue or an organ such as the heart or liver is ill or damaged, additional AST is released into the bloodstream. The amount of AST in the blood is directly related to the extent of the tissue damage. Higher LDH levels in blood has been reported to be associated with melanoma, leukemia, metastatic colon cancer, Ewing's sarcoma, and advanced NPC.[50−55] Therefore, these markers cannot function as diagnostic marker of a particular type of cancer.

The epithelial-cadherin had been reported to be the potential serum marker for diabetes, gastric carcinoma, and colorectal cancer.[56−58] The expression level of Serpin B6 was found up-regulated in serum proteome of gastric cancer patients compared with normal control.[59] In addition, annexin A1 had been detected in patient sera of idiopathic pulmonary fibrosis.[60] These proteins can be used as serum or plasma marker for screening or monitoring diseases, but lack the specificity for NPC. After eliminating the proteins that lack specificity for NPC, only two proteins, the chloride intracellular channel protein 1 (CLIC1) and collagen alpha-1(VII) chain (COL7A1), remained on our list. While COL7A1 cannot be detected in CM of NPC-TW04 or in cell lysate by Western blot (data not shown), CLIC1 remained as the most promising and the only candidate for further investigation as a potential novel biomarker of NPC.

CLIC1's protein sequence reveals that it is without a signal peptide and transmembrane domains, which was all predicted by the secretion prediction software (Table 1 and Supplementary Table S3). The exocytosis of membranous vesicles, called exosomes, was first described in professional antigen-presenting cells such as B-lymphocytes and dendritic cells,[61,62] and was later found to also occur in tumor cell lines, and presumably in a variety of different normal and transformed cell types.[63] Typical constituents of exosomes include ubiquitously expressed molecules such as intracellular metabolic enzymes (alpha enolase), cytoskeletal proteins (actin and moesin), and heat shock proteins.[30,64,65] Part of them could also be found in the NPC-TW04 secretome (Supplementary Table S3). Thus, it is possible that some of the proteins identified in this study could be released into conditioned media through the secretion of exosomes. Furthermore, it has been reported that CLIC1 could be detected in the exosomes of human urine (Supplementary Table S3).[30] We speculated that CLIC1 may be released to the CM of NPC-TW04 via exosome pathway. Although we can not completely rule out the possibility that some proteins may be released into the medium by cell death, the abundant cytosolic protein, alpha-actin, was barely detectable by Western blotting in CM from NPC-TW04.

Potential NPC Biomarker CLIC1. CLIC1, also known as NCC27, is a member of the CLIC family. The family defined by a C-terminal core segment of 230 amino acids has significant structural homology with glutathione-*S*-transferase[66] and contains seven members, including CLIC1−5, p64, and parchorin.[67] CLIC1 clearly functions as a chloride channel, much like other CLIC family members, and possesses the biological activities that regulate cell volume and acidity of intracellular organelles. CLIC1 exists in cells as an integral membrane protein as well as a soluble cytoplasm protein. These phenomena indicate that CLIC1 might cycle between membrane-inserted and soluble forms.[67]

The biological significance of CLIC1 in cancer progression remained to be understood; CLIC1, however, was found to participate in the development of Alzheimer's disease. A major characteristic of Alzheimer's disease is the accumulation of extracellular β-amyloid (Aβ) into plaques in the brain. Aβ promotes the acute translocation of CLIC1 from the cytoplasm to the microglia cell membrane, resulting in the appearance of an anion conductance within minutes. This conductance, in response to redox modulation by NADPH oxidase-derived reactive oxygen species (ROS), provides a feed-forward mechanism that facilitates sustained microglial ROS generation by the NAPDH oxidase in microglia.[68] It is plausible to presume that CLIC1 may also induce the generation of ROS during the process of carcinogenesis similar to what was discovered in the study for Alzheimer's disease. During the process of cancer progression, cancer cells can generate constitutively ROS, which are thought to promote cell proliferation, cell motility, invasion, metastasis and angiogenesis.[69,70] In terms of the evidence of a relationship between CLIC1 and ROS in Alzheimer's disease, whether the CLIC1 plays the same role in cancer is still unknown. Previous studies point out that overexpression of CLIC1 modulates cell division and/or antiapoptosis signaling,[71] and the CLIC1 protein and intracellular chloride channels have been implicated in modifying cell cycle, apoptosis, cell adhesion, and cell motility.[72] Actually, the definite role of CLIC1 in cancer progression is unclear at the present time.

Recently, Chen et al. suggested that CLIC1 is a novel potential prognostic and therapeutic target in gastric carcinoma. CLIC1 protein was significantly up-regulated in 67.9% (38 of 56) gastric carcinoma patients. The average of CLIC1 expression in those 56 patients was 1.95-fold in cancerous tissues as compared to those in the normal gastric mucosa. Elevated CLIC1 expression was strongly correlated with lymph node metastasis, lymphatic and perineural invasion, pathological staging, and poor survival rates.[21] In other types of cancers, the tissue levels of CLIC1 protein were estimated to be 1.8-fold in cervical carcinoma, 3.5-fold in liver cancer,[21] 3.3-fold in breast ductal,[68] and 1.98- to 3.04-fold in colorectal cancer.[73] CLIC1 were consistently overexpressed >3-fold in all colorectal cancer tissue samples, as compared with normal tissues by 2D-GE analysis. Furthermore, CLIC1 has been identified as a secreted protein of immature dendritic cells (the PRoteomics IDEntifications database, PRIDE, accession No. 8858−61), mature dendritic cells (PRIDE, accession No. 8875−6), and fibroblasts (PRIDE, accession No. 8931). Although CLIC1 is recognized as a potential tissue marker for various types of cancers and could be identified as a secreted protein of various cell lines, CLIC1 has not been proven as a potential blood marker for any type of disease.

