Chondrolectin is a novel diagnostic biomarker and a therapeutic target for lung cancer

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Statement of Clinical Relevance

Because there is a significant association of CHODL positivity with poor clinical outcome of lung cancer patients, CHODL positivity in resected specimens could be an index that provides valuable information to physicians in applying adjuvant therapy and intensive follow-up to the patients who are likely to relapse. Since CHODL encodes a cancer-testis antigen, it may be useful for screening of HLA-restricted epitope peptides for cancer vaccine that can induce specific immune responses by cytotoxic T cells against CHODL-positive cancers. Since CHODL plays important roles in cancer proliferation and invasion, targeting CHODL function by small molecule compounds could be a new therapeutic strategy that is expected to have a powerful anti-cancer activity with a minimal risk of adverse effects.
Abstract

Purpose: This study aims to identify molecules that might be useful as diagnostic/prognostic biomarkers and as targets for the development of new molecular therapies for lung cancer.

Experimental Design: We screened for genes that were highly transactivated in a large proportion of 120 lung cancers by means of a cDNA microarray representing 27,648 genes, and found chondrolectin (CHODL) as a candidate. Tumor tissue microarray was applied to examine the expression of CHODL protein and its clinicopathological significance in archival non-small cell lung cancer (NSCLC) tissues from 295 patients. A role of CHODL in cancer cell growth and/or survival was examined by small interfering RNA (siRNA) experiments. Cellular invasive effect of CHODL on mammalian cells was examined using Matrigel assays.

Results: Immunohistochemical staining revealed that strong positivity of CHODL protein was associated with shorter survival of patients with NSCLC (P = 0.0006), and multivariate analysis confirmed it to be an independent prognostic factor. Treatment of lung cancer cells with siRNAs against CHODL suppressed growth of the cancer cells. Furthermore, induction of exogenous expression of CHODL conferred growth and invasive activity of mammalian cells.

Conclusions: CHODL is likely to be a prognostic biomarker in the clinic and targeting CHODL might be a strategy for the development of anticancer drugs.
Introduction

Lung cancer is one of the most common cause of cancer death in the world, and non-small cell lung cancer (NSCLC) accounts for nearly 80% of those cases (1, 2). In the last two decades several newly developed cytotoxic agents including paclitaxel, docetaxel, gemcitabine, vinorelbine and irinotecan have emerged to offer multiple therapeutic choices for patients with advanced NSCLC; however, each of the new regimens can provide only modest improvements in survival and quality of life compared with traditional cisplatin-based therapies (3). Recently, molecular-targeted agents, including anti-EGFR or anti-VEGF monoclonal antibody, cetuximab or bevacizumab, and small molecule inhibitors of EGFR tyrosine kinase, such as gefitinib and erlotinib have been investigated in the clinical trials and/or were approved for clinical use. These agents are effective in the treatment of advanced NSCLC to a certain extent, but a proportion of patients who could receive a survival benefit is still limited (4, 5). Hence, new therapeutic strategies, such as the development of more selective and effective molecular-targeted agents against lung cancer are eagerly awaited.

Genome-wide cDNA microarray analysis of cancers enabled us to obtain their comprehensive gene expression profiles and to compare the gene expression levels with clinicopathological and biological information of cancers (6). This approach is also useful to identify unknown molecules involved in the carcinogenic pathway. Through the gene expression profile analysis of 120 lung cancers on a cDNA microarray consisting of 27,648 genes or expressed sequence tags (ESTs) coupled with purification of cancer cell population by laser
microdissection, and subsequent comparison of the data with the expression profile of 31 normal human tissues, we identified a number of potential molecular targets for cancer diagnosis, treatment, and/or choice of therapy (6-11). To verify the biological and clinicopathologic significance of the respective gene products, we have also been performing high-throughput screening of loss-of-function effects by means of the RNA interference technique as well as tumor tissue microarray analysis of clinical lung cancer materials (12-41). This systematic approach identified a set of molecules that appear to fall into the category of cancer-testis antigens and whose up-regulation is a frequent and important feature of the malignant nature of human cancer. These molecules identified can be regarded as potential targets for the development of highly sensitive and specific biomarkers as well as being useful in the development of small-molecular compounds, antibodies, and cancer vaccines that could have a more specific and efficient anti-cancer effect with minimal risk of adverse effects (12-41). Among them, Chondrolectin (CHODL) was commonly overexpressed in the great majority of lung cancers.

