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Brief Report

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Quantitative proteomics and protein network analysis of A549 lung cancer cells affected by miR-206

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Summary **MiR-206 acts as a potential tumor suppressor during carcinogenesis and a regulatory factor in osteoblasts differentiation, but its modulatory mechanism remains unclear. In this study, we used a quantitative proteomics method, difference gel electrophoresis (DIGE), to profile the protein variation in A549 lung cancer cells with and without miR-206 transfection. We identified a total of 17 differently expressed proteins including 5 up-regulated and 12 down-regulated proteins affected by miR-206 in A549 cells. We further constructed a protein network linked 17 differently expressed proteins with 106 computationally predicted miR-206 targets, and identified 8 "hub" genes (*CALR*, *CTSD*, *ENO1*, *HSPA5*, *CDC42*, *HSPD1*, *POLA1*, and *SMARCA4*) within the network, which may represent important miR-206 functional gene targets. In conclusion, in this study, we identified several candidate functional target genes for miR-206, which is helpful to further explore its mechanisms during carcinogenesis and osteogenesis, and we also proposed a novel proteomic strategy to identify functionally important gene targets for microRNA.**

Keywords: microRNA, gene target, proteomics, bioinformatics, miR-206

1. Introduction

MiR-206 is considered a "myomiR" as it is specifically expressed in skeletal muscle (*1*). MiR-206 acts as a positive regulator of skeletal muscle differentiation, and a negative regulator of osteoblasts differentiation (*2*). Accumulating evidence also suggests a tumor suppressor function for miR-206, as it is frequently downregulated in many human malignancies (*3*). Several oncogenes (such as estrogen receptor 1, cyclinD2) and osteogenesis regulators (such as connexin 43) have been identified and confirmed as targets of miR-206 (*3,4*). However, its exact regulatory mechanisms during carcinogenesis and osteogenesis remain to be explored further.

Identification of novel functional miRNA targets of miR-206 is central to further understand its modulation on cellular functions. Currently, most of the studies on miR-206 target identification are based on computational prediction algorithms. According to our knowledge, until now, there is still no experimental strategy for miR-206 target identification reported. Therefore, in this study, we adopted a difference gel electrophoresis (DIGE) based technology to compared the protein profiling of lung cancer A549 cells with and without miR-206. Then we bioinformatically constructed a protein network using experimentally identified miR-206-related proteins with computationally predicted targets, in order to screen functional gene targets and provide novel mechanism clues for miR-206.

2. Materials and Methods

2.1. *Cells line and miR-206 transfection*

A human lung carcinoma epithelial-like cell line A549 was obtained from the Cell Bank of Shanghai

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Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China), and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA) at 37°C in 5% CO_2. A549 cells were first cultured to reach 50-75% confluence and transfected with miR-206 mimics or negative controls (Shanghai GenePharma, Shanghai, China) with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Cells were harvested 48 h later. MiR-206-transfected cells after 48 h were collected for further analyses.

2.2. *miR-206 expression in A549 cells detected by real-time PCR assay*

Total RNA was isolated from A549 cells using Trizol® (Invitrogen). The miR-206 expression was quantified by real-time PCR using TaqMan miRNA assays according to the manufacturer's directions. U6 small nuclear RNA (snU6) was used to normalize the expression data of miR-206.

2.3. *DIGE*

A549 cells with miR-206 mimic and with vector transfection for 48 h were collected and solubilised with a lysis buffer (30 mM Tris, 7 M urea, 2 M thiourea, 4% CHAPS, pH 8.5) for protein extraction. The protein concentration was determined using a 2D Quant kit (GE Healthcare). Fifty μg of protein extracts from each group were labeled with 400 pmol Cy3 or Cy5. Fifty μg of protein extracts by combining equal amounts (25 μg) from each group was labeled with 400 pmol Cy2 as an internal standard. Then, Cy2-, Cy3-, and Cy5-labeled samples were combined and diluted with a rehydration buffer (8 M urea, 2% CHAPS, 0.5% IPG buffer, 40 mM DTT) for isoelectric focusing (IEF). After equilibration, SDS-PAGE isolation was performed. The Cydye-labeled images were scanned on a Typhoon Trio apparatus (GE Healthcare, USA), and were then post-stained by Coomassie Blue G350 (GE Healthcare, USA). Images were analysized by ImageMaster 6.0 DIGE-enable software package (GE Healthcare, USA), spots with more than 2.0-fold intensity changes between two groups were defined and selected for protein identification.

2.4. *In-gel tryptic digestion and mass spectrometry*

Excised spots of interest were destained with 25 mM NH_4CO_3/50% ACN at 37°C for 30 min, and dehydrated in 100% ACN for 10 min. Proteins were digested by sequencing grade-modified trypsin (Promega, Madison, WI, USA) overnight at 37°C. Extracted peptides were mixed with CHCA for mass spectrometry (MS). MS spectra were acquired using an ABI 4700 proteomics analyzer MALDITOF/TOF mass spectrometer (Applied Biosystems, USA) operating in a result-dependent acquisition mode. MS spectra were searched against a human subset of the Swiss-Prot database for protein identifications using GPS explorer software (Applied Biosystems).

2.5. *Network construction and "hub" gene identification*

MiR-206 targets were first computationally predicted by PicTar, miRanda, and TargetScan. Predicted target genes overlapped among at least two prediction algorithms were combined with differently expressed proteins identified in proteomic study. Then the combined gene sets was used to search for their protein-protein interaction links by accessing the STRING (Search Tool for the Retrieval of Interacting Genes/Proteins, *http://string-db.org*) and KEGG (Kyoto Encyclopedia of Genes and Genomes, *http://www.genome.jp/kegg*) databases. The links among miR-206 target or related genes were constructed into a network and visualized by Cytoscape software.

2.6. *Statistics*

The expression levels of miR-206 in real-time PCR experiment and spots intensity changes in DIGE between groups were compared with *t*-test. *p* value less than 0.05 was considered statistically significant. The connectivity of genes within the network was compared with *Z*-test.

3. Results and Discussion

For A549 cells have a relatively low level of miR-206 expression, the protein changes induced by miR-206 down-regulation would be small, therefore, in this study we only investigated the effect of ectopic overexpression of miR-206 on the protein profiling of A549 cells. By real-time PCR, we validated that the cellular level of miR-206 was significantly increased by miR-206 mimic transfection, when compared with vector transfection and the blank control.

Through a DIGE-based quantitative proteomics tool, we compared the profiling of A549 cells induced by miR-206 overexpression (Table 1). This proteomic strategy identified 17 differently expressed proteins including 5 up-regulated and 12 down-regulated proteins (Figure 1). As expected, most of the differently expressed proteins belong to indirect target genes for miR-206, except Annexin IV (ANXA4) belongs to predicted miR-206 target genes.

Next, we computationally predicted miR-206 targets by three different algorithms (PicTar, miRanda, and TargetScan), a total of 106 genes overlapped among at least two prediction sets were defined. We constructed a network linking 106 predicted genes and 17 experimentally identified proteins (Figure 2A).

Table 1. Differently expressed proteins in miR-206-transfected A549 cells compared with vector-transfected cells in DIGE analysis

Protein names	Gene names	Accession Number	Mascot scores	Fold (miR-206/vector)
GLUD1 protein	GLUD1	Q14400	78	3.0
Prelamin-A/C	LMNA	P02545	102	2.5
Cathepsin D	CTSD	P07339	131	3.5
78 kDa glucose-regulated protein	HSPA5	P11021	122	4.0
NADH dehydrogenase [ubiquinone] iron-sulfur protein 3	NDUFS3	O75489	278	2.2
Transitional endoplasmic reticulum ATPase	VCP	P55072	118	-3.1
Peroxiredoxin-6	PRDX6	P30041	240	-4.2
Eukaryotic translation initiation factor 4H	EIF4H	Q15056	86	-2.8
Galectin-1	LGALS1	P09382	150	-3.7
Sorcin	SRI	P30626	86	-2.0
Calreticulin	CALR	P27797	180	-2.5
14-3-3 protein epsilon	YWHAE	P62258	110	-3.1
Prohibitin	PHB	P35232	128	-2.2
Protein disulfide-isomerase	P4HB	P07237	280	-3.5
Stathmin	STMN1	P16949	80	-2.5
Alpha-enolase	ENO1	P06733	90	-2.3
Annexin A4	ANXA4	P09525	171	-5.0

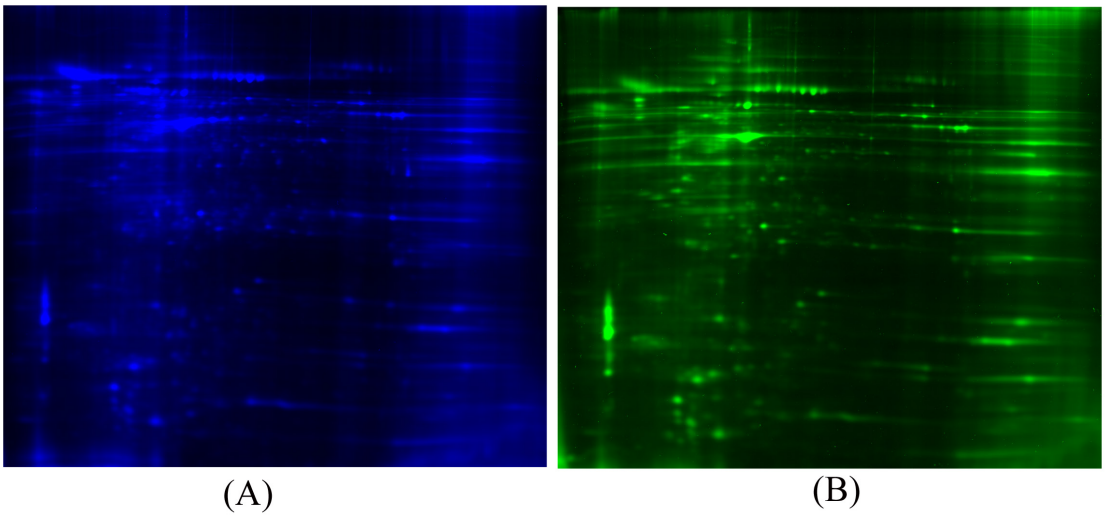

Figure 1. Representative images of DIGE analysis on A549 cells with and without miR-206. (A) Cy5 labeling A549 cells with miR-206 tranfection; **(B)** Cy3 labeling A549 cells with vector transfection.

From this network, we identified 8 highly connected "hub" genes with statistical significance ($p < 0.05$) (Figure 2B). DNA polymerase α1 catalytic subunit (POLA1) and SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4 (SMARCA4) had the highest significant correlation of gene connectivity ($p = 5.0 \times 10^{-5}$ and $p = 0.0066$, respectively) within this network.

How to identify genuinely functional target genes remains a fundamental challenge in miRNA mechanism studies (*5*). Most of the previous studies used computational programs to predict miRNA targets. There are always hundreds of target predicted by these bioinformatics tools, how to select targets for further confirmations still lacks of generally accepted criteria. In addition, computational miRNA target predictions still have a higher percentage of false-positives and false-negatives.

Recently, accumulating experimental strategies based on gene expression microarray or proteomic tools have been developed to provide more information and clues to identify genuinely functional targets for miRNA (*6*). For most targets may be repressed by a miRNA at the protein level without being affected at the mRNA level, proteomic tools represent powerful approaches in revealing the full spectrum of miRNA targets (*7,8*). However, most of the differently proteins found in proteomic studies belong to high or middle abundant proteins. For most of the functional miRNA target effectors are always low abundant proteins such as transcriptors, which is undetectable by even the most sensitive mass spectrometers. Therefore, most varied proteins identified by proteomic tools are always