Detection of CLIC1 in NPC Cell Conditioned Media and Human Tissue Samples. To determine the clinical relevance of our finding, the levels of chloride intracellular channel protein 1 (CLIC1) were further confirmed in CM of NPC cell lines and clinical specimens of NPC patients. As shown in Figure 4, CLIC1 was distinctly detected in the CM from the different types of NPC cell lines, including keratinizing carcinoma (NPC-TW02), undifferentiated carcinoma (NPC-TW04), and bone-marrow metastasized NPC (NPC-BM1), indicating CLIC1 could be secreted from different NPC cell types.

Following this, the Human Protein Atlas (HPA, version 5.0, http://www.proteinatlas.org/index.php) was consulted to evaluate the CLIC1 expression in human tissues.[74] There were two CLIC1 antibodies and eight head and neck cancer (HNC) tissues

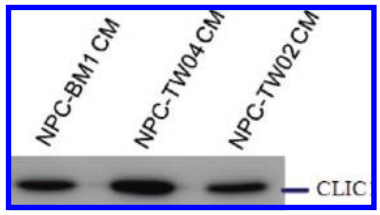

Figure 4. Western blot analysis of CLIC1 in three different type NPC cell conditioned media (CM). Twenty micrograms of proteins from conditioned media of each NPC cell lines was separated in 12% SDS gel, transferred onto a PVDF membrane, and then probed with CLIC1 antibody against the indicated target proteins. (NPC-TW02, NPC-TW04, and NPC-BM1 derived from keratinizing carcinoma, undifferentiated carcinoma, and metastasized tumor cells in the bone marrow, respectively.)

available in the HPA (Supplementary Figure S2). CLIC1 is weakly positive or negative staining in both normal nasopharynx and HNC tissues (Supplementary Figure S2). However, the eight HNC tissues in the HPA include five oral squamous carcinoma and three salivary adenocarcinoma tissues, not NPC tissues. Among most cancer tissues examined in the HPA, CLIC1 staining is also weakly positive or negative except for colon, liver, and gastric cancers (Supplementary Figure S2). It is notable that the CLIC1 staining patterns using both antibodies are inconsistent (Supplementary Figure S2); indeed, the two antibodies have gotten low scores for CLIC1 immunohistochemical analysis in the HPA.

The CLIC1 protein expression in 40 NPC tissue sections was further examined by immunohistochemistry. Positive CLIC1 staining was detected in 75% (30/40) of cancer tissue samples. Three representative cases of positive staining are shown in Figure 5 (left panels). The antibody selectively stained the cytoplasm of cancer cells, while showing little staining of adjacent noncancer epithelial cells in the majority of the samples examined (Figure 5, right panel). Among the 40 NPC tissue sections examined, 25 sections harbored noncancerous epithelial cells, 68.0% (17 sections) were negative for CLIC1 expression, 16.0% (4 sections) were weakly positive, and 16.0% (4 sections) were moderately positive. The observation infers that CLIC1 was highly expressed in NPC surgically removed samples, but rarely appeared in non-NPC tissues. Our results provide valuable evidence that the expressions of CLIC1 in cancerous and normal nasopharynx biopsies are significantly different which increases the possibility of CLIC1 to be a promising NPC biomarker candidate.

Evaluation of CLIC1 as a Plasma NPC Biomarker. As CLIC1 was overexpressed in NPC tissues and could be detected in the secretome of NPC cell lines, we speculated that we may be able to detect CLIC1 in plasma samples of NPC patients. To examine this possibility, a sensitive fluorimetric sandwich ELISA was established to detect the presence of CLIC1 in plasma. Plasma samples of 70 NPC patients and 74 healthy controls were examined. The plasma levels of CLIC1 were significantly higher in the 70 NPC patients than those of the 74 healthy controls (mean ± SD, 16.38 ± 26.53 vs 2.39 ± 5.32 μg/mL, $p = 0.00005$) (Figure 6A). A receiver operating characteristic (ROC) curve analysis was used to further evaluate the diagnostic efficacy of CLIC1. The area under the ROC curve (AUC) was determined to be 0.74 (95% CI, 0.652−0.818) for CLIC1 (Figure 6B). When a cutoff point of 2.58 μg/mL was chosen for CLIC1 to discriminate NPC patients from healthy controls, sensitivity and specificity values were 63% and 77%, respectively. There were