    CHODL, a human ortholog of mouse chondrolectin (chodl) encodes a single-pass transmembrane protein with one carbohydrate recognition domain (CRD) of C-type lectins (42). C-type lectin family is classified into 17 groups according to their domain architecture. They are divided into two major types; transmembrane C-type lectins and soluble ones. These lectins have a wide variety of functions at cytoplasm, cytoplasmic membrane, and extracellular space. Mouse chodl was mainly expressed in embryonic tissues, and its expression level was tightly controlled during embryonic development. Chodl
transcripts were present at the stage of 7 days post-conception (dpc), and elevated up to the stage of 15 dpc, followed by decrease at the stage of 17 dpc (43). To date, the biological function of CHODL and its activation in human cancers were not reported.

Here we report evidences that CHODL might play a significant role in lung carcinogenesis, and is a potential diagnostic and prognostic marker for lung cancer.
Materials and Methods

Cell lines and clinical tissue samples. The human lung-cancer cell lines used in this study included five adenocarcinomas (ADCs; A549, LC319, NCI-H1373, PC14, and NCI-H1781), five squamous cell carcinomas (SCCs; LU61, NCI-H520, NCI-H1703, NCI-H2170, SK-MES-1), one large cell carcinoma (LCC; LX1), and four small cell lung cancers (SCLCs; DMS114, DMS273, SBC-3, and SBC-5). A human bronchial epithelial cell line (BEAS-2B) was used as a control (Supplementary Table S1). All cells were grown in monolayers in appropriate media supplemented with 10% fetal calf serum (FCS) and were maintained at 37°C in an atmosphere of humidified air with 5% CO2. Primary lung cancers were obtained with informed consent along with adjacent normal lung tissue samples from patients, as described previously (7, 11). All tumors were staged on the basis of the pTNM pathological classification of the UICC (International Union Against Cancer) (44). Formalin-fixed primary NSCLCs and adjacent normal lung tissue samples used for immunostaining on tissue microarrays had been obtained from 295 patients undergoing curative surgery at Saitama Cancer Center (Saitama, Japan). To be eligible for this study, tumor samples were selected from patients who fulfilled all of the following criteria: (a) patients suffered from primary NSCLC with confirmed stage (only pT1 to pT3, pN0 to pN2, and pM0), (b) patients underwent curative surgery, but did not receive any preoperative treatment, (c) among them NSCLC patients with lymph node metastasis positive (pN1, pN2) tumors were treated with platinum-based adjuvant chemotherapies after surgery, whereas patients with pN0 did not receive adjuvant chemotherapies, and (d) patients whose clinical follow-up data
were available. This study and the use of all clinical materials mentioned were approved by individual institutional ethics committees.

**Semi-quantitative RT-PCR analysis.** Total RNA was extracted from cultured cells and clinical tissues using Trizol reagent (Life Technologies, Inc.) according to the manufacturer’s protocol. Extracted RNAs and normal human-tissue polyA RNAs were treated with DNase I (Roche Diagnostics) and then reverse-transcribed using oligo (dT) primer and SuperScript II reverse transcriptase (Life Technologies, Inc.). Semi-quantitative RT-PCR experiments were carried out with synthesized CHODL gene-specific primers (5’-GGAAGGAAAGGAACTACGAAATC-3’ and 5’-GTTAAAAGGAGCACAGGGACATA-3’), or with beta-actin (ACTB)-specific primers (5’-ATCAAGATCATTGCTCCTCCT-3’ and 5’-CTGCAGTTGGTTTTTCTG-3’) as an internal control. All PCR reactions involved initial denaturation at 95°C for 2 min followed by 22 (for ACTB) or 30 cycles (for CHODL) of 95°C 30 s, 54-60°C for 30 s, and 72°C for 60 s on a GeneAmp PCR system 9700 (Applied Biosystems).

**Immunocytochemical analysis.** Immunocytochemical analyses were done as previously described (13), using 1 μg/mL of a goat polyclonal anti-CHODL antibody (Catalog No. sc-54867; Santa Cruz Biotechnology) for detecting endogenous CHODL and 4 μg/mL of a rabbit polyclonal anti-Calreticulin antibody (Catalog No. SPA-600; StressGen) for detecting endogenous calreticulin in the endoplasmic reticulum (ER) as a primary antibody. The cells
were incubated with these primary antibodies for 1 h at room temperature, followed by incubation with Alexa 488–conjugated donkey anti-goat secondary antibodies (Molecular Probe) and an Alexa 594–conjugated donkey anti-rabbit secondary antibodies (Molecular Probe). Each stained specimen was mounted with Vectashield (Vector Laboratories, Inc.) containing 4′, 6-diamidino-2-phenylindole and visualized with Spectral Confocal Scanning Systems (TSC SP2 AOBS; Leica Microsystems). On immunocytochemical analyses, we confirmed that the antibody was specific for CHODL protein, using NSCLC cell lines that endogenously expressed CHODL as well as cell lines derived from NSCLC or bronchial epithelia that did not express it. We also confirmed the specificity of the antibody by immunostaining NSCLC cells that were transfected with siRNAs against CHODL that could suppress CHODL expression or control siRNAs that could not (Supplementary Fig. S1).