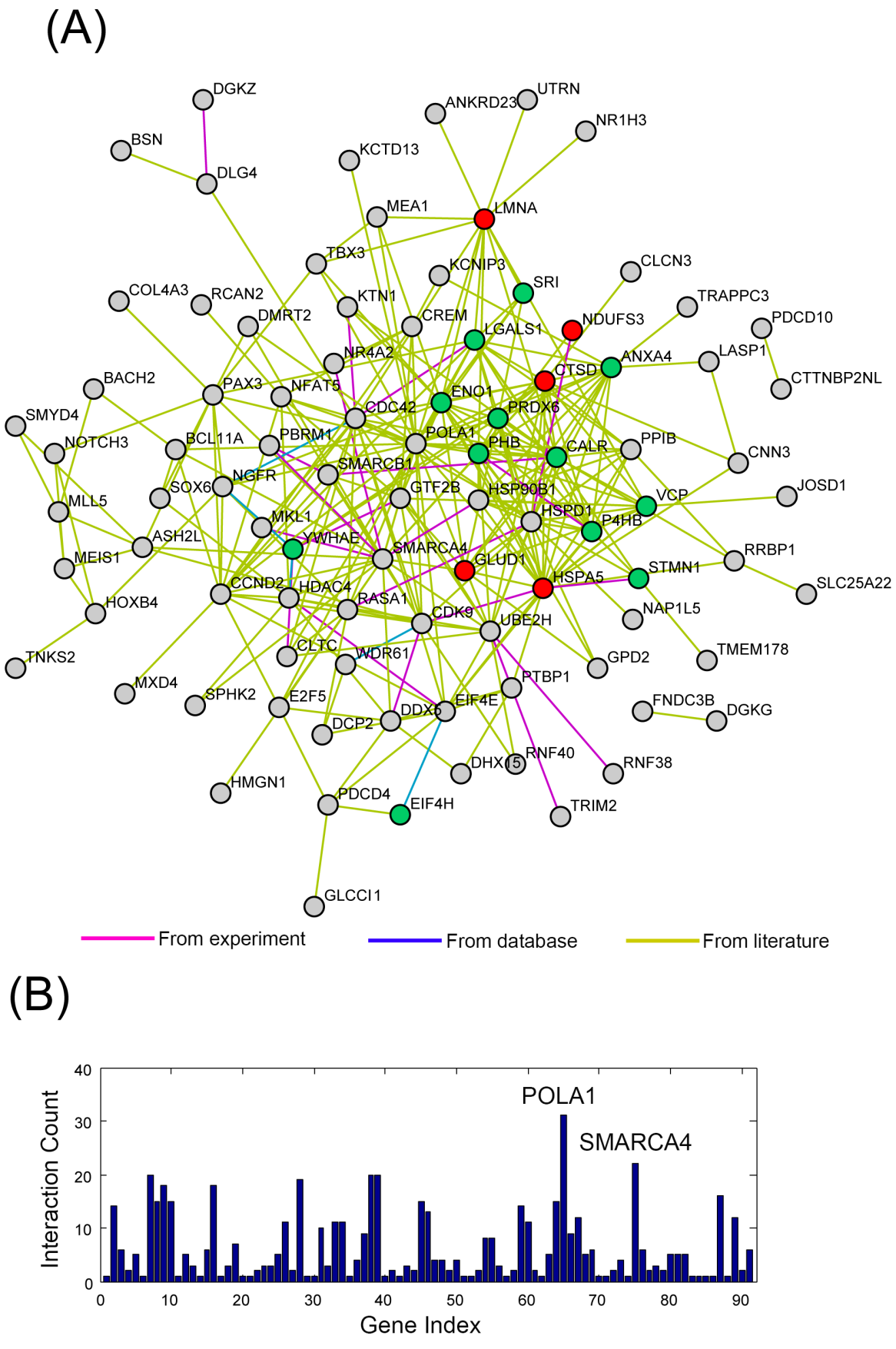

Figure 2. Gene network and hub gene analysis. (A) Protein network of 17 experimentally identified differently expressed proteins in proteomic studies combined with 106 computationally predicted miR-206 targets; **(B)** "Hub" genes in miR-206-related network.

indirect targets. Among the differently expressed proteins in this study, only ANXA4 is a direct predict target.

Different from previous studies, this study proposed a novel strategy to combine both of predicted and experimental data to identify functional miRNA targets. Using the networks composed, we rank the direct and indirect candidate targets by the connectivity in a miR-206 regulatory network. Absence of "hub" genes would be expected to affect many more other miRNA targeted proteins in gene network (*9*); therefore, these "hub" genes might represent important regulators for miRNA in mechanism studies.

Among the "hub" genes, *CALR* (*calreticulin*), *CTSD* (*cathepsin D*), *ENO1* (*enolase 1*), *HSPA5* (*heat shock 70kDa protein 5*) were from experimental data, while *CDC42* (*cell division cycle 42*), *HSPD1* (*heat shock 60kDa protein 1*), *POLA1*, *SMARCA4* from the predicted genes. Most of these targets have been proved to be associated with cancer progressions. *CDC42* is a critical β-catenin signaling driver in osteoblasts (*10*). *HSPD1* has been identified to play an important role in gene networks underlying bone development (*11*). Therefore, we proposed that these "hub" target genes might help to account for the role of miR-206 as a potential tumor suppressor and osteogenesis inhibitor.

In conclusion, in this study, using miR-206 as an example, we constructed a network using the predicted genes and experimentally identified proteins by a DIGE-based proteomic method. Using this network, we developed a bioinformatic strategy to rank the importance of targets according to their potential role in the miRNA-mediated gene profiling. And the "hub" genes we found for miR-206 in this study deserve further investigation in future.

Acknowledgements

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Brief Report

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SCUBE3 overexpression predicts poor prognosis in non-small cell lung cancer

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Summary **Signal peptide-CUB-EGF-like domain-containing protein 3 (SCUBE3) is highly expressed in invasive lung cancers. In vitro investigation indicated that SCUBE3 may play a critical role in lung cancer invasion and metastasis. The current study immunohistochemically investigated the expression of SCUBE3 in 119 cases of non-small cell lung cancer (NSCLC) tumors and this study evaluated its clinical-pathological and prognostic significance. SCUBE3 was found to be up-regulated in NSCLC tissue samples compared to adjacent normal tissue. High SCUBE3 expression was noted in 84/119 (70.6%) of NSCLC tissue samples and was positively correlated with lymph node involvement (p = 0.001) and advanced stages of tumor/lymph node metastasis (TNM) (p = 0.014). Furthermore, high SCUBE3 expression was significantly associated with loss of the epithelial marker E-cadherin (p = 0.0015) and acquisition of expression of the mesenchymal marker vimentin (p = 0.005). Patients with high SCUBE3 expression had significantly a shorter survival time compared to patients with low SCUBE3 expression (p = 0.001), and SCUBE3 expression served as an independent prognostic factor for NSCLC patients. Results indicated that SCUBE3 might be involved in regulating the epithelial-mesenchymal transition (EMT) and malignant progression in NSCLC. Results also indicated that SCUBE3d may be a potential therapeutic target for lung cancers.**

Keywords: Non-small cell lung cancer, SCUBE3, epithelial-mesenchymal transition, prognosis, metastasis

1. Introduction

Lung cancer is the most commonly occurring type of cancer and the leading cause of cancer-related deaths worldwide. In China, the incidence of lung cancer is still rapidly increasing, and lung cancer mortality has increased by 465% over the past three decades (*1*). Non-small cell lung cancer (NSCLC) is the most frequent (approximately 85%) type of lung cancer. Due to the lack of effective biomarkers, at least 40% of patients with lung cancer are diagnosed in an advanced stage (*2*). Despite efforts at treatment, the prognosis for patients with NSCLC remains poor, with only a 5-year survival rate of 15% after patients have received the standard therapies (*3*). The pathological stage is currently the most commonly accepted prognostic factor for NSCLC but is not enough to significantly improve the management of patients. Therefore, reliable and independent prognostic or predictive markers are needed for further intervention.

Signal peptide-CUB-EGF-like domain-containing protein 3 (SCUBE3) is a secreted cell-surface glycoprotein that has been implicated in murine

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embryogenesis and development (*4*). Recently, Wu *et al*. (*5*) found that SCUBE3 was also highly expressed in extremely invasive lung cancers. Further investigation indicated that SCUBE3 may play a critical role in lung cancer invasion and metastasis, mainly *via* triggering the transforming growth factor-β (TGF-β) pathway and subsequently promoting tumor angiogenesis and the epithelial-mesenchymal transition (EMT) (*5,6*). These findings suggest that SCUBE3 might be a potential oncotarget for pharmacological intervention.

Recent studies have noted that methylation of SCUBE3 is significantly associated with an increased risk of cancer recurrence or death (*7*). Although *in vitro* studies have confirmed that SCUBE3 plays an important regulatory role in the development of lung cancer (*5,6*), its clinicopathological and prognostic significance and its association with EMT in NSCLC specimens have yet to be clarified. The current study immunohistochemically analyzed the expression of SCUBE3 in archived NSCLC tissue samples. Moreover, this study investigated the relationship between levels of SCUBE3 expression and expression of three EMT markers in NSCLC samples. Furthermore, this study assessed its potential clinicopathological and prognostic value in patients with NSCLC.

2. Materials and Methods

2.1. *Patients and samples*

Archived formalin-fixed paraffin-embedded (FFPE) NSCLC samples with corresponding adjacent non-tumor tissue were obtained from 119 patients undergoing surgery from January 2004 to December 2008. Clinical and pathological characteristics including gender, age, histological type, grade, stage, tumor size, differentiation, and status of lymph node metastasis are summarized in Table 1. None of the 119 patients received chemotherapy or radiation therapy before surgery. Survival was calculated from the date of surgery until the date of death or last follow-up appointment. The median follow-up was 39 months (range 4 to 89 months). This study was approved by local research ethics committees.

2.2. *Immunohistochemistry*

Immunostaining was performed as previously described. Briefly, 4 μm deparaffinized slices were incubated with 3% H_2O_2 in phosphate-buffered saline to block endogenous peroxidase. Antigen retrieval was performed by a combination of heat and pressure in sodium citrate buffer. Slides were incubated with rabbit polyclonal anti-SCUBE3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; at 1:100 dilution), mouse monoclonal anti-human E-cadherin antibody (Santa Cruz Biotechnology; at 1:100 dilution), and mouse monoclonal anti-human vimentin antibody (Santa Cruz Biotechnology; at 1:100 dilution). Detection was performed using the DAKO EnVision system (DAKO, Carpinteria, CA, USA) with diaminobenzidine (DAB) as the chromogen. Normal mouse IgG was used instead of the primary antibodies as a negative control.

2.3. *Evaluation of immunohistochemical results*

Immunostaining was evaluated by two independent pathologists who were blinded to clinical data. The percentage of SCUBE3-positive cells was determined and a score of 0 to 3 was assigned: 0 for 0%, 1 for 1-33%, 2 for 34-66%, and 3 for 67-100%. The intensity of SCUBE3 staining was scored from 0 to 3 for no staining, 1 for weak staining, 2 for moderate staining, and 3 for strong staining. SCUBE3 immunostaining results were scored by multiplying the percentage of positive cells by their intensity and expression was classified as low (0-3) or high (4-9). For selected EMT makers (E-cadherin and vimentin), immunohistochemistry staining was evaluated using the method described in a previous study: no staining or positive staining of < 10% of tumor cells was deemed to be negative, whereas positive staining of ≥ 10% of tumor cells was considered to be positive (*8*).

2.4. *Statistical analysis*

The association between SCUBE3 expression and clinicopathologic variables and between SCUBE3 expression and expression of EMT markers was examined using the chi-square test. Overall survival curves were calculated using the Kaplan-Meier method and were compared using the log-rank test. Factors with statistical prognostic significance in univariate models were included in multivariate analysis using a multivariate Cox regression model. Statistical analyses were completed using SPSS 16.0, and a p value of less than 0.05 was considered statistically significant.

3. Results and Discussion

3.1. *Immunohistochemical results for SCUBE3 and EMT markers in NSCLC tissue samples*

Representative immunohistochemical staining patterns observed for SCUBE3 and two EMT markers proteins are shown in Figure 1. Different levels of positive staining for SCUBE3 were noted mainly in the cytoplasm and membrane of cancer cells from NSCLC tissue samples. SCUBE3 immunostaining was not observed in the stroma of these tissue samples. High SCUBE3 expression was noted in 84/119 (70.6%) of NSCLC tissue samples and was noted in 18/119 (15.1%) of adjacent matched non-cancerous tissue samples

(Pearson's chi square test, $p < 0.001$).

The pattern of E-cadherin expression was predominantly cytoplasmic and membranous in the area of the tumor. Positive immunohistochemical staining for vimentin in cancer cells was observed in the cytoplasm of cancer cells or stromal fibroblasts in NSCLC tissue samples.

3.2. *Relationship between expression of SCUBE3 proteins and clinicopathological parameters in NSCLC*

The association between SCUBE3 expression and clinicopathological features of NSCLC is shown in Table 1. SCUBE3 expression was higher in patients with lymph node metastasis and subgroups with advanced stages of cancer than in patients with no lymph node metastasis or an early stage tumor; differences in the levels of SCUBE3 expression were statistically significant ($p = 0.001$ and $p = 0,014$, respectively). No significant associations with age, gender, tumor size, histological type, or grade were noted.

Table 1. Correlation of high SCUBE3 expression with clinicopathologic characteristics of NSCLC

Variable	No.	SCUBE3 expression		*p* value
		Low ($n = 35$)	High ($n = 84$)	
Age (years)				
<60	36	11	25	0.805
≥60	83	24	59	
Gender				
Male	93	28	65	0.753
Female	26	7	19	
Pathological type				
Squamous cell carcinoma	58	19	39	0.435
Adenocarcinoma	61	16	45	
Tumor size				
T1-T2	102	32	70	0.250
T3	17	3	14	
Lymph node status				
Negative	67	28	39	0.001
Positive	52	7	45	
Tumor grade				
G1-2	73	19	54	0.307
G3	46	16	30	
TNM stage				
1-2	83	30	53	0.014
3-4	36	5	31	
E-cadherin				
Negative	64	13	51	0.019
Positive	55	22	33	
Vimentin				
Negative	88	32	56	0.005
Positive	31	3	28	

3.3. *Correlation of SCUBE3 expression with expression of the EMT indicator proteins*

The relationship between SCUBE3 expression and expression of EMT indicator proteins was analyzed (Table 1). In the 119 NSCLC tissue samples, epithelial protein loss occurred at a rate of 52.9% for E-cadherin. In the same samples, expression of abnormal mesenchymal proteins occurred at a rate of 26.1% for vimentin. Results also indicated that high expression of SCUBE3 correlated with a loss of E-cadherin expression (Pearson's Chi-Square = 5.522, $p = 0.019$) and anomalous positivity of vimentin (Pearson Chi-Square = 7.864, $p = 0.005$) in clinical NSCLC samples.