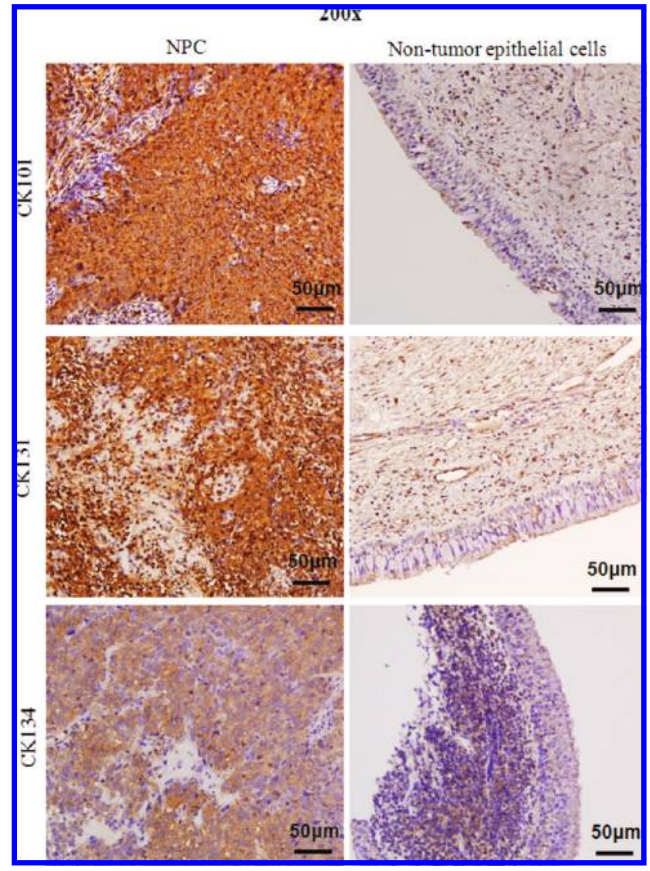

Figure 5. Immunohistochemical staining of CLIC1 in NPC tissues derived from 3 of 40 NPC patients. Tumor cells with positive for CLIC1 staining, as well as adjacent benign epithelial cells of with negative for CLIC1 staining. Original magnification: 200×.

no statistical differences between the plasma CLIC1 levels of the primary tumor stage T1/T2 ($N = 34$) and T3/T4 ($N = 36$) (13.3 ± 22.22 vs 19.28 ± 30.06 μg/mL, $p = 0.246$), nor is there any difference between lymph node metastasis stage N0/N1 ($N = 35$) and N2/N3 ($N = 35$) (14.2 ± 22.47 vs 18.55 ± 30.22 μg/mL, $p = 0.44$). The overall tumor satge I/II ($N = 22$) and III/IV ($N = 48$) (13.67 ± 26.13 vs 17.62 ± 26.89 μg/mL, $p = 0.198$) shows no statistical difference in this case-control study either (Figure 6C).

To test whether CLIC1 could be a marker specific to NPC, we have determined the plasma level of CLIC1 in patients suffering from other diseases, including lung cancer and colon cancer. Compared with healthy controls ($N = 70$, 2.39 ± 5.32 μg/mL), the plasma CLIC1 levels were not statistically different in the patients suffering from lung ($N = 43$, 4.15 ± 5.12 μg/mL, $p = 0.081$) and colon cancer ($N = 45$, 2.96 ± 4.92 μg/mL, $p = 0.557$) (Figure 6A). The results suggest that CLIC1 might represent a relatively specific marker for NPC. The potential of CLIC1 as a NPC marker remains to be evaluated using a larger series of plasma specimens, but these early results seem promising.

NPC produces few symptoms in its primary stage, and hence, most NPC patients are diagnosed at advanced stages during their first visit to an otorhinolaryngologist.[2] As an insidious tumor, NPC often metastasizes to lymph nodes, or other organs, before it is discovered, and distant metastasis is the leading cause of mortality for NPC patients.[4] Therefore, the plasma biomarker for early detection of NPC is critical for