**Northern blot analysis.** Human multiple tissue blots (23 normal tissues including heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, colon, leukocyte, stomach, thyroid, spinal cord, lymph node, trachea, adrenal gland, bone marrow; BD Biosciences) were hybridized with a $^{32}$P-labeled PCR product of CHODL cDNA. The cDNA probe of CHODL was prepared by reverse transcriptase-PCR (RT-PCR) using primers CHODL-F (5′-GGTGCATAAACACTAATGCAGTC-3′) and CHODL-R (5′-GTTAAAAGGAGCACAGGGACATA-3′). Prehybridization, hybridization, and washing were performed according to the supplier's recommendations. The blots were autoradiographed with intensifying screens.
at -80°C for 7 days.

**Tissue microarray and Immunohistochemistry.** Tumor tissue microarrays were constructed using 295 formalin-fixed primary lung cancers, as published previously (45-47). The tissue area for sampling was selected based on visual alignment with the corresponding HE-stained section on a slide. Three, four, or five tissue cores (diameter 0.6 mm; height 3-4 mm) taken from a donor tumor block were placed into a recipient paraffin block using a tissue microarrayer (Beecher Instruments, Sun Prairie, WI). A core of normal tissue was punched from each case, and 5 µm sections of the resulting microarray block were used for immunohistochemical analysis.

To investigate the presence of CHODL protein in clinical samples that had been embedded in paraffin blocks, we stained the sections in the following manner. Briefly, 4 µg/ml of a goat polyclonal anti-human CHODL antibody (Catalog No. sc-54867; Santa Cruz Biotechnology) was added after blocking of endogenous peroxidase and proteins. The sections were incubated with HRP-labeled anti-goat IgG as the secondary antibody. Substrate-chromogen was added and the specimens were counterstained with hematoxylin. On immunohistochemical analyses, we confirmed that the antibody was specific for CHODL protein by antigen blocking assays using CHODL antigen peptides (Catalog No. sc-54867P; Santa Cruz Biotechnology) that were used for immunization of goats to produce polyclonal anti-human CHODL antibodies (Supplementary Fig. S2). Three independent investigators assessed CHODL positivity semi-quantitatively without prior knowledge of clinicopathological data.
The intensity of CHODL staining was evaluated using the following criteria: strong positive (scored as 2+), brown staining in > 50% of tumor cells completely obscuring cytoplasm; weak positive (1+), any lesser degree of brown staining appreciable in tumor cell cytoplasm; and absent (scored as 0), no appreciable staining in tumor cells. Cases were accepted as strongly positive if two or more investigators independently defined them as such.

**Statistical analysis.** Statistical analyses were performed using the StatView statistical program (SAS) to compare patient characteristics with responses to therapy. Associations between clinicopathological variables and positivity for CHODL were compared by Fisher's exact tests. Tumor-specific survival and 95% confidence intervals (CIs) were evaluated with the Kaplan-Meier method, and differences between the two groups were evaluated with the log-rank test. Factors associated with the prognosis were evaluated using Cox's proportional-hazard regression model with a step-down procedure. Only those variables with statistically significant results in univariate analysis were included in a multivariate analysis. The criterion for removing a variable was the likelihood ratio statistic, which was based on the maximum partial likelihood estimate (default P value of 0.05 for removal from the model).

**Western blot analysis.** Cells were lysed in lysis buffer [50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 0.5% NP40, 0.5% sodium deoxycholate, and Protease Inhibitor Cocktail Set III (EMD Biosciences, Inc.). Protein samples were separated by SDS-polyacrylamide gels and electroblotted onto
Hybond-ECL nitrocellulose membranes (GE Healthcare Bio-Sciences). Blots were incubated with a mouse monoclonal anti-myc antibody. Antigen-antibody complexes were detected using secondary antibodies conjugated to horseradish peroxidase (GE Healthcare Bio-Sciences). Protein bands were visualized by enhanced chemiluminescence Western blotting detection reagents (GE Healthcare Bio-Sciences).