3.4. *Survival analysis*

Survival was plotted using the Kaplan-Meier method. As seen in Table 2 and Figure 2, results revealed that patients with high SCUBE3 expression had a significantly shorter survival time than those with low SCUBE3 expression. Furthermore, loss of E-cadherin and acquired vimentin protein expression were also significantly associated with overall survival according to univariate analysis. SCUBE3 expression and these two genetic markers were included in a multivariate Cox regression model along with two other clinical prognostic factors (status of lymph node metastasis and stage). Results revealed that high SCUBE3 expression and a loss of E-cadherin expression were independent prognostic factors for a shorter survival time in patients with NSCLC (Table 2).

In this study, immunohistochemistry showed that SCUBE3 was up-regulated in NSCLC tissue samples compared to adjacent normal tissue, and

Table 2. Univariate and multivariate analysis of the prognosis for NSCLC

Variable	Univariate analyses HR for death (95% CI)	*p* value	Multivariate analysis HR for death (95% CI)	*p* value
Age	0.965 (0.515-1.809)	0.912		
Gender	0.951 (0.472-1.919)	0.889		
Pathological type	1.160 (0.650-2.069)	0.616		
Tumor size	1.669 (0.804-3.467)	0.169		
Lymph node status	3.546 (1.894-6.637)	< 0.001	2.426 (1.019-5.780)	0.045
Tumor grade	1.040 (0.578-1.872)	0.895		
TNM stage	2.611 (1.453-4.690)	0.001	1.054 (0.495-2.244)	0.891
E-cadherin	0.359 (0.188-0.687)	0.002	0.472 (0.244-0.914)	0.026
Vimentin	2.210 (1.210-4.039)	0.010	1.012 (0.520-1.968)	0.973
SCUBE3	4.520 (1.784-11.452)	0.001	2.962 (1.141-7.689)	0.026

HR: Hazard ratio; CI: Confidence intervals.

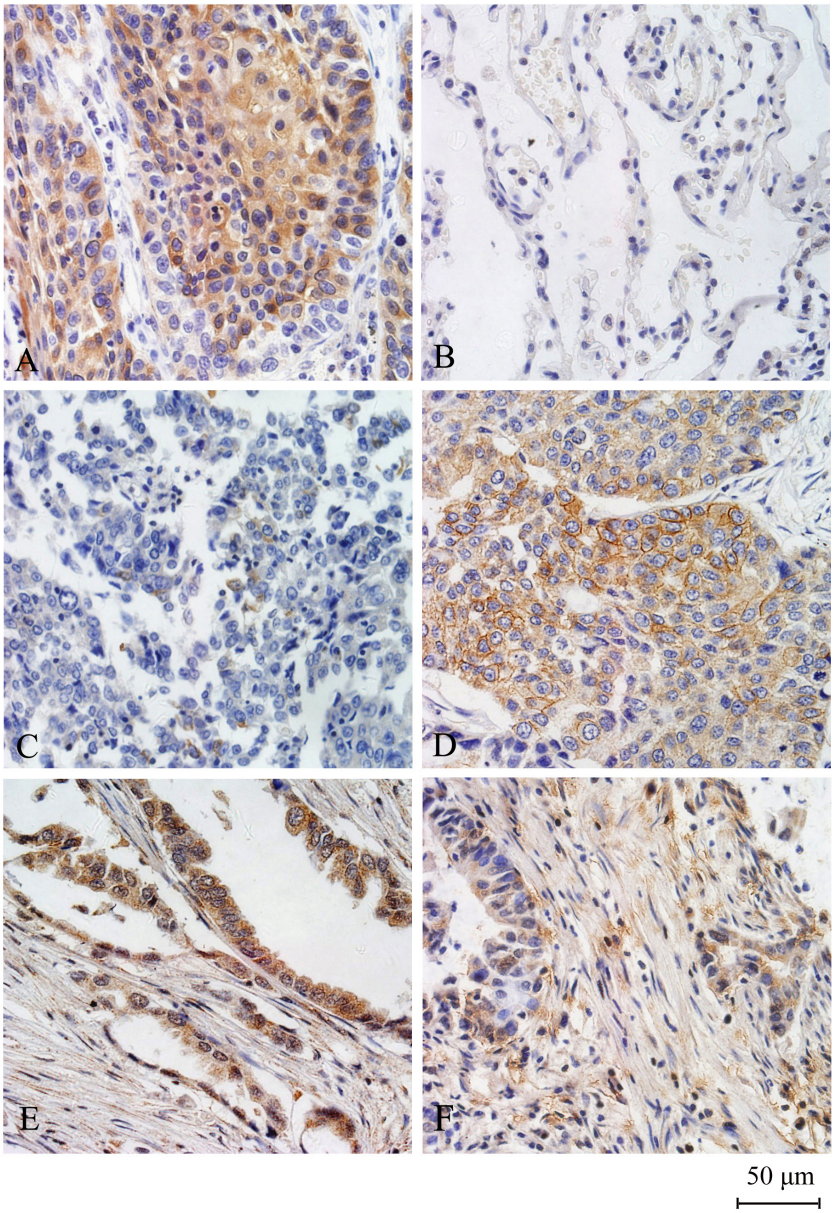

Figure 1. Representative immunohistochemical images of SCUBE3 and EMT markers. (A) High SCUBE3 expression in NSCLC tissue samples; **(B)** Negative SCUBE3 expression in adjacent lung tissue; **(C)** Loss of epithelial marker E-cadherin in NSCLC tissue samples; **(D)** Positive expression of E-cadherin in NSCLC tissue samples; **(E)** Acquisition of expression of the mesenchymal marker vimentin in NSCLC cells; **(F)** Vimentin expression was absent in cancer cells but present in stromal cells from NSCLC tissue samples.

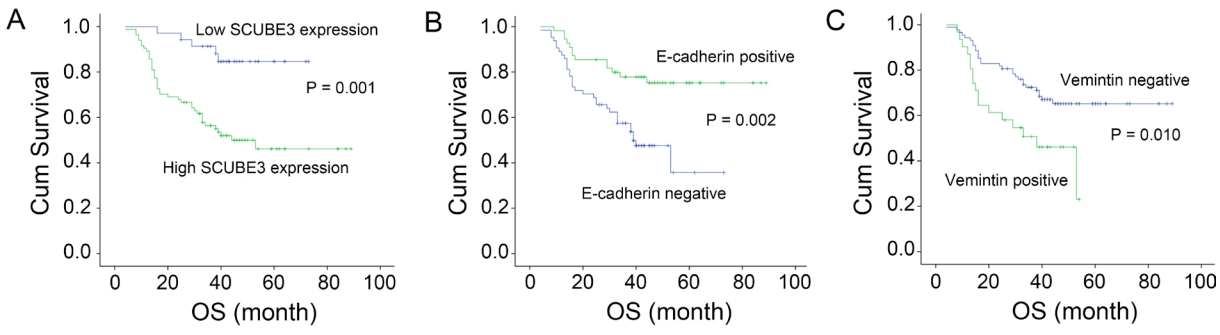

Figure 2. Kaplan-Meier survival curves in accordance with SCUBE3 expression and expression of EMT markers. (A) A subgroup with high SCUBE3 expression had a significantly shorter survival than a subgroup with low SCUBE3 expression; **(B)** Curves calculated for E-cad expression indicated that patients with decreased E-cadherin had a shorter survival time than patients who were positive for E-cadherin; **(C)** Curves calculated for vimentin expression indicated that a subgroup testing positive for vimentin had a shorter survival time than the negative control.

immunohistochemistry also indicated that high levels of SCUBE3 expression were associated with aggressive traits, shorter survival, and expression of EMT indicator proteins in NSCLC. Furthermore, high expression of SCUBE3 was shown to be an independent prognostic factor for patients with NSCLC. To the extent known, this is the first study that has focused on the clinicopathological and prognostic value of SCUBE3 expression and its association with EMT phenomena in clinical NSCLC tissue samples.

There is considerable proof that presence of the EMT phenomenon is related to more aggressive behaviors and shorter survival in lung cancer (*9-11*). The current results revealed that loss of E-cadherin and acquired vimentin expression were associated with outcomes in NSCLC, but E-cadherin was the only EMT marker that independently predicted prognosis. These findings are consistent with those of a previous study (*12*). Mounting *in vitro* evidence suggests that SCUBE3 plays an important role in regulating the EMT and progression in lung cancer. Wu *et al*. found that exogenous SCUBE3 treatment promoted lung cancer cell mobility and invasiveness. Through the C-terminal CUB domain, SCUBE3 binds to the TGF-β type II receptor and then induces Smad2/3 phosphorylation, increasing Smad2/3 transcriptional activity and up-regulating the expression of target genes involved in EMT and cancer progression. The current study expanded on those findings and this study indicated that high SCUBE3 was associated with lymph node metastasis and advanced stages of tumor/lymph node metastasis (TNM) in clinical NSCLC samples. In this study, the TNM stage failed to retain prognostic significance in multivariate analysis. This may be due to differences in the clinical status of patients and their tolerance of treatment. High levels of expression for SCUBE3 were found to be correlated with decreased E-cadherin protein expression and increases in vimentin protein expression in NSCLC. Therefore, the current results support the contention that the regulatory role of SCUBE3 in the EMT process accounts for its positive association with aggressive behaviors such as metastasis and poor prognosis.

Wu *et al*. and Chou *et al*. found that knockdown of SCUBE3 expression suppressed tumorigenesis and effectively inhibited the metastatic potential of NSCLC in an *in vivo* model. Microarray analysis revealed that SCUBE3-knockdown tumors had decreased expression of several genes involved in angiogenesis and EMT. Given the current findings with regard to the clinicopathological and prognostic significance of SCUBE3 in NSCLC, SCUBE3 might be a potential therapeutic target for lung cancer treatment, and particularly for the prevention of metastatic progression and invasion.

In conclusion, SCUBE3 was found to be closely associated with tumor progression and a poor prognosis in NSCLC. Highly expressed SCUBE3 could serve as an independent prognostic factor for NSCLC. Furthermore, results also indicated that SCUBE3 was correlated with expression of EMT-related genes in clinical NSCLC samples. SCUBE3 could serve as a potential therapeutic target in patients with lung cancers.

Acknowledgements

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Original Article

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A high-salinity solution with calcium chloride enables RNase-free, easy plasmid isolation within 55 minutes

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Summary **We dramatically improved a plasmid-isolation protocol based on the popular alkaline-sodium dodecyl sulfate plasmid isolation method. Our modified method provides significant time and cost savings. We used a modified solution during the neutralization step, which allowed us to skip several subsequent handling steps, saving a great amount of time. The plasmids purified by this method were of high quality, and the optical density ratio 260 and 280 was approximately 1.8. Plasmid DNA isolated by our method was of sufficient quality to perform subsequent restriction enzyme cuts and other downstream experiments, including budding yeast transformation, cultured cell transfection, and *Caenorhabditis elegans* injection experiments.**

Keywords: Plasmid isolation, calcium chloride, polyethylene glycol, RNase-free

1. Introduction

Plasmid isolation from *Escherichia coli* is an indispensable step in most routine laboratory experiments for molecular biology, biochemistry, and cell biology. There are several published plasmid-isolation methods (*1-8*). Among them, the alkaline-sodium dodecyl sulfate (SDS) method (*1*) is the most popular procedure for purifying plasmid DNA. In this method, the DNA denaturation step (using Solution II) and neutralization step (using Solution III) are very effective and sophisticated techniques for separating plasmid DNA from *E. coli* genomic DNA. Moreover, insoluble cellular debris, including proteins, is separated together with genomic DNA from plasmids. One of the difficulties of this popular method is that a huge amount of RNA is collected along with the plasmid DNA. Therefore, RNase is always required to remove unwanted RNA from the plasmid solution. Then a hazardous organic solvent (phenol/chloroform) is added to inactivate and remove the RNase protein. This process requires several additional steps and extra time.

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Many commercial kits are available for plasmid isolation. Two major kits with different principles are widely used. One is based on an anion-exchange resin (*9*). Plasmid DNA is adsorbed onto the resin by the negative charge of DNA and then eluted by adding a high-salinity solution. Another method employs a silica membrane with chaotropic solutions (*10,11*). Under chaotropic conditions, nucleic acids are adsorbed onto silica particles and eluted using pure water. In both major commercial kits, the principle of separating plasmid DNA from bacterial genomic DNA is still based on the popular alkaline-SDS method. Moreover, both kits require RNase to digest unwanted RNA. Therefore, a large amount of RNase is added to the kit solution. These kits are very easy to use, but rather expensive. Thus, another time- and cost-saving protocol for high-quality and high-quantity plasmid isolation is needed for everyday experiments in the laboratory. Furthermore, RNase is widely known as a robust, stable protein. RNase protein contamination results in the degradation of RNA in the laboratory and disrupts RNA experiments. It is best not to use RNase protein in laboratories that handle RNA molecules.

Calcium chloride ($CaCl_2$) is an effective reagent that selectively removes RNA from a mixture of DNA and RNA (*12,13*). That is, RNA can be precipitated by centrifugation in the presence of $CaCl_2$ (RNase is not needed). However, this requires several centrifugation

steps and takes quite a long time. Hence, we established a modified plasmid purification method using $CaCl_2$ (called the Super Sol III method or Sasagawa method) that is based on standard alkaline-SDS isolation but is much easier and less time consuming. Our method eliminates several steps, allowing us to isolate plasmid DNA in much less time, and the total isolation time is around 55 min.