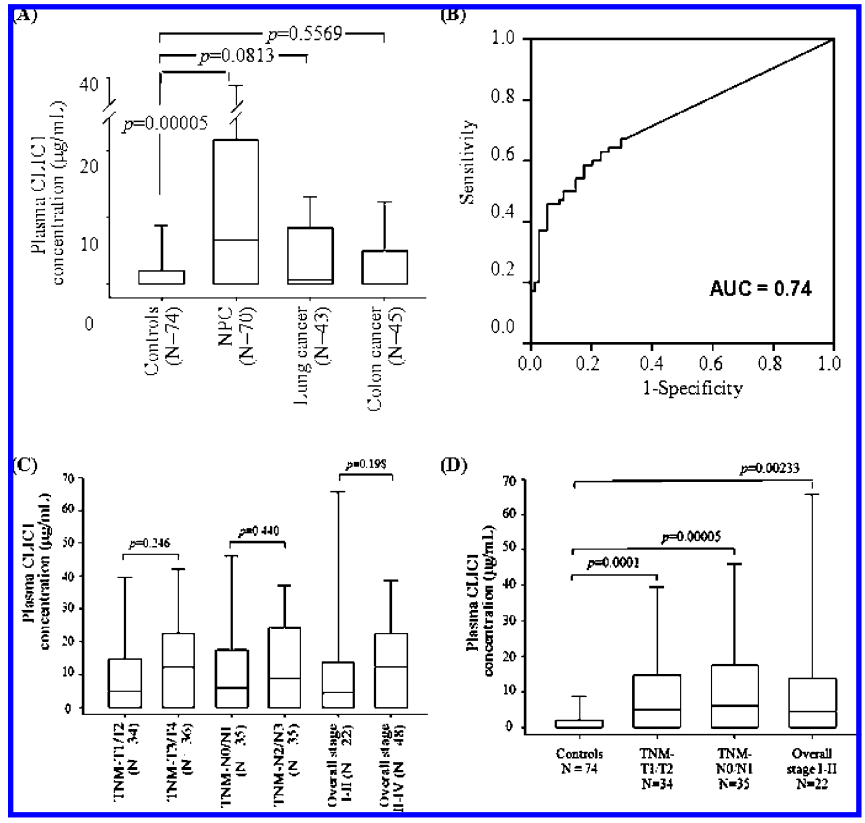

Figure 6. Elevated CLIC1 levels in NPC plasma samples. (A) The plasma CLIC1 levels of healthy controls ($N = 74$), NPC patients ($N = 70$), lung cancer patients ($N = 43$), and colon cancer patients ($N = 45$) were measured by sandwich ELISA using 5 μL of plasma samples. (B) ROC curve analysis of the diagnostic efficacy of CLIC1. (C) The correlation of CLIC1 plasma levels between the early and late TNM stages of NPC. (D) The correlation of CLIC1 plasma levels between the healthy controls and the early TNM stages of NPC.

management of this disease. Further analysis of the plasma CLIC1 level revealed that its level was significantly higher in the early stages of primary tumor (TNM-T1/T2, $N = 34$, 13.3 ± 22.22 μg/mL, $p = 0.0001$), lymph node metastasis (TNM-N0/N1, $N = 35$, 14.2 ± 22.47 μg/mL, $p = 0.00005$), and overall early TNM stage (TNM-I/II, $N = 22$, 13.67 ± 26.13 μg/mL, $p = 0.00233$) compared to the healthy controls ($N = 74$, 2.39 ± 5.32 μg/mL) (Figure 6D). The results suggest that CLIC1 could be a potential plasma marker for NPC early detection.

This is the first report for the detection of CLIC1 in blood samples and used for the purpose as NPC plasma marker. Our results suggest that CLIC1 may be a novel biomarker for NPC diagnosis, or prognosis, and even for early screening. The possible use of CLIC1 as an NPC biomarker remains to be evaluated with larger clinical tests.

Conclusion

The established strategy of the HFC system combined with proteomics technology for cell secretome analysis was used to discover NPC plasma biomarkers in this study. A total of 66 proteins were identified as released from NPC cells. With the assistance of bioinformatics analysis, CLIC1 was sieved out as a NPC biomarker candidate, and was further demonstrated in the expression levels of tissue and plasma samples. Approximately 75% of the examined NPC tumor tissue specimens were positive for CLIC1 staining, and the plasma levels of CLIC1 were significantly higher in the NPC patients, as compared to the healthy controls. The AUC was determined to be 0.74 for CLIC1 and sensitivity and specificity values were 63% and 77%, respectively, with threshold set at 2.58 μg/mL. The correlation between the CLIC1 plasma levels of the healthy controls and that of the NPC patients with early TNM stages had statistically significant differences. The outcome may illustrate how CLIC1 could apply as an early screening marker. This is the first time that CLIC1 was identified by proteomics analysis of NPC cell secretome collected with the HFC system and also detected in plasma levels. These results collectively support that the analytical platform of NPC cell secretome could be a practical tactic for NPC plasma biomarker discovery, and in addition, exhibited that CLIC1 may represent a target for further development of diagnostic markers and/or therapeutic strategies for NPC.

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Supporting Information Available: Figures of the peptide spectra of 66 identified proteins and CLIC1 expression profiles in the Human Protein Atlas using CAB020825 and CAB020825 antibodies. Tables of clinicopathological characteristics of cancer patients and healthy controls in CLIC1 ELISA, characteristics of NPC patients in CLIC1 immunohistochemical

analysis, complete list of 66 identified proteins, and the peptides information of 66 identified proteins. this material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Chan, A. T.; Teo, P. M.; Johnson, P. J. Nasopharyngeal carcinoma. *Ann. Oncol.* **2002**, *13* (7), 1007–15.

(2) Wei, Y. S.; Zheng, Y. H.; Liang, W. B.; Zhang, J. Z.; Yang, Z. H.; Lv, M. L.; Jia, J.; Zhang, L. Identification of serum biomarkers for nasopharyngeal carcinoma by proteomic analysis. *Cancer* **2008**, *112* (3), 544–51.

(3) Liao, Q.; Zhao, L.; Chen, X.; Deng, Y.; Ding, Y. Serum proteome analysis for profiling protein markers associated with carcinogenesis and lymph node metastasis in nasopharyngeal carcinoma. *Clin. Exp. Metastasis* **2008**, *25* (4), 465–76.

(4) Wang, C. T.; Cao, K. J.; Li, Y.; Xie, G. F.; Huang, P. Y. [Prognosis analysis of nasopharyngeal carcinoma patients with distant metastasis]. *Aizheng* **2007**, *26* (2), 212–5.

(5) Cho, W. C. Nasopharyngeal carcinoma: molecular biomarker discovery and progress. *Mol. Cancer* **2007**, *6*, 1.

(6) Gallagher, A.; Armstrong, A. A.; MacKenzie, J.; Shield, L.; Khan, G.; Lake, A.; Proctor, S.; Taylor, P.; Clements, G. B.; Jarrett, R. F. Detection of Epstein-Barr virus (EBV) genomes in the serum of patients with EBV-associated Hodgkin's disease. *Int. J. Cancer* **1999**, *84* (4), 442–8.