**RNA interference assay.** To evaluate the biological functions of CHODL in lung cancer cells, we used small interfering RNA (siRNA) duplexes against the target genes. The target sequences of the synthetic oligonucleotides for RNA interference were as follows: control 1 (si-LUC: siRNA against *Photinus pyralis* luciferase gene), 5′-CGUACGCGGAAUCUUCGA-3′; control 2 (si-SCR: siRNA against chloroplast *Euglena gracilis* gene coding for 5S and 16S (rRNAs), 5′-GCGCGCUUUGUAGGAUUCG-3′; si-CHODL-#A, 5′-AAAGUGGCAUGGAAGUAUA-3′; si-CHODL-#B, 5′-GGUAUAAUCCCAUCAAU-3′. Lung cancer cell lines, SBC-5 and NCI-H2170 were plated onto 10 cm dishes (1.0 × 10⁶ per dish), and transfected with either of the siRNA oligonucleotides (100 nmol/L) using 24 µL of Lipofectamine 2000 (Invitrogen) according to the manufacturers' instructions. After 7 days of incubation, cell viability was assessed by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. The number of colonies stained with Giemsa was also counted by colony formation assay using colony counting software (ImageJ software 1.42, NIH).
**Cell growth assay.** COS-7, LC319 and BEAS-2B cells which scarcely expressed endogenous CHODL were plated at densities of $1 \times 10^6$ cells/10 cm dish and transfected with plasmids designed to express myc-tagged CHODL or mock plasmids. Cells were selected in medium containing 0.4 mg/mL of geneticin (Invitrogen) for 7 days, and cell viability was assessed by MTT assay (cell counting kit-8 solution; Dojindo Laboratories).

**Matrigel invasion assay.** COS-7, LC319 and BEAS-2B cells transfected either with plasmids expressing myc-tagged CHODL or with mock plasmids were grown to near confluence in DMEM or RPMI containing 10% FCS. The cells were harvested by trypsinization, washed in medium without addition of serum or protease inhibitor, and suspended in medium at a concentration of $2 \times 10^5$/mL. Before preparing the cell suspension, the dried layer of Matrigel matrix (Becton Dickinson Labware) was rehydrated with medium for 2 h at room temperature. Medium (0.75 mL) containing 10% FCS was added to each lower chamber in 24 well Matrigel invasion chambers, and 0.5 mL ($1 \times 10^5$ cells) of cell suspension was added to each insert of the upper chamber. The plates of inserts were incubated for 22 h at 37°C, in which condition, no growth promoting effect by exogenous CHODL expression was also confirmed by MTT assay. Then the chambers were processed, and cells invading through the Matrigel were fixed and stained by Giemsa as directed by the supplier (Becton Dickinson Labware).

**Results**

**CHODL expression in lung cancers and normal tissues.** To search for
novel target molecules for the development of therapeutic agents and/or diagnostic biomarkers for NSCLC, we first screened genes that showed more than a 5-fold higher level of expression in cancer cells than in normal cells, in more than 50% of the lung cancers analyzed by cDNA microarray. Among 27,648 genes or ESTs screened, we identified that the expression of CHODL transcript showed 5-folds or higher in 63% of NSCLCs compared with normal lung (control). Moreover, our cDNA microarray database indicated that CHODL was hardly detectable in normal tissues except testis. Therefore, we determined to select CHODL as a good candidate for further analyses. We confirmed its transactivation by semi-quantitative RT-PCR experiments in 7 of 15 additional lung cancer tissues and 10 of 15 lung cancer cell lines (Figs. 1A and 1B). To determine the subcellular localization of endogenous CHODL in lung cancer cells, we performed immunocytochemical analysis using anti-CHODL polyclonal antibodies; CHODL protein was localized in cytoplasm with granular appearance in A549 and NCI-H2170 cells, which expressed endogenous CHODL, but it was not detectable in CHODL-negative LC319 and BEAS-2B cells (Fig. 1C). Furthermore, we confirmed that CHODL protein was mainly co-localized with calreticulin that was used as an ER marker (Fig. 1D), but not co-localized with Golgi and endosome markers (data not shown). We confirmed that the positive signal by anti-CHODL antibody obtained in lung cancer A549 cells was markedly reduced by suppression of CHODL expression by siRNA against CHODL, which proved the specificity of antibody to CHODL protein (Supplementary Fig. S1).