2. Materials and Methods

2.1. *E. coli, liquid medium, and plasmid DNA*

We used *E. coli* JM109 or XL-1 blue, which we routinely use for cloning experiments. Bacteria were grown in LB medium supplemented with ampicillin (final concentration, 50 μL/mL). *E. coli* was incubated in 15-50-mL tubes with 5-10 mL LB medium in a shaking air incubator. The plasmids pBluescript and pUC118, and their derivatives, were also tested.

2.2. *Reagents and equipment*

For all of our experiments, the purest grade reagents available were purchased from Wako Pure Chemical Industries (Osaka, Japan) or Nacalai Tesque (Kyoto, Japan). Solutions I and II were prepared according to a standard protocol (*14*). We purchased restriction enzymes from Takara-Bio (Shiga, Japan) and/or Toyobo (Osaka, Japan) to cut plasmid DNA. Sample quality and quantity were determined using a NanoDrop 2000 (Thermo Fisher Scientific, Yokohama, Japan). A Narishige micromanipulator (Tokyo, Japan) was used to inject plasmid into *C. elegans*.

2.3. *Super Sol III solution*

The modified Solution III (Super sol III or Sol III-Ca) consisted of 1 mL Solution III (*14*), 1 mL 5 M $CaCl_2$, and 0.5 mL H_2O. A warming step to dissolve the solution might be needed (*i.e.*, 37°C or above).

2.4. *Handmade filtration column*

We made a filtration column as follows (Figure 1). The bottom of a 5-mL polystyrene round-bottom tube (*e.g.*, Falcon 352058) was drilled using a heated ice pick to create a pinhole (Figure 1a). A polypropylene centrifuge tube (15 mL) was used for sample collection. We made a hole in a 15-mL screw cap tube using a cork borer (#6, Φ12 mm). The round-bottom tube was inserted through the cap hole, and the top of the round-bottom tube was taped with electrical tape as a stopper (Figure 1b). A piece of tissue paper was pushed firmly into a 5-mL polystyrene round-bottom tube (Figure 1c). Before use, 5 mL H_2O was applied and the filter was washed using centrifugation. After centrifugation, filtered H_2O was discarded.

2.5. *Budding yeast, cultured cells, and C. elegans*

The budding yeast *Saccharomyces cerevisiae* strain PJ69-4A was used for transformation. A derivative of plasmid p426ADH (*15*) was transformed into the yeast using URA3 as a selectable marker. A pEGFP plasmid was transfected into HeLa cells as a model of mammalian cell transfection. *C. elegans* (N2 strain) were injected with plasmids pRF4, in which a mutated collagen gene is coded.

3. Results

3.1. *Removal of high-molecular-weight RNA in a single step*

Our first challenge was to modify Solution III by adding $CaCl_2$. We named the new mixture Super Sol III. We tested it and found that it neutralized as well as traditional Solution III. Moreover, it greatly reduced the amount of RNA in the cleared lysate. Higher molecular weight RNA precipitates out with protein and genomic DNA. Only small RNAs, such as tRNA, seem to remain in the lysate (Figure 2, lane 3).

Centrifugation was thought to be necessary to precipitate RNA in the presence of $CaCl_2$. However, surprisingly, we found that centrifugation was not absolutely necessary to remove high-molecular-weight RNA. To test the effect of Super Sol III on RNA removal from the lysate, we first filtered the neutralized sample solution, which contained a large amount of debris, without using centrifugation. We then added 2-propanol to the filtered lysate and centrifuged the

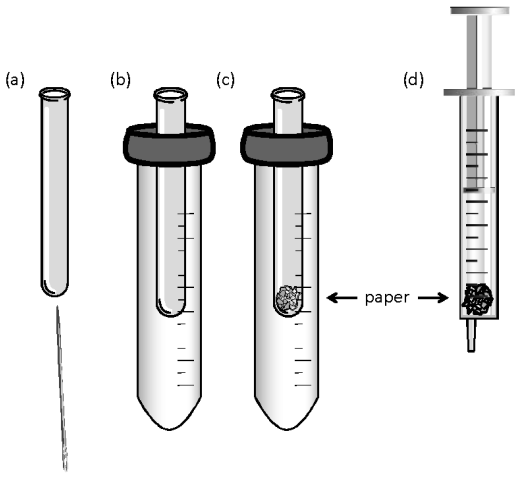

Figure 1. A handmade filtration column. (a) A hole was made in the bottom of a round-bottom 5-mL polystyrene tube using a heated needle. **(b)** A hole was made in the top of a 15-mL tube screw cap, and a 5-mL tube **(a)** was inserted through the hole. The top of the round-bottom tube was taped with electrical tape as a stopper. **(c)** A piece of tissue paper was pushed into a 5-mL polystyrene round-bottom tube. **(d)** A 2.5-10-mL disposable syringe with tissue paper could also be used as a handmade filter in this experiment.

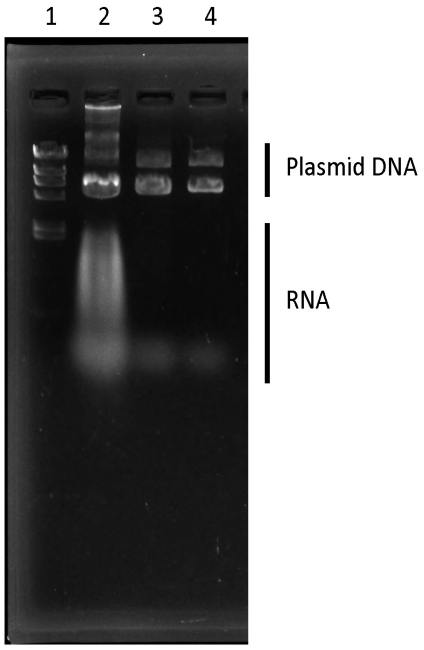

Figure 2. Effect of RNA removal by $CaCl_2$, which is premixed into Solution III. Lane 1: molecular weight marker. Lane 2: negative control (standard alkali-SDS plasmid purification without RNase). Lane 3: a solution neutralized by Super Sol III was centrifuged, and then 2-propanol precipitation was performed. Lane 4: a solution neutralized by Super Sol III was filtered without centrifugation, and then 2-propanol precipitation was performed.

sample to precipitate nucleic acids. This procedure resulted in the recovery of plasmid DNA and a small amount of RNA (Figure 2, lane 4). These results indicate that a large amount of RNA became insoluble, and was removed with insoluble proteins and debris as well as genomic DNA during the filtration step.

3.2. *A combination of Super Sol III and polyethylene glycol precipitation*

Based on the above results, we further improved the protocol by adding polyethylene glycol directly to the filtered lysate to precipitate plasmid DNA. Polyethylene glycol precipitates plasmid DNA, but not small-molecular-weight RNA (*16,17*). We added polyethylene glycol to a final concentration of 0-12% to filtered lysate and centrifuged the sample to precipitate plasmid DNA. Pure plasmid DNA without unwanted RNA was obtained. Even small-molecular-weight RNA disappeared from the sample (Figure 3). A final concentration of 6-12% polyethylene glycol produced good results. Therefore, we decided to precipitate plasmid DNA to a final concentration of 8% polyethylene glycol by adding 32% polyethylene glycol solution. Both polyethylene glycol #4,000 and #6,000 worked well for precipitating clear plasmid DNA (data not shown).

Prior to polyethylene glycol precipitation, the debris (*i.e.*, insoluble proteins and genomic DNA) should be completely removed from the lysate. For this purpose,

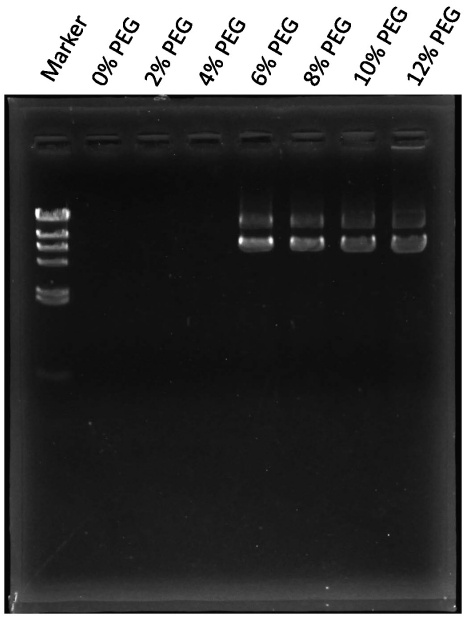

Figure 3. A combination of Super Sol III and polyethylene glycol precipitation. Lane 1: molecular weight marker. Lanes 2–8 represent various final concentrations of polyethylene glycol (PEG) during the plasmid precipitation step. Lane 2: 0% PEG. Lane 3: 2% PEG. Lane 4: 4% PEG. Lane 5: 6% PEG. Lane 6: 8% PEG. Lane 7: 10% PEG. Lane 8: 12% PEG.

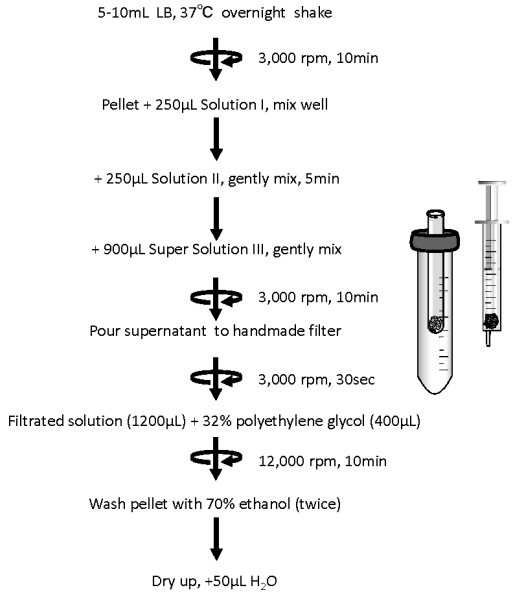

Figure 4. The complete 55-min protocol established in this study.

we made a handmade filtration column (Figure 1). Based on these results, we established our complete plasmid purification protocol (Figure 4).

3.3. *Quality and quantity check of plasmid DNA by spectrophotometer*

We checked the quality of plasmid DNA purified by our Super Sol III method. As shown in Table 1, we obtained very high quality plasmid DNA. The optical

Table 1. Spectrophotometric DNA quality and quantity check

	A_{260}	Conc.(ng/μL)	Total plasmid (μg)	A_{260}/A_{280}
Average	3.58	179	8.95	1.85
S.D	1.81	91	4.54	0.04

Data are presented as average ± S.D.

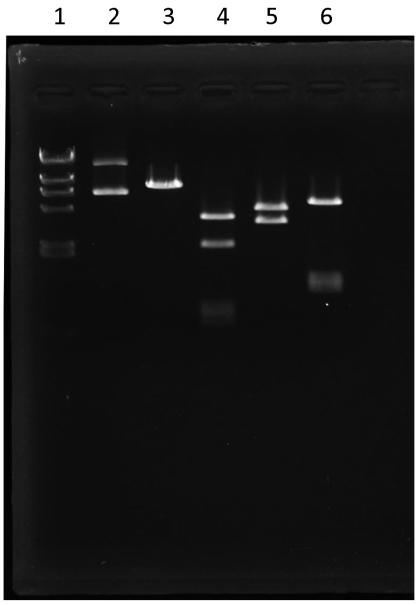

Figure 5. Restriction enzyme check. Lane 1: molecular weight marker. Lane 2: uncut plasmid DNA. Lane 3: *Kpn* I cut (Low-salt buffer). Lane 4: *Hind* III cut (Medium-salt buffer). Lane 5: *Eco* R I cut (High-salt buffer). Lane 6: *Bam* H I cut (High-salt buffer with potassium).

density ratio at 260 and 280 (A_{260}/A_{280}) was around 1.8, indicating that there was no protein contamination in the isolated plasmid. The quantity of plasmid DNA was almost 1 μg/mL in LB medium, which is sufficient for downstream experiments. We found that both handmade-columns and syringe filters have good qualities (Table 1).

3.4. *Restriction enzyme check*

The quality of purified plasmid DNA was also checked by using restriction enzymes. A purified plasmid was cut with *Eco* R I (High-salt buffer), *Bam* H I (High-salt buffer with potassium), *Hind* III (Medium-salt buffer), and *Kpn* I (Low-salt buffer). All of these enzymes successfully cut plasmid DNA (Figure 5).

3.5. *Injection into C. elegans*

To check the quality of the purified plasmid, we injected plasmids into *C. elegans* following a previous study (*18*). Generally, we obtain F1 transformants in the injection experiments, but the F2 transformant (*i.e.* stable transformant) appears only when the transgene is provided in the F1 germline. That is, we can conclude the stable transformation experiment was successful

Table 2. Results of DNA injection into *C. elegans*

Injected worm	9
F1 transformant	6
F2 transformant	3

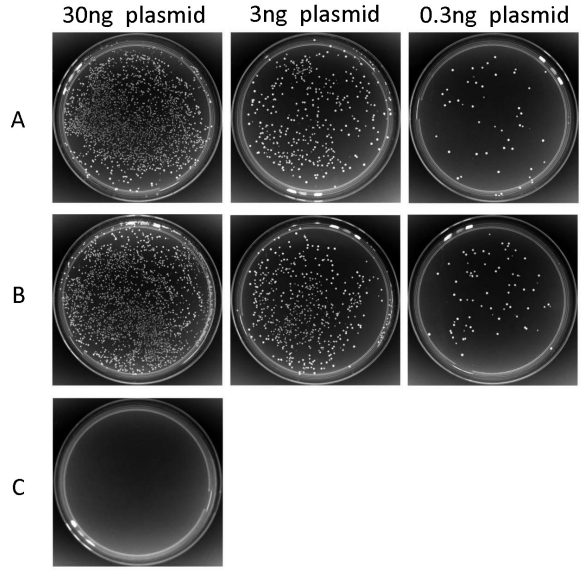

Figure 6. Results of yeast transformation. In total 30 ng, 3 ng, and 0.3 ng plasmid per plate were transformed into the budding yeast. **(A)**, plasmid DNA isolated by our protocol. **(B)**, plasmid DNA isolated by a standard silica membrane kit (positive control). **(C)**, no plasmid DNA (negative control).

only when we obtained F2 transformants. We injected plasmid DNA from our method into nine worms, and obtained six F1 transformants. And then, one of the F1 transformants had F2 transformants, indicating that our injection experiments were successful using the plasmid purified by our method (Table 2).