(7) Gan, Y. J.; Sullivan, J. L.; Sixbey, J. W. Detection of cell-free Epstein-Barr virus DNA in serum during acute infectious mononucleosis. *J. Infect. Dis.* **1994**, *170* (2), 436–9.

(8) Lei, K. I.; Chan, L. Y.; Chan, W. Y.; Johnson, P. J.; Lo, Y. M. Diagnostic and prognostic implications of circulating cell-free Epstein-Barr virus DNA in natural killer/T-cell lymphoma. *Clin. Cancer Res.* **2002**, *8* (1), 29–34.

(9) Lo, Y. M.; Chan, W. Y.; Ng, E. K.; Chan, L. Y.; Lai, P. B.; Tam, J. S.; Chung, S. C. Circulating Epstein-Barr virus DNA in the serum of patients with gastric carcinoma. *Clin. Cancer Res.* **2001**, *7* (7), 1856–9.

(10) Yamamoto, M.; Kimura, H.; Hironaka, T.; Hirai, K.; Hasegawa, S.; Kuzushima, K.; Shibata, M.; Morishima, T. Detection and quantification of virus DNA in plasma of patients with Epstein-Barr virus-associated diseases. *J. Clin. Microbiol.* **1995**, *33* (7), 1765–8.

(11) Bast, R. C., Jr.; Xu, F. J.; Yu, Y. H.; Barnhill, S.; Zhang, Z.; Mills, G. B. CA 125: the past and the future. *Int. J. Biol. Markers* **1998**, *13* (4), 179–87.

(12) Duffy, M. J.; Bonfrer, J. M.; Kulpa, J.; Rustin, G. J.; Soletormos, G.; Torre, G. C.; Tuxen, M. K.; Zwirner, M. CA125 in ovarian cancer: European Group on Tumor Markers guidelines for clinical use. *Int. J. Gynecol. Cancer* **2005**, *15* (5), 679–91.

(13) Anderson, N. L.; Anderson, N. G.; Haines, L. R.; Hardie, D. B.; Olafson, R. W.; Pearson, T. W. Mass spectrometric quantitation of peptides and proteins using stable isotope standards and capture by anti-peptide antibodies (SISCAPA). *J. Proteome Res.* **2004**, *3* (2), 235–44.

(14) Wu, H. Y.; Chang, Y. H.; Chang, Y. C.; Liao, P. C. Proteomics analysis of nasopharyngeal carcinoma cell secretome using a hollow fiber culture system and mass spectrometry. *J. Proteome Res.* **2009**, *8* (1), 380–9.

(15) Lin, C. T.; Chan, W. Y.; Chen, W.; Huang, H. M.; Wu, H. C.; Hsu, M. M.; Chuang, S. M.; Wang, C. C. Characterization of seven newly established nasopharyngeal carcinoma cell lines. *Lab. Invest.* **1993**, *68* (6), 716–27.

(16) Nesvizhskii, A. I.; Keller, A.; Kolker, E.; Aebersold, R. A statistical model for identifying proteins by tandem mass spectrometry. *Anal. Chem.* **2003**, *75* (17), 4646–58.

(17) Bendtsen, J. D.; Nielsen, H.; von Heijne, G.; Brunak, S. Improved prediction of signal peptides: SignalP 3.0. *J. Mol. Biol.* **2004**, *340* (4), 783–95.

(18) Nielsen, H.; Krogh, A. Prediction of signal peptides and signal anchors by a hidden Markov model. *Proc. Int. Conf. Intell. Syst. Mol. Biol.* **1998**, *6*, 122–30.

(19) Bendtsen, J. D.; Jensen, L. J.; Blom, N.; Von Heijne, G.; Brunak, S. Feature-based prediction of non-classical and leaderless protein secretion. *Protein Eng. Des. Sel.* **2004**, *17* (4), 349–56.

(20) Moller, S.; Croning, M. D.; Apweiler, R. Evaluation of methods for the prediction of membrane spanning regions. *Bioinformatics* **2001**, *17* (7), 646–53.

(21) Chen, C. D.; Wang, C. S.; Huang, Y. H.; Chien, K. Y.; Liang, Y.; Chen, W. J.; Lin, K. H. Overexpression of CLIC1 in human gastric carcinoma and its clinicopathological significance. *Proteomics* **2007**, *7* (1), 155–67.

(22) Wu, C. C.; Chien, K. Y.; Tsang, N. M.; Chang, K. P.; Hao, S. P.; Tsao, C. H.; Chang, Y. S.; Yu, J. S. Cancer cell-secreted proteomes as a basis for searching potential tumor markers: nasopharyngeal carcinoma as a model. *Proteomics* **2005**, *5* (12), 3173–82.

(23) Ravn, V.; Rasmussen, B. B.; Hojholt, L.; Barfoed, M.; Heiberg, I.; Thorpe, S. M. Reproducibility of subjective immunohistochemical estrogen- and progesterone receptor determination in human endometrium. *Pathol. Res. Pract.* **1993**, *189* (9), 1015–22.

(24) Hanley, J. A.; McNeil, B. J. The meaning and use of the area under a receiver operating characteristic (ROC) curve. *Radiology* **1982**, *143* (1), 29–36.