Northern blot analysis identified a 2.8-kb CHODL transcript to be highly
expressed in the testis; however it was hardly detectable in other normal tissues examined (Fig. 2A). We subsequently examined expression of CHODL protein in five normal tissues (heart, liver, lung, kidney, and testis), as well as lung cancers using anti-CHODL antibody, and found that it was hardly detectable in the former four tissues, whereas positive CHODL staining was detected at cytoplasm of testis and lung cancer tissues (Fig. 2B). We confirmed that the positive signal by anti-CHODL antibody obtained in lung cancer tissues was markedly diminished by preincubation of the antibody with the CHODL antigen peptide used for immunization of goat, which also independently indicated its specificity to CHODL protein (Supplementary Fig. S2). The expression levels of CHODL protein in lung cancer were significantly higher than those in testis. We also evaluated CHODL staining in lung cancers and adjacent normal lung tissues, and confirmed CHODL protein to be positively stained in the majority of NSCLC tissues, but not in their corresponding normal lungs (Fig. 2C). Furthermore, CHODL protein was hardly detectable in benign lung disease of emphysema (Supplementary Fig. S3).

**Association of CHODL protein expression with poor clinical outcome of NSCLC patients.** To verify the biological and clinicopathological significance of CHODL, we also examined the expression of CHODL protein by means of tissue microarrays containing lung cancer tissues from 295 NSCLC patients who underwent surgical resection. We classified a pattern of CHODL expression on the tissue array ranging from absent (scored as 0) to weak/strong positive (scored as 1+ or 2+; Fig. 2D). As shown in Table 1A, the number of NSCLC
tissues scored as 2+, 1+, and 0 were 98 (33.2%), 131 (44.4%), and 66 (22.4%), respectively. We then evaluated the association between CHODL status and clinicopathologic variables among the 295 patients. The median survival time of NSCLC patients with strong CHODL-staining (score 2+) was significantly shorter than that with absent/weak CHODL expression (score 0-1+) \((P = 0.0006\) by log-rank test; **Fig. 2E**). We also used univariate analysis to evaluate associations between patient prognosis and other factors including age (\(\geq 65\) versus 65+), gender (male versus female), histological classification (non-adenocarcinoma versus adenocarcinoma), smoking status (current/former smoker versus never smoker), pathological T stage (T2+T3 versus T1), pathological N stage (N1+N2 versus N0), and CHODL positivity (strong positive versus weak positive/absent) (**Table 1B**). Among those parameters, strong CHODL-positivity, elderly, male gender, non-adenocarcinoma histology, advanced pT stage and advanced pN stage were significantly associated with poor prognosis. In multivariate analysis of prognostic factors, CHDOL-positivity, age, larger tumor size and lymph node metastasis were significant and independent prognostic factors for NSCLC patients.

**Growth-promoting effect of CHODL.** Using siRNA oligonucleotides for CHODL, we attempted to knock down the expression of endogenous CHODL in lung cancer cell lines SBC-5 and NCI-H2170, which showed high levels of endogenous CHODL expression. Two CHODL-specific siRNAs (si-CHODL-#A and si-CHODL-#B) significantly suppressed expression of CHODL transcripts compared with control siRNAs (si-LUC and si-SCR) (**Fig. 3A**). MTT and colony
Formation assays revealed that reduction of CHODL expression by the two siRNAs significantly suppressed the growth of SBC-5 and NCI-H2170 cells (Figs. 3B and 3C; Supplementary Fig. S4).

To further disclose a potential role of CHODL in tumorigenesis, we prepared plasmids designed to express either CHODL (pcDNA3.1/myc-His vector) or mock vector, and transfected them into COS-7, LC319 and BEAS-2B cells which scarcely expressed endogenous CHODL. Cells that transiently expressed exogenous CHODL revealed significant growth promotion compared with the mock-transfected cells (Figs. 3D and 3E).

Enhanced cell invasion by CHODL. Since strong expression of CHODL in NSCLC tissues was associated with poor prognosis of patients, we next examined a possible role of CHODL in cellular invasion by Matrigel assays using COS-7, LC319 and BEAS-2B cells. Transfection of CHODL cDNA into either of the cells significantly enhanced their invasive activity (Figs. 4A-4C). This result also suggests that CHODL could contribute to the more malignant phenotype of cells.