3.6. *Yeast transformation*

We performed yeast transformation in *S. cerevisiae* to determine plasmid DNA quality. A derivative of plasmid p426ADH (*15*) was purified using our plasmid isolation method, and then transformed into *S. cerevisiae* with a standard lithium chloride protocol (*19*). As shown in Figure 6, transformation was efficient with our plasmid, and was similar to the plasmid transformation efficiency achieved when using a Sigma commercial kit (*10*). Transformation efficiency was calculated as colony forming units per 1 μg plasmid (cfu/μg). According to this calculation, the efficiency of our system was 1.8×10^5 cfu/μg, while the efficiency using the Sigma kit was 2.5×10^5 cfu/μg. Generally, around 10^5 cfu/μg is an acceptable result.

3.7. *Transfection into cultured cells*

Our plasmid-isolation method also provided plasmids of sufficient quality for transfection into cultured cells.

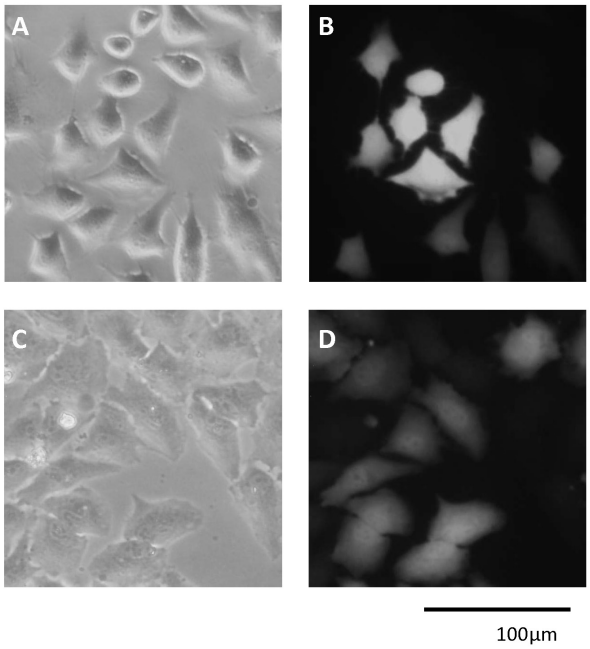

Figure 7. Result of cultured cell transfection. Upper panels **(A, B)** are cells transfected using a plasmid described in this manuscript, and lower panels **(C, D)** are cells using a commercial kit. **(A)** and **(C)**, phase-contrast image; **(B)** and **(D)**, EGFP fluorescence detection.

Using the standard Fugene 6.0 (Promega) protocol (*20*), 0.5 μg pEGFP plasmid was transfected into cells seeded in a 20-mm dish. As shown in Figure 7, we successfully observed EGFP fluorescence in HeLa cells. This indicates that plasmid DNA isolated by our method is of sufficient quality for use in cell transfection. We counted cells in microscopic images and calculated transfection efficiencies. The transfection efficiency of our plasmid isolation method was 20%, whereas the efficiency was 50% when we transfected a plasmid with a Sigma kit.

4. Discussion

The advantage of the traditional alkaline-SDS method is that chromosomal *E. coli* DNA is removed, along with insoluble debris, by several simple steps, leaving plasmid DNA in the cleared lysate. The basic steps of this traditional method include cell lysis and protein/DNA denaturation by the alkaline solution (Sol II) and a sudden pH change to neutrality by neutralization buffer (Sol III). This sudden pH change is essential to transform genomic DNA and proteins into insoluble debris.

The difficulty of this method lies in separating RNA from the cleared lysate; RNA and plasmid DNA react similarly to pH changes. Therefore, an RNA-removal step using RNase is always needed, which requires additional processes such as phenol/chloroform extraction.

Our new plasmid-purification protocol greatly improved on previous methods in two ways. First, we modified Solution III (into Super Sol III) by adding calcium chloride to directly remove a large amount of RNA during the neutralization step. This allowed us to purify plasmid high quality DNA in fewer steps. It also did not require RNase incubation or a hazardous phenol/chloroform extraction step. Our modified Solution III removed unwanted RNA in the neutralization step without centrifugation. A small amount of RNA still remained in the cleared lysate, which was easily removed by simple polyethylene glycol precipitation. Second, we added a filtration step. Unlike an anion-exchange column and/or a silica membrane column, our column simply filtered and separated insoluble debris. In our protocol (Figure 4), centrifugation was performed before filtration not to precipitate RNA but simply to reduce debris. Use of a commercial filter and/or gel filtration resin (such as Sephadex) may lead to a much better result, although our handmade column was sufficient for our experiments. The syringe filter was easier to handle, although the total quantity was better in the handmade-column than the syringe (Table 1). This might be due to the dead volume of the syringe filter. The syringe filter is still applicable because the plasmid purified using the syringe filter had a quality good enough for downstream experiments. In our manuscript, Figure 2, Table 1 (in part) and Table 2 were data using the syringe filter, and others were from the handmade-column. We tested both filters and concluded that they worked well in our daily experiments (data not shown).

Too large of an amount of *E. coli* at the start (*i.e.* too much *E. coli* cells for reagent volumes) results in insoluble impurities in the final plasmid solution. It is important to keep a volume balance between solutions and *E. coli*. In large-scale experiments, simply dividing *E. coli* samples into several test tubes will give a good result. An option for scaling up is to use a 50-mL polypropylene centrifuge tubes and 15-mL polystyrene round-bottom tubes for the handmade-column, instead of the tubes indicated in Figure 1. We checked and confirmed that this scaled-up protocol worked well up to 50-mL LB medium (data not shown).

Generally, super-high-quality plasmid DNA is required for injection into *C. elegans* or transfection into cultured cells. Our data strongly suggests that plasmid DNA isolated by our protocol is of high enough quality for use in biochemical reactions and transformations. A Sigma commercial kit showed better transfection efficiency for cultured cells than our method, but it is very surprising that we can prepare a transfection-grade plasmid by such a simple protocol as described in this manuscript. This commercial kit describes that up to 15 μg of plasmid DNA can be purified from 1-5 mL of *E. coli* culture (*10*), which is a better quantity than our method. Nevertheless, our method has good quality and quantity for downstream experiments (Table 1). Besides, our method has significant advantages that we

do not need RNase, any special reagents or equipment. The column and syringe are recyclable, so that we do not need to take these costs into account.

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Original Article

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Increased serum leukocyte cell-derived chemotaxin 2 (LECT2) levels in obesity and fatty liver

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Summary **Leukocyte cell-derived chemotaxin 2 (LECT2) is a signaling molecule expressed in the liver and regulated by Wnt/β-catenin pathways implicated in hepatic metabolism. However, the clinical relevance of LECT2 in obesity and fatty liver is unknown. The objective of this study was to determine whether serum LECT2 levels are affected by of obesity and fatty liver. A cross sectional study comprising 231 Japanese adult subjects were tested for LECT2 using a highly sensitive assay. We evaluated the associations between LECT2 and the anthropometric or clinical markers of obesity and fatty liver. The mean serum LECT2 levels were 43.5 ± 13.6 ng/mL. LECT2 positively correlated with all the anthropometric measures of obesity: body mass index, waist circumference, waist-to-hip ratio, and waist-to-height ratio (W/Ht). Multiple regression analysis revealed that LECT2 is independently related to γ-glutamyl transpeptidase (γ-GTP), triglyceride, and age in males, whereas in females it was related to the homeostasis model assessment ratio, blood urea nitrogen, high-density lipoprotein cholesterol, and γ-GTP. Receiver operating characteristics curve analyses revealed that LECT2 correlated with obesity [area under the curve (AUC) 0.655, 95% confidence interval (CI) = 0.551-0.758, $p = 0.002$ in males; AUC 0.670, 95% CI = 0.570-0.770, $p < 0.001$ in females] and fatty liver (AUC 0.646, 95% CI = 0.544-0.749, $p = 0.004$ in males; AUC 0.733, 95% CI = 0.621-0.844, $p < 0.001$ in females). The present study indicates that serum LECT2 levels are increased by obesity and fatty liver, and suggests that LECT2 is a novel obesity-related protein.**

Keywords: Leukocyte cell-derived chemotaxin 2, obesity related protein, enzyme-linked immunosorbent assay

1. Introduction

The rapidly growing incidence of obesity worldwide is alarming because it is a major cause of morbidity and mortality. Obesity increases the risk of developing serious complications, including type 2 diabetes mellitus, hypertension, steatohepatitis, fatty liver, and dyslipidemia (*1*). Fatty liver is characterized by excessive triglyceride (TG) accumulation in the liver and is one of the most common forms of liver disease associated with obesity (*2*). Obesity alters hepatic metabolism as well as cytokine and growth factor production, which contribute to the development of obesity-associated complications. In a mouse model of high-fat diet-induced obesity, the Wnt/β-catenin signaling pathway was demonstrated to regulate hepatic metabolism (*3*). In contrast, in another study, liver-specific β-catenin knockout mice revealed increased susceptibility to methionine- and choline-deficient diet-induced steatohepatitis (*4*). These reports

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suggest that this pathway plays an important role in hepatic metabolism.

Leukocyte cell-derived chemotaxin 2 (LECT2) is one of the proteins regulated by hepatic β-catenin (*5*) and is primarily expressed by hepatocytes (*6,7*). It was initially identified *in vitro* as a neutrophil chemotactic factor (*8*). More recently, functional studies have reported that LECT2 may function in immunological events such as hepatitis (*9*) and arthritis (*10*), antihepatocarcinogenesis (*11,12*), and the negative regulation of the Wnt receptor signaling pathway in the small intestine (*13*). There is accumulating evidence indicating that LECT2 is a pleiotropic protein, as are many cytokines; however, the possible involvement of LECT2 in metabolic diseases has not been investigated.

The aim of the present study was to determine whether serum LECT2 levels may be affected by obesity and its related diseases such as fatty liver. We developed a highly sensitive enzyme-linked immunosorbent assay (ELISA) protocol to evaluate the association between serum LECT2 levels and anthropometric or clinical variables. Moreover, this study sought to provide evidence that LECT2 is specific for metabolic diseases and is not a predictor of other disorders such as chronic kidney disease (CKD).

2. Materials and Methods

2.1. *Study subjects*

The study included 231 Japanese subjects (113 males and 118 females; aged 40-69 years) who visited the Department of General Medicine at the National Center for Global Health and Medicine (Tokyo, Japan) between August 2010 and September 2012 for an annual health check-up. Subjects were categorized into obese [waist-to-height ratio (W/Ht) ≥ 0.5, n = 113] and lean (W/Ht < 0.5, n = 118) groups. The subjects receiving medication for diabetes mellitus, hypertension, or dyslipidemia or those with a provisional diagnosis of diabetes mellitus [fasting plasma glucose ≥ 126 mg/dL or hemoglobin A_{1c} (HbA1c) ≥ 6.5%] were excluded. This study was conducted with the approval from the ethical committee of the National Center for Global Health and Medicine. Written informed consent was obtained from each subject prior to study enrollment.

2.2. *Data collection*

Anthropometric measurements and blood sampling were performed after overnight fasting (for at least 12 h). Waist circumference (WC) at the level of the umbilicus and hip circumference at the level of the maximum protuberance of the buttocks were recorded with a measuring tape. Body mass index (BMI), waist-to-hip ratio (WHR), and W/Ht were calculated using the anthropometric measures. For the insulin resistance index, the homeostasis model assessment ratio (HOMA-R) was calculated from fasting plasma glucose (FPG) and fasting insulin values using the following equation: HOMA-R = [FPG (mg/dL) × fasting insulin (mU/L)]/405. A physician documented the medical history and alcohol consumption during a personal interview. A diagnosis of fatty liver was made on the basis of the results of an abdominal ultrasound performed by trained technicians. Fatty liver was defined as liver parenchyma with echogenicity higher than that of the kidney cortex, the presence of vascular blurring, and deep attenuation of ultrasound waves (*14*). Subjects were assessed for kidney disease by calculating the estimated glomerular filtration rate (eGFR) according to the Japanese formula using serum creatinine (SCr) (*15*). A diagnosis of CKD was made if eGFR was < 60 mL/min/1.73 m^2 (*16*).