(25) Zweig, M. H.; Campbell, G. Receiver-operating characteristic (ROC) plots: a fundamental evaluation tool in clinical medicine. *Clin. Chem.* **1993**, *39* (4), 561–77.

(26) Bjorndahl, M.; Cao, R.; Eriksson, A.; Cao, Y. Blockage of VEGF-induced angiogenesis by preventing VEGF secretion. *Circ. Res.* **2004**, *94* (11), 1443–50.

(27) Wahrenbrock, M. G.; Varki, A. Multiple hepatic receptors cooperate to eliminate secretory mucins aberrantly entering the bloodstream: are circulating cancer mucins the "tip of the iceberg. *Cancer Res.* **2006**, *66* (4), 2433–41.

(28) Mears, R.; Craven, R. A.; Hanrahan, S.; Totty, N.; Upton, C.; Young, S. L.; Patel, P.; Selby, P. J.; Banks, R. E. Proteomic analysis of melanoma-derived exosomes by two-dimensional polyacrylamide gel electrophoresis and mass spectrometry. *Proteomics* **2004**, *4* (12), 4019–31.

(29) Nickel, W. The mystery of nonclassical protein secretion. A current view on cargo proteins and potential export routes. *Eur. J. Biochem.* **2003**, *270* (10), 2109–19.

(30) Pisitkun, T.; Shen, R. F.; Knepper, M. A. Identification and proteomic profiling of exosomes in human urine. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101* (36), 13368–73.

(31) Kozlowski, L.; Wojtukiewicz, M. Z.; Ostrowska, H. Cathepsin A activity in primary and metastatic human melanocytic tumors. *Arch. Dermatol. Res.* **2000**, *292* (2−3), 68–71.

(32) Podgorski, I.; Sloane, B. F. Cathepsin B and its role(s) in cancer progression. *Biochem. Soc. Symp.* **2003**, (70), 263–76.

(33) Milton, R. H.; Abeti, R.; Averaimo, S.; DeBiasi, S.; Vitellaro, L.; Jiang, L.; Curmi, P. M.; Breit, S. N.; Duchen, M. R.; Mazzanti, M. CLIC1 function is required for beta-amyloid-induced generation of reactive oxygen species by microglia. *J. Neurosci.* **2008**, *28* (45), 11488–99.

(34) Sasaki, T.; Brakebusch, C.; Engel, J.; Timpl, R. Mac-2 binding protein is a cell-adhesive protein of the extracellular matrix which self-assembles into ring-like structures and binds beta1 integrins, collagens and fibronectin. *EMBO J.* **1998**, *17* (6), 1606–13.

(35) Ulmer, T. A.; Keeler, V.; Loh, L.; Chibbar, R.; Torlakovic, E.; Andre, S.; Gabius, H. J.; Laferte, S. Tumor-associated antigen 90K/Mac-2-binding protein: possible role in colon cancer. *J. Cell. Biochem.* **2006**, *98* (5), 1351–66.

(36) Gumbiner, B. M. Cell adhesion: the molecular basis of tissue architecture and morphogenesis. *Cell* **1996**, *84* (3), 345–57.

(37) Perl, A. K.; Wilgenbus, P.; Dahl, U.; Semb, H.; Christofori, G. A causal role for E-cadherin in the transition from adenoma to carcinoma. *Nature* **1998**, *392* (6672), 190–3.

(38) Birchmeier, W.; Behrens, J. Cadherin expression in carcinomas: role in the formation of cell junctions and the prevention of invasiveness. *Biochim. Biophys. Acta* **1994**, *1198* (1), 11–26.

(39) Lee, Y. C.; Hwang, Y. C.; Chen, K. C.; Lin, Y. S.; Huang, D. Y.; Huang, T. W.; Kao, C. Y.; Wu, H. C.; Lin, C. T.; Huang, C. Y. Effect of Epstein-Barr virus infection on global gene expression in nasopharyngeal carcinoma. *Funct. Integr. Genomics* **2007**, *7* (1), 79–93.

(40) Weng, L. P.; Wu, C. C.; Hsu, B. L.; Chi, L. M.; Liang, Y.; Tseng, C. P.; Hsieh, L. L.; Yu, J. S. Secretome-based identification of Mac-2 binding protein as a potential oral cancer marker involved in cell growth and motility. *J. Proteome Res.* **2008**, *7* (9), 3765–75.

(41) Wu, C. C.; Chen, H. C.; Chen, S. J.; Liu, H. P.; Hsieh, Y. Y.; Yu, C. J.; Tang, R.; Hsieh, L. L.; Yu, J. S.; Chang, Y. S. Identification of collapsin response mediator protein-2 as a potential marker of colorectal carcinoma by comparative analysis of cancer cell secretomes. *Proteomics* **2008**, *8* (2), 316–32.

(42) Pardo, M.; Garcia, A.; Antrobus, R.; Blanco, M. J.; Dwek, R. A.; Zitzmann, N. Biomarker discovery from uveal melanoma secretomes: identification of gp100 and cathepsin D in patient serum. *J. Proteome Res.* **2007**, *6* (7), 2802–11.

(43) Cooper, E. H. Neuron-specific enolase. *Int. J. Biol. Markers* **1994**, *9* (4), 205–10.

(44) Kato, K.; Satoh, T. Changes in the concentration of enolase isozymes and S-100 protein in degenerating and regenerating rat sciatic nerve. *J. Neurochem.* **1983**, *40* (4), 1076–81.