Discussion
Several molecular-targeting drugs have been developed and proved their efficacy in cancer therapy. However, the proportion of patients showing good response is still limited. Therefore, further development of molecular-targeting drugs for cancer is urgently awaited. We have screened diagnostic and therapeutic target molecules by whole-genome gene expression profile analysis.
as well as subsequent tissue microarray and functional analyses (7-41). Using this approach, we have shown here that CHODL is frequently overexpressed in clinical lung cancer samples and cell lines, and that its gene product plays indispensable roles in the growth and progression of lung cancer cells.

CHODL appears to belong to C-type lectin-containing protein family that is classified into 17 groups. They are involved in diverse processes including cell recognition and communication, cell-cell adhesion, and extracellular matrix-cell interactions (48). For example, of proteoglycans belong to soluble C-type lectin family member without transmembrane domain, play an important role in the structure and stability of the extracellular matrix (ECM) and the interaction between the ECM and the cell (49). Some lectins are also important in embryonic development and immune responses. They function as recognition molecules in important processes in the immune system: for example, the selectines, which belong to C-type lectin with a transmembrane domain, function at cell surface in leukocyte-leukocyte and leukocyte-endothelial interactions (49). Some transmembrane C-type lectins such as calnexin play important roles as molecular chaperones like calreticulin in the ER. These proteins bind to misfolded proteins and prevent them from being exported from the ER to the Golgi apparatus (50). Since we confirmed that CHODL was partly localized in the ER of cancer cells, it might have a similar function in control system for some oncogenic proteins in the ER.

In this study, we demonstrated that CHODL was expressed only in testis among the 23 normal tissues examined and was highly expressed in surgically resected samples from NSCLC patients, indicating CHODL to be a typical
cancer-testis antigen. Clinicopathological evidences obtained through our tissue microarray experiments indicated that NSCLC patients with strong CHODL-positive tumors had shorter cancer-specific survival periods than those with weak/absent expression tumors. Functional assays by knockdown of CHODL expression with siRNA or exogenous expression of CHODL revealed that CHODL was important for cellular growth and invasion. Although the precise molecular mechanism underlying our observations is unknown, the combined results strongly suggest that CHODL is likely to function as an oncogenic protein in lung carcinogenesis and tumor progression. Elucidation of the mechanism implied by these observations should reveal important new information about cancer cell proliferation and cancer progression.

To date, several prognostic biomarkers for lung cancer were reported, however, it is difficult to measure the malignant level of tumors in individual patients using only a single marker. To quantitatively predict the prognosis of NSCLC patients who had undergone curative surgery, we next attempted combined assays as a pilot study using the positivity data of CHODL and the available data of 2 other prognostic biomarkers for NSCLC (FGFR1OP and DLX5) which had been previously identified by our group using the same set of lung cancer tissues used in this study (23, 25). NSCLC patients were divided into four groups; (group-1) all three markers were scored as strong positive in each patient, (group-2) either of two markers were strong positive, (group-3) either of one marker was strong positive, and (group-4) no marker was strong positive. There is a significant correlation between the increased number of strong positive markers and poorer prognosis of NSCLC patients.
(Supplementary Fig. S5). Although further screening and validation analyses that determine the best combination of promising prognostic biomarkers providing the best separation of long survivors and short ones are necessary, the results demonstrate that this type of diagnostic approach combined with more quantitative scoring system of immunohistochemical staining may be a useful and practical index in the clinic for application of adjuvant therapy to the patients who are likely to have poor prognosis after surgery.

In summary, we have shown that over-expressed CHODL is likely to be one of essential contributors to a growth-promoting pathway and to aggressive features of lung cancers. CHODL is a possible prognostic biomarker in the clinic.

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References


Figure legends

Figure 1. CHODL expression in lung cancers.
A, Expression of CHODL in clinical lung cancers, examined by semi-quantitative RT-PCR. B, Expression of CHODL in lung-cancer cell lines, examined by semi-quantitative RT-PCR. C, Subcellular localization of endogenous CHODL protein in lung cancer cells. CHODL was stained in the cytoplasm of cells with granular appearance in CHODL-positive A549 and NCI-H2170 cells, while CHODL was not stained in LC319 and BEAS-2B cells that did not express endogenous CHODL. D, Co-localization of endogenous CHODL with calreticulin as endoplasmic reticulum marker in A549 cells.