2.3. *Measurement of serum LECT2 by ELISA*

The serum LECT2 levels were measured using a commercially available ELISA kit (Ab-Match ASSEMBLY Human LECT2 Kit with Ab-Match UNIVERSAL Kit; Medical & Biological Laboratories, Nagoya, Japan) with modifications to improve sensitivity. The LECT2 standard and serum samples (1:10 dilution) were diluted with sample diluent (provided with the kit) in the presence of 300 mM NaCl, which is an optimal concentration for sensitivity (Table S1, *http://www.biosciencetrends.com/docindex.php?year=2013&kanno=6*). These samples were incubated for 60 min at room temperature before adding to an ELISA plate. These conditions consistently generated LECT2 values that were 2-fold higher than those of the standard protocol (Table S2, *http://www.biosciencetrends.com/docindex.php?year=2013&kanno=6*).

2.4. *Statistical analyses*

The results were presented as mean ± standard deviation (S.D.). Data were analyzed using the IBM SPSS Statistics version 20 (IBM Corp., Armonk, NY, USA) and R version 2.8.1 (R Foundation for Statistical Computing, *http://www.r-project.org*). The continuous variables were analyzed using the Shapiro-Wilk test. A two-tailed unpaired Student's *t*-test was used to evaluate the differences in serum LECT2 levels between males and females and between obese and lean subjects. Comparisons of clinical parameters between males and females were performed using the two-tailed unpaired Student's *t*-test or the nonparametric Mann-Whitney *U* test, as appropriate. The relationship between serum LECT2 levels and anthropometric measures was evaluated using the two-tailed Pearson's correlation coefficient, whereas the interrelationships between the serum LECT2 levels and metabolic parameters, including age, were analyzed using the Spearman's

rank correlation coefficient. A partial correlation was used to evaluate these relationships, independent of the other variables. With regard to stepwise multiple linear regression analysis, because the levels of TG, high-density lipoprotein (HDL) cholesterol, C-reactive protein (CRP), aspartate transaminase (AST), alanine transaminase (ALT), and γ-glutamyl transpeptidase (γ-GTP) were not normally distributed, logarithmic transformations were used to approach normal distribution and to obtain equal variances. p values < 0.05 were considered statistically significant.

3. Results

3.1. *Clinical characteristics of the subjects*

A total of 231 subjects were included in this study, which included approximately equal numbers of males (n = 113) and females (n = 118), who were in their mid 50s (Table 1). Analysis of the anthropomorphic parameters revealed significantly lower BMI, WC, and WHR in females ($p < 0.001$). The mean systolic and diastolic blood pressures were within the normal range but significantly higher in males. With regard to systemic disease, there was no significant gender difference in the CRP levels. However, there were gender differences with regard to white blood cell counts. The other parameters were selected to characterize the subject population with regard to diabetes/obesity, dyslipidemia/fatty liver, and CKD. None of the subjects were diagnosed with diabetes mellitus. However, half of the subjects were classified as obese on the basis of W/Ht ≥ 0.5 (*17*), with a higher occurrence rate in males. With regard to liver diseases, the levels of AST, ALT, and γ-GTP were slightly higher in males. These findings are consistent with the fact that approximately 36% subjects were diagnosed with fatty liver, with a 2-fold higher incidence observed in males than females. Overall, these findings are consistent with

Table 1. Clinical characteristics of study subjects

Characteristics	Total (n = 231)	Males (n = 113)	Females (n = 118)	Gender tests p values
Serum LECT2 (ng/mL)	43.5 ± 13.6	43.6 ± 12.9	43.4 ± 14.3	NS
Age (years)	55.1 ± 8.7	55.0 ± 8.9	55.1 ± 8.4	NS
Anthropometric parameters				
Body mass index (BMI) (kg/m^2)	22.2 ± 3.1	23.4 ± 2.7	21.1 ± 3.0	< 0.001
Waist circumference (cm)	81.3 ± 9.0	84.7 ± 7.9	78.1 ± 8.9	< 0.001
Waist-to-hip ratio (WHR)	0.89 ± 0.06	0.91 ± 0.05	0.88 ± 0.07	< 0.001
Waist-to-height ratio (W/Ht)	0.50 ± 0.05	0.50 ± 0.04	0.50 ± 0.06	NS
Hypertension				
Systolic blood pressure (mm Hg)	122.1 ± 15.9	124.8 ± 16.1	119.5 ± 15.4	0.011
Diastolic blood pressure (mm Hg)	76.3 ± 11.3	80.1 ± 10.3	72.7 ± 11.0	< 0.001
Inflammation				
CRP (mg/dL)	0.114 ± 0.425	0.163 ± 0.585	0.067 ± 0.152	NS
White blood cells (10^3/μL)	5.32 ± 1.40	5.77 ± 1.31	4.90 ± 1.36	< 0.001
Diabetes mellitus				
Fasting plasma glucose (mg/dL)	92.2 ± 9.4	93.8 ± 9.1	90.7 ± 9.5	0.012
HbA1c (%)	5.6 ± 0.3	5.6 ± 0.3	5.6 ± 0.3	NS
Insulin (mU/L)	4.2 ± 2.6	4.4 ± 2.5	3.9 ± 2.6	NS
HOMA-R	0.96 ± 0.65	1.03 ± 0.62	0.89 ± 0.68	NS
Dyslipidemia				
Total cholesterol (mg/dL)	218.0 ± 36.1	211.1 ± 32.5	224.5 ± 38.3	0.005
TG (mg/dL)	102.2 ± 55.1	118.3 ± 58.5	86.8 ± 46.8	< 0.001
HDL cholesterol (mg/dL)	67.1 ± 18.0	60.2 ± 15.5	73.7 ± 17.8	< 0.001
LDL cholesterol (mg/dL)	126.9 ± 31.7	127.0 ± 28.4	126.9 ± 34.7	NS
Liver disease				
AST (IU/L)	22.9 ± 6.1	23.9 ± 6.6	21.9 ± 5.3	0.013
ALT (IU/L)	21.5 ± 8.8	23.5 ± 9.0	19.6 ± 8.2	< 0.001
γ-GTP (IU/L)	35.2 ± 28.1	44.6 ± 32.6	26.1 ± 19.1	< 0.001
Kidney disease				
UA (mg/dL)	5.4 ± 1.3	6.2 ± 1.1	4.6 ± 1.1	< 0.001
BUN (mg/mL)	13.7 ± 3.4	14.1 ± 3.1	13.3 ± 3.6	NS
SCr (mg/dL)	0.74 ± 0.16	0.85 ± 0.13	0.64 ± 0.11	< 0.001
eGFR (mL/min/1.73 m^2)	76.2 ± 14.3	76.2 ± 14.4	76.2 ± 14.3	NS
Diagnosis				
Obesity (W/Ht ≥ 0.5)	113 (48.9)	63 (55.8)	50 (42.4)	0.042
Fatty liver (by ultrasonic)	83 (35.9)	56 (49.6)	27 (22.9)	< 0.001
Chronic kidney disease (eGFR < 60)	21 (9.1)	8 (7.1)	13 (11.0)	NS
Alcohol consumption (male ≥40 g/day, female ≥20 g/day, ≥10 yr)	105 (45.5)	67 (59.3)	38 (32.2)	< 0.001

Data are presented as mean ± S.D. or proportion (%). LECT2, leukocyte cell-derived chemotaxin 2; CRP, C-reactive protein; HbA1c, hemoglobin A_{1c}; HOMA-R, homeostasis model assessment ratio; TG, triglyceride; HDL, high-density lipoprotein; LDL, low-density lipoprotein; AST, aspartate transaminase; ALT, alanine transaminase; γ-GTP, γ-glutamyl transpeptidase; UA, uric acid; BUN, blood urea nitrogen; SCr, serum creatinine; eGFR, estimated glomerular filtration rate. NS, nonsignificant for p values > 0.05.

the results of the general Japanese population (Report of National Health and Nutrition Survey in 2011; Ministry of Health, Labour and Welfare of Japan). Therefore, we separately analyzed the relationship between serum LECT2 and anthropometric and metabolic parameters in males and females.

3.2. *Impact of obesity on serum LECT2 levels*

Serum LECT2 levels were comparable in males and females (43.6 ± 12.9 *vs*. 43.4 ± 14.3 ng/mL) with an overall average of 43.5 ± 13.6 ng/mL. The average LECT2 levels were 47.4 ± 13.1 ng/mL and 39.7 ± 13.1 ng/mL for the obese and lean subjects, respectively ($p < 0.001$). When the data were further segregated on the basis of gender, we observed similar effects in males (46.8 ± 11.9 *vs.* 39.5 ± 13.1 ng/mL, $p = 0.002$) and females (48.2 ± 14.6 *vs.* 39.8 ± 13.1 ng/mL, $p = 0.001$), respectively. These data suggest that serum LECT2 levels presented no gender difference and were higher in obese subjects. Therefore, we conducted regression analyses between serum LECT2 and the anthropometric parameters: BMI, WC, WHR, and W/Ht. All the four parameters positively correlated with serum LECT2 levels (Figure 1). These data indicate that serum LECT2 levels increase in an obesity-dependent manner. The strongest correlation was observed between LECT2 and W/Ht adjusted for age and gender ($r = 0.349$, $p < 0.001$). As a result, W/Ht was subsequently employed as the sole anthropometric measure to simplify further analyses on obesity.

3.3. *Impact of liver and kidney diseases on serum LECT2*

Because several subjects were diagnosed with secondary complications of obesity, the Spearman's rank correlation analyses were conducted to determine whether LECT2 associations were selective for clinical parameters indicative of hypertension, inflammation, diabetes mellitus, dyslipidemia, liver diseases, or kidney disease (Table 2). Among the markers of obesity, high LECT2 levels simply correlated with CRP and AST levels only in males, and with HbA1c, LDL cholesterol, BUN, and SCr levels only in females. In addition, a positive correlation was observed between LECT2 and the markers of diabetes mellitus, dyslipidemia, liver disease, and kidney disease. These included, insulin, HOMA-R, TG, HDL cholesterol, ALT, γ-GTP, and uric acid (UA). These correlations suggest that high serum LECT2 levels are associated with the development of liver and kidney diseases.

3.4. *Multiple linear regression analysis of serum LECT2 levels*

All the parameters having significant simple correlation with LECT2 for at least one gender (Table 2) were

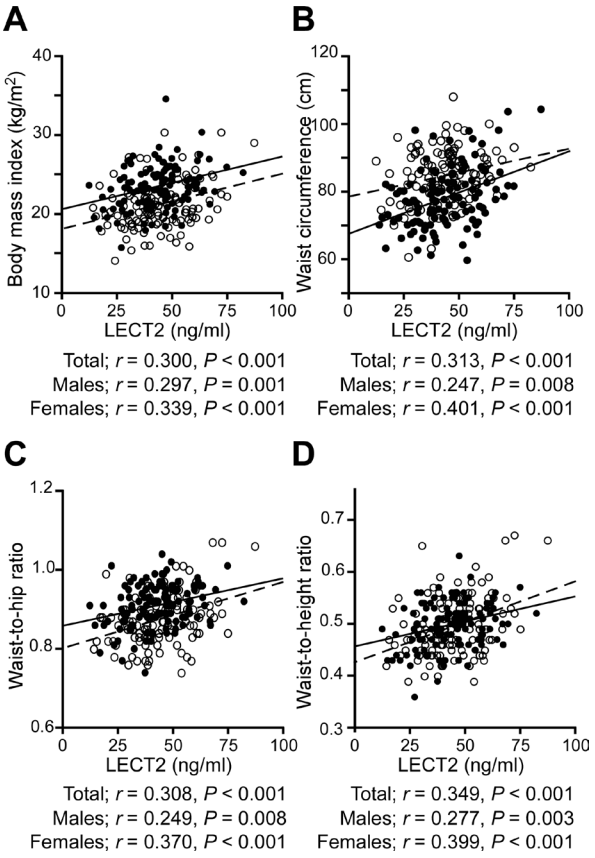

Figure 1. Relationship between serum LECT2 and anthropometric measurements. Distribution of serum LECT2 levels of males and females aged 40-69 years with regard to **(A)** body mass index (BMI), **(B)** waist circumference (WC), **(C)** waist-to-hip ratio (WHR), and **(D)** waist-to-height ratio (W/Ht). Males, filled circles and solid line; females, open circles and dashed line.

assessed for an independent relationship with LECT2 (CRP, HbA1c, insulin, HOMA-R, TG, HDL cholesterol, LDL cholesterol, AST, ALT, γ-GTP, UA, BUN, and SCr) as well as age and W/Ht. These multiple linear regression analyses were performed with serum LECT2 as a dependent variable for each gender (Table 3). In males, γ-GTP, TG, and age remained significant determinant of serum LECT2. On the other hand, in females, HOMA-R, BUN, HDL cholesterol, and γ-GTP were observed to significantly contribute to the determinant of serum LECT2 levels. These multiple regression analyses indicate that serum LECT2 is associated not only with obesity but also with liver diseases in both genders and with kidney dysfunction in females. In addition, these findings suggested that γ-GTP levels were strongly associated with serum LECT2 levels. We considered that this observation may arise if the serum LECT2 levels were also correlated with alcohol consumption. Therefore, we analyzed whether serum LECT2 and γ-GTP levels were correlated with alcohol consumption. Spearman's rank correlation analysis showed that serum LECT2 levels were not statistically correlated with alcohol consumption ($\rho = 0.065$, $p = 0.327$ in males, $\rho =$

Table 2. Spearman's rank correlation coefficient between serum LECT2 and the markers of obesity-related complications