(45) Niklinski, J.; Furman, M. Clinical tumour markers in lung cancer. *Eur. J. Cancer Prev.* **1995**, *4* (2), 129–38.
(46) Pahlman, S.; Esscher, T.; Bergh, J.; Steinholtz, L.; Nou, E.; Nilsson, K. Neuron-specific enolase as a marker for neuroblastoma and small-cell carcinoma of the lung. *Tumour Biol.* **1984**, *5* (2), 119–26.
(47) Royds, J. A.; Parsons, M. A.; Taylor, C. B.; Timperley, W. R. Enolase isoenzyme distribution in the human brain and its tumours. *J. Pathol.* **1982**, *137* (1), 37–49.
(48) Ito, S.; Honma, T.; Ishida, K.; Wada, N.; Sasaoka, S.; Hosoda, M.; Nohno, T. Differential expression of the human alpha-enolase gene in oral epithelium and squamous cell carcinoma. *Cancer Sci.* **2007**, *98* (4), 499–505.
(49) Hsu, P. P.; Sabatini, D. M. Cancer cell metabolism: Warburg and beyond. *Cell* **2008**, *134* (5), 703–7.
(50) Agarwala, S. S.; Keilholz, U.; Gilles, E.; Bedikian, A. Y.; Wu, J.; Kay, R.; Stein, C. A.; Itri, L. M.; Suciu, S.; Eggermont, A. M. LDH correlation with survival in advanced melanoma from two large, randomised trials (Oblimersen GM301 and EORTC 18951). *Eur. J. Cancer* **2009**, *45* (10), 1807–14.
(51) Hafiz, M. G.; Mannan, M. A. Serum lactate dehydrogenase level in childhood acute lymphoblastic leukemia. *Bangladesh Med. Res. Counc. Bull.* **2007**, *33* (3), 88–91.
(52) Hafiz, M. G.; Rahman, M. M.; Mannan, M. A. Serum lactate dehydrogenase as a prognostic marker of childhood acute lymphoblastic leukemia. *Mymensingh Med. J.* **2008**, *17* (2), 169–73.
(53) Azuma, M.; Shi, M.; Danenberg, K. D.; Gardner, H.; Barrett, C.; Jacques, C. J.; Sherod, A.; Iqbal, S.; El-Khoueiry, A.; Yang, D.; Zhang, W.; Danenberg, P. V.; Lenz, H. J. Serum lactate dehydrogenase levels and glycolysis significantly correlate with tumor VEGFA and VEGFR expression in metastatic CRC patients. *Pharmacogenomics* **2007**, *8* (12), 1705–13.
(54) Bacci, G.; Balladelli, A.; Forni, C.; Longhi, A.; Serra, M.; Fabbri, N.; Alberghini, M.; Ferrari, S.; Benassi, M. S.; Picci, P. Ewing's sarcoma family tumours. Differences in clinicopathological characteristics at presentation between localised and metastatic tumours. *J. Bone Joint Surg. Br.* **2007**, *89* (9), 1229–33.
(55) Turen, S.; Ozyar, E.; Altundag, K.; Gullu, I.; Atahan, I. L. Serum lactate dehydrogenase level is a prognostic factor in patients with locoregionally advanced nasopharyngeal carcinoma treated with chemoradiotherapy. *Cancer Invest.* **2007**, *25* (5), 315–21.
(56) Chan, A. O.; Lam, S. K.; Chu, K. M.; Lam, C. M.; Kwok, E.; Leung, S. Y.; Yuen, S. T.; Law, S. Y.; Hui, W. M.; Lai, K. C.; Wong, C. Y.; Hu, H. C.; Lai, C. L.; Wong, J. Soluble E-cadherin is a valid prognostic marker in gastric carcinoma. *Gut* **2001**, *48* (6), 808–11.
(57) Jiang, H.; Guan, G.; Zhang, R.; Liu, G.; Cheng, J.; Hou, X.; Cui, Y. Identification of urinary soluble E-cadherin as a novel biomarker for diabetic nephropathy. *Diabetes Metab. Res. Rev.* **2009**, *25* (3), 232–41.
(58) Wilmanns, C.; Grossmann, J.; Steinhauer, S.; Manthey, G.; Weinhold, B.; Schmitt-Graff, A.; von Specht, B. U. Soluble serum E-cadherin as a marker of tumour progression in colorectal cancer patients. *Clin. Exp. Metastasis* **2004**, *21* (1), 75–8.
(59) Liu, C.; Liang, Y.; Wang, H.; Pan, C. Application of proteome technology in screening biomarkers associated with gastric cancer. *Chin. Ger. J. Clin. Oncol.* **2008**, *7* (7), 394–7.
(60) Kurosu, K.; Takiguchi, Y.; Okada, O.; Yumoto, N.; Sakao, S.; Tada, Y.; Kasahara, Y.; Tanabe, N.; Tatsumi, K.; Weiden, M.; Rom, W. N.; Kuriyama, T. Identification of annexin 1 as a novel autoantigen in acute exacerbation of idiopathic pulmonary fibrosis. *J. Immunol.* **2008**, *181* (1), 756–67.
(61) Zitvogel, L.; Regnault, A.; Lozier, A.; Wolfers, J.; Flament, C.; Tenza, D.; Ricciardi-Castagnoli, P.; Raposo, G.; Amigorena, S. Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell-derived exosomes. *Nat. Med.* **1998**, *4* (5), 594–600.