Figure 2. Expression of CHODL in normal tissues and lung tumors.
A, Northern blot analysis of the CHODL transcript in 23 normal adult human tissues. B, Immunohistochemical evaluation of CHODL protein using anti–CHODL antibody in lung ADC, lung SCC, lung LCC and five normal tissues. C, Immunohistochemical staining of CHODL protein using anti-CHODL antibody in four representative paired lung tumors and adjacent normal lung tissues. D, Immunohistochemical evaluation of CHODL expression on tissue microarrays (X100). Examples are shown of strong, weak, absent, and normal lung tissue. E, Kaplan-Meier analysis of tumor-specific survival in patients with NSCLCs according to CHODL expression (P = 0.0006; Log-rank test). Number of cases at risk is shown in Supplementary Table S2.

Figure 3. Growth effect of CHODL expression.
A-C, Response of SBC-5 and NCI-H2170 cells to si-CHODL#A, si-CHODL#B or control siRNAs (si-LUC or si-SCR). A, The level of CHODL mRNA expression detected by semi-quantitative RT-PCR in cells transfected with either control siRNAs or si-CHODLs. B, The effect of siRNA against CHODL on cell viability, evaluated by MTT assay. C, The effect of siRNA against CHODL on colony formation, evaluated by colony formation assays. All assays were performed thrice and in triplicate wells. D-E, Growth promoting effects of CHODL. D, Transient expression of CHODL in COS-7, LC319 and BEAS-2B cells, detected...
by western blot analysis. **E**, Effect of CHODL on growth of COS-7, LC319 and BEAS-2B cells. At each time point, cell viability was evaluated by MTT assay.

**Figure 4.** Enhancement of cellular invasiveness by CHODL.
**A,** Transient expression of CHODL in COS-7, LC319 and BEAS-2B cells, detected by western blot analysis. **B** and **C**, Assays demonstrating the invasive nature of COS-7, LC319 and BEAS-2B cells in Matrigel matrix after transfection with expression plasmids for human *CHODL*. The relative number of cells migrating through the Matrigel-coated filters (**B**) and Giemsa staining (**C**; magnification, X100). Assays were done thrice and in triplicate wells.
# Table 1A. Association between CHODL-status in NSCLC and patients' characteristics (n=295)

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<td>T1</td>
<td>120</td>
<td>33</td>
<td>53</td>
<td>34</td>
<td>0.1018</td>
</tr>
<tr>
<td>T2+T3</td>
<td>175</td>
<td>65</td>
<td>78</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>pN factor</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>193</td>
<td>57</td>
<td>88</td>
<td>48</td>
<td>0.0699</td>
</tr>
<tr>
<td>N1+N2</td>
<td>102</td>
<td>41</td>
<td>43</td>
<td>18</td>
<td></td>
</tr>
</tbody>
</table>

ADC, adenocarcinoma, SCC, squamous cell carcinoma
Others, large cell carcinoma(LCC) plus adenosquamous cell carcinoma(ASC)
*ADC versus non-ADC

# Table 1B. Cox's proportional hazards model analysis of prognostic factors in patients with NSCLCs

<table>
<thead>
<tr>
<th>Variables</th>
<th>Unfavorable/Favorable</th>
<th>(P)-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHODL</td>
<td>1.841</td>
<td>1.294-2.620</td>
</tr>
<tr>
<td>Age(years)</td>
<td>1.699</td>
<td>1.188-2.431</td>
</tr>
<tr>
<td>Gender</td>
<td>1.639</td>
<td>1.092-2.463</td>
</tr>
<tr>
<td>Histological type</td>
<td>1.558</td>
<td>1.108-2.190</td>
</tr>
<tr>
<td>Smoking status</td>
<td>1.310</td>
<td>0.872-1.968</td>
</tr>
<tr>
<td>pT factor</td>
<td>2.985</td>
<td>1.984-4.484</td>
</tr>
<tr>
<td>pN factor</td>
<td>2.451</td>
<td>1.745-3.448</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Variables</th>
<th>Unfavorable/Favorable</th>
<th>(P)-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHODL</td>
<td>1.660</td>
<td>1.161-2.372</td>
</tr>
<tr>
<td>Age(years)</td>
<td>1.869</td>
<td>1.303-2.680</td>
</tr>
<tr>
<td>Gender</td>
<td>0.829</td>
<td>0.530-1.298</td>
</tr>
<tr>
<td>Histological type</td>
<td>1.195</td>
<td>0.821-1.739</td>
</tr>
<tr>
<td>pT factor</td>
<td>2.288</td>
<td>1.495-3.497</td>
</tr>
<tr>
<td>pN factor</td>
<td>2.160</td>
<td>1.522-3.058</td>
</tr>
</tbody>
</table>