	Total (n = 231)				Males (n = 113)				Females (n = 118)			
	Simple correlation		Age-, gender-, and W/Ht-adjusted		Simple correlation		Age- and W/Ht-adjusted		Simple correlation		Age- and W/Ht-adjusted	
	ρ	p value	Partial ρ	p value	ρ	p value	Partial ρ	p value	ρ	p value	Partial ρ	p value
Hypertension												
Systolic blood pressure	0.023	NS	− 0.032	NS	− 0.026	NS	− 0.072	NS	0.060	NS	− 0.005	NS
Diastolic blood pressure	0.057	NS	0.004	NS	0.041	NS	− 0.044	NS	0.070	NS	0.032	NS
Inflammation												
CRP	0.212	0.001	0.133	0.046	0.262	0.005	0.197	0.040	0.128	NS	0.054	NS
White blood cell	0.089	NS	0.050	NS	0.029	NS	0.002	NS	0.138	NS	0.102	NS
Diabetes mellitus												
Fasting plasma glucose	0.082	NS	0.038	NS	0.009	NS	− 0.014	NS	0.149	NS	0.090	NS
HbA1c	0.123	NS	0.079	NS	− 0.002	NS	− 0.039	NS	0.213	0.021	0.147	NS
Insulin	0.330	< 0.001	0.207	0.001	0.313	< 0.001	0.231	0.016	0.338	< 0.001	0.220	0.019
HOMA-R	0.337	< 0.001	0.217	0.001	0.308	< 0.001	0.229	0.017	0.352	< 0.001	0.239	0.011
Dyslipidemia												
Total cholesterol	0.063	NS	0.051	NS	0.066	NS	0.065	NS	0.071	NS	0.013	NS
TG	0.277	< 0.001	0.248	< 0.001	0.307	< 0.001	0.261	0.006	0.250	0.006	0.205	0.028
HDL cholesterol	− 0.274	< 0.001	− 0.204	0.002	− 0.205	0.030	− 0.141	NS	− 0.319	< 0.001	− 0.210	0.025
LDL cholesterol	0.190	0.004	0.136	0.042	0.128	NS	0.105	NS	0.236	0.010	0.137	NS
Liver disease												
AST	0.194	0.003	0.226	< 0.001	0.207	0.028	0.234	0.014	0.178	NS	0.231	0.015
ALT	0.261	< 0.001	0.215	0.001	0.220	0.019	0.174	NS	0.297	0.001	0.253	0.008
γ-GTP	0.270	< 0.001	0.270	< 0.001	0.340	< 0.001	0.300	0.002	0.261	0.004	0.274	0.004
Kidney disease												
UA	0.240	< 0.001	0.232	< 0.001	0.278	0.003	0.211	0.028	0.312	< 0.001	0.258	0.006
BUN	0.215	0.001	0.209	0.002	0.110	NS	0.137	NS	0.307	< 0.001	0.283	0.002
SCr	0.167	0.011	0.201	0.002	0.158	NS	0.180	NS	0.266	0.004	0.242	0.009

NS, nonsignificant for p values > 0.05.

Table 3. Multiple linear regression analyses with serum LECT2 as a dependent variable in Japanese subjects

Variables	B	(95% confidence interval)	Standardized β	t	p value	Partial correlation
Males (n = 113, R^2 = 0.214, adjusted R^2 = 0.192, p < 0.001)						
γ-GTP*	15.067	(6.188, 23.946)	0.295	3.363	0.001	0.307
TG*	16.346	(4.904, 27.788)	0.248	2.831	0.006	0.262
Age	− 0.258	(− 0.502, − 0.015)	− 0.178	− 2.100	0.038	− 0.197
Females (n = 118, R^2 = 0.355, adjusted R^2 = 0.332, p < 0.001)						
HOMA-R	5.217	(1.834, 8.601)	0.248	3.055	0.004	0.276
BUN	1.238	(0.629, 1.847)	0.308	4.030	< 0.001	0.354
HDL cholesterol*	− 41.955	(− 63.515, − 20.394)	− 0.306	− 3.855	< 0.001	− 0.341
γ-GTP*	14.299	(4.698, 23.900)	0.232	2.951	0.004	0.267

*, log scale.

Table 4. Receiver operating characteristic curve analysis for serum LECT2

Disease	Gender	cutoff values (ng/mL)	Area under the curve (95% confidence interval)	Significance	Sensitivity (%)	Specificity (%)
Obesity	Males	41.8	0.655 (0.551–0.758)	0.002	71.4	62.0
	Females	45.0	0.670 (0.570–0.770)	< 0.001	64.0	72.1
Fatty liver	Males	43.3	0.646 (0.544–0.749)	0.004	67.9	59.7
	Females	46.4	0.733 (0.621–0.844)	< 0.001	70.4	69.2

−0.064, $p = 0.488$ in females), whereas γ-GTP level was positively correlated with alcohol consumption in both genders ($\rho = 0.363$, $p < 0.001$ in males, $\rho = 0.193$, $p = 0.037$ in females). These results suggest that an increase in serum LECT2 level does not directly reflect liver damage due to alcohol abuse.

When the 231 subjects were divided on the basis of the diagnosis of fatty liver, the average LECT2 levels were 48.7 ± 13.6 ng/mL and 40.5 ± 12.8 ng/mL in subjects with and without fatty liver ($p < 0.001$), respectively. When categorized on the basis of gender, the average LECT2 levels were 46.8 ± 12.8 ng/mL and 40.4 ± 12.3 ng/mL ($p = 0.007$) in males with and without fatty liver, respectively, and 52.6 ± 14.7 ng/mL and 40.6 ± 13.1 ng/mL ($p < 0.001$) in females with and without fatty liver, respectively. However, no statistically significant difference was observed when the subjects were divided into a CKD group (48.1 ± 14.4 ng/mL) and a non-CKD group (43.0 ± 13.5 ng/mL) according to eGFR, even separated by gender (45.1 ± 12.9 ng/mL and 43.5 ± 13.0 ng/mL in males, 50.0 ± 15.5 ng/mL and 42.6 ± 14.1 ng/mL in females). Taken together, these results suggest that high serum LECT2 levels may indicate higher risks of developing obesity-related liver diseases.

3.5. *Receiving operating characteristic curve analyses*

Considering the relationships established between LECT2 levels and the markers of obesity and fatty liver, receiver operating characteristic (ROC) curve analysis was performed to check whether serum LECT2 levels could discriminate between lean and obese subjects, and subjects without and with fatty liver (Table 4). With regard to obesity, the area under curve (AUC) values for LECT2 levels were significant both in males (0.655, $p = 0.002$) and females (0.670, $p < 0.001$). The optimal cutoff values were 41.8 ng/mL (sensitivity, 71.4%; specificity, 62.0%) and 45.0 ng/mL (sensitivity, 64.0%; specificity, 72.1%) for males and females, respectively. Moreover, as presented in Table 4, ROC curve analysis revealed that AUCs of LECT2 levels for fatty liver were 0.646 ($p = 0.004$) and 0.733 ($p < 0.001$) for males and females, respectively. From these data, the optimal cutoff values of 43.3 ng/mL (sensitivity, 67.9%; specificity, 59.7%) and 46.4 ng/mL (sensitivity, 70.4%; specificity, 69.2%) were determined for males and females, respectively. Overall, these results indicated that serum LECT2 levels have predictive values with regard to the occurrence of obesity and fatty liver.

4. Discussion

The worldwide obesity epidemic is raising concerns because of the serious complications developing in these patients, including cardiovascular disease, diabetes mellitus, and liver diseases. In fact, the development of better preventive measures based on education and early detection and diagnosis have become a priority in the medical community. Unfortunately, the best predictors of obesity are anthropometric measures, which do not take the obesity-related complications into account. The present study demonstrates, for the first time, that serum LECT2 levels positively correlate with the markers of obesity and fatty liver.

The sensitivity of the ELISA assay currently available for serum LECT2 is too low to distinguish between different patient populations. Studies using this ELISA protocol reported the presence of LECT2 levels in human serum or plasma in the range of 1-15 ng/mL (*18-20*). Recently, Ando *et al*. reported LECT2 levels of 19.7 ± 3.4 ng/mL in heparinized plasma from healthy volunteers with the conventional ELISA protocol (*21*). In addition, we preliminarily determined that the serum LECT2 levels measured by conventional ELISA were lower than the estimated levels obtained by immunoblotting using recombinant LECT2 protein as a standard (data not revealed). These studies suggested that electrostatic interactions with other serum proteins may interfere with the LECT2 assay. One such candidate protein is transferrin, whose interaction with LECT2 has been reported in fish and mice (*22*). Another candidate protein is LECT2 itself, which can oligomerize *in vitro* under certain conditions as previously reported (*23*). Therefore, we resolved the electrostatic interference by adding 300 mM NaCl to the sample diluent, which increased the sensitivity of the assay by more than 2-fold. Using this modified protocol, we report overall LECT2 levels of 43.5 ± 13.6 ng/mL in human serum in a subject group representative of the general Japanese population. When the 231 subjects included in the present study were segregated on the basis of a diagnosis of obesity or fatty liver, the average LECT2 levels were 47.4 ± 13.1 ng/mL *vs.* 39.7 ± 13.1 ng/mL in the obese and lean subjects, respectively, and 48.7 ± 13.6 ng/mL *vs.* 40.5

± 12.8 ng/mL in subjects with and without fatty liver, respectively. The fact that differences between these values were statistically significant testifies to the high sensitivity of this modified ELISA assay. These results indicate a positive correlation between serum LECT2 levels and obesity and fatty liver.

Simple regression analysis established a positive correlation between serum LECT2 and all the four major anthropometric measures of obesity: BMI, WC, WHR, and W/Ht. In addition, the ROC analysis revealed the predictive accuracy of obesity detection in both genders. The hepatic Wnt/β-catenin signaling pathway plays an important role in the metabolism of hepatic glucose, glycogen, and lipids (*3*). Together with the Wnt/β-catenin signaling pathway that increases LECT2 expression (*5*), these studies and the present data are comparable with regard to the notion that LECT2 participates in the events leading to obesity.

The LECT2 levels of the subjects with fatty liver were significantly higher compared with those in the subjects without fatty liver. Moreover, the ROC curve analysis showed that serum LECT2 levels significantly discriminated between subjects with and without fatty liver in both genders. In addition, these data are consistent with the studies reporting an increase in hepatic CYP2E1 in obesity (*24*) and fatty liver (*25*), which is a direct hepatic β-catenin target gene as well as *LECT2* gene (*5*). In addition, insulin resistance is promoted and plays a key role during the progression of a nonalcoholic fatty liver disease. HOMA-R, an indicator of insulin resistance, was correlated with serum LECT2 levels. Therefore, further studies are proposed to investigate the association between serum LECT2 levels and steatohepatitis, cirrhosis, and hepatocarcinoma.

Furthermore, LECT2 levels were associated with the kidney disease-related factors UA, BUN, and SCr. However, in this study, LECT2 could not be determined as a predictor of renal function. These findings are supportive evidence that LECT2 is specific for obesity and obesity-related liver disease.

In conclusion, this study demonstrates that the more sensitive ELISA protocol, which we developed for human serum LECT2, will now allow discriminative studies among different patient populations. Using this assay, we demonstrated that serum LECT2 levels vary between subjects diagnosed with obesity and fatty liver. This clinical study may lead to the development of a new population screening strategy for the worldwide obesity epidemic and its major secondary complications.

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Original Article

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High prevalence of HIV-associated neurocognitive disorder in HIV-infected patients with a baseline CD4 count ≤ 350 cells/μL in Shanghai, China

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Summary **This study sought to determine the prevalence and risk factors of HIV-associated neurocognitive disorder (HAND) in HIV-infected patients with a baseline CD4 count ≤ 350 cells/μL in Shanghai, China. Subjects were 309 HIV-infected patients with a baseline CD4 count ≤ 350 cells/μL. General demographic and clinical information were collected by patient interview. Patients' cognitive function was assessed using the Montreal Cognitive Assessment (MoCA), combined with a questionnaire on cognitive complaints. The median age of patients was 34 years (IQR: 28-43.5). In terms of sex, 272 (88.0%) of the patients were male. Of the patients, 236 (76.4%) had been on antiretroviral treatment (ART) (for a median duration of 14 months, IQR: 1-29 months) before the study. Of the patients, 183 (59.2%) mentioned having a cognitive disorder. MoCA screening revealed that the prevalence of HAND was 48.2% and that HAND was more prevalent in patients with cognitive complaints (53.0%) than in patients with no such complaints (41.3%) ($p = 0.042$). Multivariate analysis indicated that HAND was associated with being female ($p = 0.006$), being older ($p < 0.001$), having a lower level of education ($p < 0.001$), and longer use of efavirenz in an ART regimen ($p = 0.040$). This study found that HAND frequently developed in HIV-infected patients with a baseline CD4 count ≤ 350 cells/μL in Shanghai, China. Being older, being female, having a low level of education, and receiving efavirenz treatment for a longer period may be associated with a greater risk of developing HAND. This study suggests that HAND should be routinely screened for in all newly diagnosed HIV-positive patients, and especially in those with the aforementioned risk factors for developing HAND.**

Keywords: Prevalence, HIV-associated neurocognitive disorder, HIV

1. Introduction

Human immunodeficiency virus (HIV) can cause dysfunction and damage in the central nervous system (CNS), leading to a wide range of neurocognitive complications, known as HIV-associated neurocognitive disorder (HAND). Patients with HAND typically exhibit abnormalities in cognition, motor function, and behavior. Cognitive impairment predominantly consists of mental slowing, memory loss, and attention deficit. Motor symptoms include slowness and loss of balance. Behavioral changes are characterized by apathy, social withdrawal, and mood disturbances. Three forms of HAND are, in order of increasing severity, HIV-associated asymptomatic neurocognitive impairment (ANI), HIV-associated mild neurocognitive disorder (MND), and HIV-associated dementia (HIV-D) (*1*).