(62) Escola, J. M.; Kleijmeer, M. J.; Stoorvogel, W.; Griffith, J. M.; Yoshie, O.; Geuze, H. J. Selective enrichment of tetraspan proteins on the internal vesicles of multivesicular endosomes and on exosomes secreted by human B-lymphocytes. *J. Biol. Chem.* **1998**, *273* (32), 20121–7.
(63) Thery, C.; Zitvogel, L.; Amigorena, S. Exosomes: composition, biogenesis and function. *Nat. Rev. Immunol.* **2002**, *2* (8), 569–79.
(64) Thery, C.; Boussac, M.; Veron, P.; Ricciardi-Castagnoli, P.; Raposo, G.; Garin, J.; Amigorena, S. Proteomic analysis of dendritic cell-derived exosomes: a secreted subcellular compartment distinct from apoptotic vesicles. *J. Immunol.* **2001**, *166* (12), 7309–18.
(65) Van Niel, G.; Raposo, G.; Candalh, C.; Boussac, M.; Hershberg, R.; Cerf-Bensussan, N.; Heyman, M. Intestinal epithelial cells secrete exosome-like vesicles. *Gastroenterology* **2001**, *121* (2), 337–49.
(66) Harrop, S. J.; DeMaere, M. Z.; Fairlie, W. D.; Reztsova, T.; Valenzuela, S. M.; Mazzanti, M.; Tonini, R.; Qiu, M. R.; Jankova, L.; Warton, K.; Bauskin, A. R.; Wu, W. M.; Pankhurst, S.; Campbell, T. J.; Breit, S. N.; Curmi, P. M. Crystal structure of a soluble form of the intracellular chloride ion channel CLIC1 (NCC27) at 1.4-A resolution. *J. Biol. Chem.* **2001**, *276* (48), 44993–5000.
(67) Tulk, B. M.; Kapadia, S.; Edwards, J. C. CLIC1 inserts from the aqueous phase into phospholipid membranes, where it functions as an anion channel. *Am. J. Physiol. Cell. Physiol.* **2002**, *282* (5), C1103–12.
(68) Tomonaga, T.; Matsushita, K.; Yamaguchi, S.; Oh-Ishi, M.; Kodera, Y.; Maeda, T.; Shimada, H.; Ochiai, T.; Nomura, F. Identification of altered protein expression and post-translational modifications in primary colorectal cancer by using agarose two-dimensional gel electrophoresis. *Clin. Cancer Res.* **2004**, *10* (6), 2007–14.
(69) Hu, Y.; Rosen, D. G.; Zhou, Y.; Feng, L.; Yang, G.; Liu, J.; Huang, P. Mitochondrial manganese-superoxide dismutase expression in ovarian cancer: role in cell proliferation and response to oxidative stress. *J. Biol. Chem.* **2005**, *280* (47), 39485–92.
(70) Trachootham, D.; Zhou, Y.; Zhang, H.; Demizu, Y.; Chen, Z.; Pelicano, H.; Chiao, P. J.; Achanta, G.; Arlinghaus, R. B.; Liu, J.; Huang, P. Selective killing of oncogenically transformed cells through a ROS-mediated mechanism by beta-phenylethyl isothiocyanate. *Cancer Cell* **2006**, *10* (3), 241–52.
(71) Huang, J. S.; Chao, C. C.; Su, T. L.; Yeh, S. H.; Chen, D. S.; Chen, C. T.; Chen, P. J.; Jou, Y. S. Diverse cellular transformation capability of overexpressed genes in human hepatocellular carcinoma. *Biochem. Biophys. Res. Commun.* **2004**, *315* (4), 950–8.
(72) Suh, K. S.; Mutoh, M.; Gerdes, M.; Crutchley, J. M.; Mutoh, T.; Edwards, L. E.; Dumont, R. A.; Sodha, P.; Cheng, C.; Glick, A.; Yuspa, S. H. Antisense suppression of the chloride intracellular channel family induces apoptosis, enhances tumor necrosis factor {alpha}-induced apoptosis, and inhibits tumor growth. *Cancer Res.* **2005**, *65* (2), 562–71.
(73) Wulfkuhle, J. D.; Sgroi, D. C.; Krutzsch, H.; McLean, K.; McGarvey, K.; Knowlton, M.; Chen, S.; Shu, H.; Sahin, A.; Kurek, R.; Wallwiener, D.; Merino, M. J.; Petricoin, E. F., III; Zhao, Y.; Steeg, P. S. Proteomics of human breast ductal carcinoma in situ. *Cancer Res.* **2002**, *62* (22), 6740–9.
(74) Berglund, L.; Bjorling, E.; Oksvold, P.; Fagerberg, L.; Asplund, A.; Szigyarto, C. A.; Persson, A.; Ottosson, J.; Wernerus, H.; Nilsson, P.; Lundberg, E.; Sivertsson, A.; Navani, S.; Wester, K.; Kampf, C.; Hober, S.; Ponten, F.; Uhlen, M. A genecentric Human Protein Atlas for expression profiles based on antibodies. *Mol. Cell. Proteomics* **2008**, *7* (10), 2019–27.

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