ADC, adenocarcinoma
* \(P < 0.05\) (Fisher's exact test)
A

Clinical lung cancers

<table>
<thead>
<tr>
<th>ADC</th>
<th>SCC</th>
<th>SCLC</th>
<th>Normal lung</th>
</tr>
</thead>
<tbody>
<tr>
<td>LC1</td>
<td>LC7</td>
<td>LC5</td>
<td>LC6</td>
</tr>
<tr>
<td>LC2</td>
<td>LC8</td>
<td>LC9</td>
<td>LC10</td>
</tr>
<tr>
<td>LC3</td>
<td>LC11 LC12</td>
<td>LC13</td>
<td>LC14</td>
</tr>
<tr>
<td>LC4</td>
<td>LC15</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

CHODL

ACTB

B

Lung cancer cell lines

<table>
<thead>
<tr>
<th>ADC</th>
<th>SCC</th>
<th>LCC</th>
<th>SCLC</th>
<th>Airway epithelia</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCI-H1781</td>
<td>NCI-H520</td>
<td>NCI-H2170</td>
<td>LU61</td>
<td>DMS114</td>
</tr>
<tr>
<td>NCI-H1373</td>
<td>NCI-H1703</td>
<td>NCI-H2170</td>
<td>LX1</td>
<td>DMS273</td>
</tr>
<tr>
<td>LC 319</td>
<td>PC14</td>
<td></td>
<td></td>
<td>SBC-3</td>
</tr>
<tr>
<td>A549</td>
<td>SK-MES-1</td>
<td></td>
<td></td>
<td>SBC-5</td>
</tr>
<tr>
<td>PC14</td>
<td></td>
<td></td>
<td></td>
<td>BEAS-2B</td>
</tr>
</tbody>
</table>

CHODL

ACTB

Figure 1
Figure 1

CHODL negative
Airway epithelia

CHODL positive

A549
NCI-H2170

LC319
BEAS-2B
Figure 2

Normal tissues

(Kb)

heart  brain  placenta  lung  liver  skeletal muscle  pancreas  spleen  thymus  prostate  testis  ovary  small intestine  colon  leukocyte  stomach  thyroid  spinal cord  lymph node  trachea  adrenal gland  bone marrow
Figure 2
Figure 2

Lung cancer

Normal lung

CASE1

CASE2

CASE3

CASE4
**Figure 2**

**D**

Lung adenocarcinoma

<table>
<thead>
<tr>
<th>Strong positive</th>
<th>Weak positive</th>
<th>Absent</th>
<th>Normal lung</th>
</tr>
</thead>
</table>

**E**

Lung cancer mortality

- Weak CHODL positive or absent (n=197)
- Strong CHODL positive (n=98)

Survival rate (%) vs. postoperative days

- *P* = 0.0006
Figure 3

**A**

<table>
<thead>
<tr>
<th></th>
<th>SBC-5</th>
<th>NCI-H2170</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anti-CHODL siRNA</strong></td>
<td>si-#A</td>
<td>si-#B</td>
</tr>
<tr>
<td><strong>CHODL</strong></td>
<td>[Image]</td>
<td>[Image]</td>
</tr>
<tr>
<td><strong>ACTB</strong></td>
<td>[Image]</td>
<td>[Image]</td>
</tr>
</tbody>
</table>

**B**

**Relative absorbance (490/630nm)**

- **SBC-5**
  - si-#A: 0.5 ± 0.1
  - si-#B: 1.5 ± 0.2
  - si-LUC: 1.0 ± 0.1
  - si-SCR: 0.5 ± 0.1

- **NCI-H2170**
  - si-#A: 0.5 ± 0.1
  - si-#B: 1.5 ± 0.2
  - si-LUC: 1.0 ± 0.1
  - si-SCR: 0.5 ± 0.1

**P-values**

- **SBC-5**
  - si-#A vs. si-LUC: *P < 0.0001*
  - si-#B vs. si-LUC: *P < 0.0001*

- **NCI-H2170**
  - si-#A vs. si-LUC: *P < 0.0001*

**C**

**Images**

- si-#A
- si-#B
- si-LUC
- si-SCR
Figure 4
Clinical Cancer Research

Chondrolectin is a novel diagnostic biomarker and a therapeutic target for lung cancer

Ken Masuda, Atsushi Takano, Hideto Oshita, et al.

Clin Cancer Res Published OnlineFirst October 20, 2011.

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