Highly active antiretroviral therapy (HAART) has significantly decreased the incidence of HAND (*2*). However, HIV-positive patients are living much longer than before the advent of HAART, so the prevalence

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of HAND has increased, and this is especially true for minor cognitive disorders that have a reported prevalence of 20-50% (*2,3-5*).

HAND is associated with a decrease in the quality of life, diminished compliance with HAART, and increased mortality. Therefore, an important step is to try and identify HAND, and especially in individuals with a minor cognitive disorder. Generally, HAND is assessed using a combination of neuropsychological tests, neuroimaging, and tests on the cerebrospinal fluid (CSF) that are usually expensive and time-consuming. Less expensive and less time consuming standardized symptom questionnaires (regularly administered) also play a role in clinical screening. The HIV Dementia Scale (HDS) and International HDS (IHDS) have been developed to identify HIV-D, but their usefulness at detecting milder HAND is still being studied (*6,7*). The Montreal Cognitive Assessment (MoCA) has been validated in instances of mild cognitive impairment (MCI) and is now widely used to measure cognitive functions in a variety of diseases (*8-12*). Studies have shown that the Chinese version of the MoCA has a high sensitivity and specificity at screening for MCI among the Chinese population (*13,14*).

The prevalence of and risk factors for HAND among Chinese patients have yet to be documented. This study attempted to use the MoCA to determine the frequency of HAND in a Chinese outpatient population. Risk factors associated with HAND were also assessed, potentially providing useful clinical indicators.

2. Materials and Methods

2.1. *Selection of study subjects*

A cross-sectional survey of HIV-1 positive patients treated in the outpatient facility of the Shanghai Public Health Clinical Center (Shanghai, China) was conducted from May 2012 to February 2013. Potential subjects were 309 patients who had their cognitive function tested. Inclusion criterion were a recent diagnosis with HIV-1 infection or already being on HAART, age ≥ 18 years, and having a baseline $CD4^+$ T cell count ≤ 350 cells/μL. Exclusion criteria were: age < 18 years, suffering a major opportunistic infection of the brain in the past 3 years, psychoactive drug use, alcohol addiction, physical disability (*e.g.* amputation), major depression, having a severe systemic disease that might affect testing, and being pregnant. Of the potential subjects, 118 declined to participate in the study either because they thought the tests were troublesome and time consuming or because they did not think that they had neurocognitive problems.

2.2. *Data collection*

General demographic and clinical information, including sex, age, educational level, medical history, and previous HAART (when HAART was started and the details of the HAART regimen), were collected by patient interview.

Cognitive function assessment: The tests below were used to assess the cognitive function of all of the patients studied.

Cognitive complaints questionnaire: A short questionnaire asked four questions to screen for cognitive complaints: "Do you experience frequent memory loss (*e.g.* do you forget the occurrence of certain events, like appointments, and especially more recent ones)," "Do you feel that you are slower when reasoning, planning activities, or solving problems," "Have you lost interest in previous activities and hobbies," and "Do you have difficulty managing your daily affairs." For each question, answers were "never," "hardly ever," or "yes, definitely." Patients answering "yes, definitely" to at least one question were deemed to have cognitive complaints.

The Chinese version of the MoCA: The MoCA (*http://www.mocatest.org*) assesses several cognitive domains. The short-term memory recall task (5 points) involves two learning trials of five nouns and delayed recall after approximately 5 minutes. Visualspatial abilities are assessed using a clock-drawing task (3 points) and a three-dimensional cube copy (1 point). Multiple aspects of executive functions are assessed using an alternation task adapted from the trail-making B task (1 point), a phonemic fluency task (1 point), and a two-item verbal abstraction task (2 points). Attention, concentration, and working memory are evaluated using a sustained attention task (target detection using tapping; 1 point), a serial subtraction task (3 points), and digits forward and backward (1 point each). Language is assessed using a three-item confrontation naming task with low-familiarity animals (lion, camel, rhinoceros; 3 points), repetition of two syntactically complex sentences (2 points), and the aforementioned fluency task. Finally, orientation to time and place is evaluated (6 points). The MoCA has a total score of 30 points, and < 26 points is the cutoff point for cognitive impairment. An adjustment was made for educational level by adding one point to the final score if the patient was educated for less than 12 years.

2.3. *Statistical methods*

Data were analyzed using Stata v. 10.0. Continuous data with a normal distribution were expressed as means ± standard deviation (mean ± S.D.) and compared using *t*-tests; continuous data with a skewed distribution were expressed as medians (inter-quartile range, IQR) and compared using the Wilcoxon rank sum test. Categorical data were expressed in frequencies and percentages and compared using chi-square (χ^2) tests, with a $p < 0.05$ considered to be statistically significant.

3. Results

3.1. *General information of the patients studied*

Subjects for this study were 309 patients who had their neurocognitive function tested. Of these, 272 (88.0%) were male and 37 (12.0%) were female. The median age was 34 years (IQR: 28-43.5), and 236 (76.4%) patients had undergone antiretroviral treatment (ART) for a median duration of 14 months (IQR: 1-29). The ART regimens were: zidovudine or stavudine or tenofovir + lamivudine + efavirenz or nevirapine or lopinavir/r. There were no statistically significant differences in median age, age distribution, number of patients on ART, and ART duration between male and female patients ($p = 0.87$, $p = 0.28$, $p = 0.92$, $p = 0.05$). However, male patients had a significantly higher educational level compared to female patients ($p < 0.01$) (Table 1).

3.2. *Prevalence of cognitive complaints in the patients studied*

Based on the devised questionnaire, the prevalence of cognitive complaints among HIV-1 positive patients was 59.2% (183/309): 38.8% (120/309) had memory difficulties, 34.3% (106/309) had mental slowing, 24.6% (76/309) had difficulty managing daily affairs, and 10.4% (32/309) had lost interest in previous activities and hobbies. In terms of the number of complaints, 89 patients had only one complaint, 54 patients had 2 complaints, 23 had three complaints, and 17 had four complaints.

3.3. *Prevalence of HAND in the patients studied and comparison of HIV-1 positive patients with and without HAND*

Overall, 160 patients (51.8%) had normal MoCA scores (≥ 26) and 149 patients (48.2%) had abnormal MoCA scores (< 26). The prevalence of HAND was 48.2% (149/309) in HIV-1 positive patients overall, 53.0% (97/183) in patients with a cognitive complaint, and 41.3% (52/126) in patients without a cognitive complaint. HAND was more prevalent in patients with cognitive complaints than in patients with no such complaints ($\chi^2 = 4.116$, $p = 0.042$). Compared to patients without HAND ($n = 160$), patients with HAND ($n = 149$) were more likely to be women ($p = 0.004$), of older age ($p < 0.001$), less educated ($p < 0.001$), had undergone ART for a longer period ($p = 0.006$), and had been treated with efavirenz (EFV) for longer ($p = 0.019$). No significant differences were observed in MoCA scores based on ART initiation ($p = 0.960$) and central nervous system (CNS) penetration-effectiveness (CPE) of HAART ($p = 0.830$) (*15*). Cognitive complaints were more prevalent in patients with HAND, but the difference was not statistically significant ($p = 0.117$). However, a larger proportion of patients with HAND had ≥ 3 complaints compared to patients without HAND ($p = 0.003$) (Table 2).

3.4. *Multivariate analysis of the MoCA score*

The results of multivariate analysis are shown in Table 3. These results indicate that HAND was associated with sex ($\beta = -1.052$, $p = 0.006$, OR = 0.35), age ($\beta = -0.109$, $p < 0.001$) , level of education < 12 years ($\beta = -1.415$, $p < 0.001$, OR = 4.12), and duration of ART ($\beta = -0.020$, $p = 0.040$). Since the side effects of EFV on CNS may influence the MoCA score, the correlation between duration of treatment with EFV and HAND was analyzed, and no association between the total duration of treatment with EFV and HAND was noted ($\beta = -0.026$, $p = 0.060$). However, patients treated with EFV ≥ 24 months had a greater risk of developing HAND ($\beta = -0.745$, $p = 0.020$, OR = 2.11). Being female and having a lower level of education were also possible risk factors for developing HAND. An older age and longer duration of ART were positively correlated with development of HAND.

Table 1. Clinical and demographic information on the patients studied

Items	Male ($n = 272$)	Female ($n = 37$)	Total ($n = 309$)	Statistic	p-value
Age (years)*	34 (28.3-43)	33 (28-48.5)	34 (28-43.5)	$Z = 0.163$[a]	0.87
< 30	78 (28.7%)	13 (35.1%)	91(29.5%)	$\chi^2 = 5.06$[b]	0.28
30-40	104 (38.2%)	10 (27.0%)	114 (36.9%)		
40-50	54 (19.9%)	5 (13.5%)	59 (19.1%)		
50-60	29 (10.6%)	7 (18.9%)	36 (11.7%)		
> 60	7 (2.6%)	2 (5.4%)	9 (2.9%)		
Educational level (years)					
≤ 12	86 (31.6%)	24 (64.9%)	110 (35.6%)	$\chi^2 = 15.703$[c]	< 0.01
On ART	208 (76.5%)	28 (75.7%)	236 (76.4%)	$\chi^2 = 0.011$[c]	0.92
ART duration (months)*	12 (1-27.8)	24 (4-37.5)	14 (1-29)	$Z = -1.991$[a]	0.05

*, Data are presented as the median (IQR); ART, antiretroviral therapy; [a], Wilcoxon rank sum test; [b], CMH chi-square test; [c], chi-square test.

Table 2. Comparison of patients with HAND and patients without HAND

Items	Without HAND (n = 160)	With HAND (n = 149)	Statistic	p-value
Sex				
Male	149 (54.8%)	123 (45.2%)	$\chi^2 = 8.185^a$	0.004
Female	11 (29.73%)	26 (70.27%)		
Age (years)*	31.5 (27-36)	40 (31-49)	$Z = -5.870^b$	< 0.001
< 30	61 (67.0%)	30 (33.0%)	$\chi^2 = 39.95^c$	< 0.001
30-40	71 (62.3%)	43 (37.7%)		
40-50	18 (30.5%)	41 (69.5%)		
50-60	8 (22.2%)	28 (77.8%)		
> 60	2 (22.2%)	7 (77.8%)		
Educational level (years)				
≤ 12	33 (30.0%)	77 (70.0%)	$\chi^2 = 32.451^a$	< 0.001
> 12	127 (63.8%)	72 (36.2%)		
ART initiation				
Yes	122 (51.7%)	114 (48.3%)	$\chi^2 = 0.003^a$	0.960
No	38 (52.1%)	35 (47.9%)		
Duration of ART (months)*	10 (0.8-22.8)	17 (1-36.5)	$Z = -2.755^b$	0.006
ART regimen including EFV				
Yes	90 (56.3%)	80 (53.7%)	$\chi^2 = 0.204^a$	0.651
No	70 (43.7%)	69 (46.3%)		
Duration of treatment with EFV (months)(n = 170)				
≤24	62 (60.2%)	41(39.8%)	$\chi^2 = 5.518^a$	0.019
> 24	28 (41.8%)	39 (58.2%)		
CPE score of HAART*	9 (7-9)	9 (7-9)	$Z = 0.214^b$	0.830
Cognitive complaints (n = 183)	88 (55.0%)	95 (63.8%)	$\chi^2 = 2.451^a$	0.117
≤ 2	78 (48.8%)	67 (45.0%)	$\chi^2 = 9.106^a$	0.003
≥ 3	10 (6.3%)	28 (18.8%)		

*, Data are presented as the median (IQR); EFV, efavirenz; ART, antiretroviral therapy; [a], chi-square test; [b], Wilcoxon rank sum test; [c], CMH chi-square test; CPE, central nervous system penetration-effectiveness rank, the CPE score was calculated by adding up the scores of each antiretroviral drug in the regimen (tenofovir = 1, lamivudine and stavudine = 2, efavirenz and lopinavir/r = 3, and zidovudine and nevirapine = 4).

Table 3. Results of multivariate analysis of the MoCA score

Items	β	p-value	OR	95% CI
Sex (female)*	-1.052	0.006	0.35	0.17-0.74
Age#	-0.109	< 0.001	/	/
Educational level < 12 years*	-1.415	< 0.001	4.12	2.5-6.79
Duration of ART#	-0.020	0.040	/	/
Duration of treatment with EFV#	-0.026	0.060	/	/
Duration of treatment with EFV ≥ 24 months*	-0.745	0.020	2.11	1.13-3.94

*, Logistic regression analysis; #, Linear regression; EFV, efavirenz; ART, antiretroviral therapy.

4. Discussion

Results indicated that 59.2% of HIV-1 positive patients with a baseline CD4 count ≤ 350 cells/μL complained of neurocognitive symptoms, including memory difficulties, mental slowing, difficulty managing daily affairs, and losing interest in activities one "used to do." Screening *via* the MoCA score revealed that 48.2% of the patients overall presented with HAND, with a higher prevalence among patients with neurocognitive complaints (53.0%) than among patients with no such complaints (41.3%). This finding shows that there is a high prevalence of cognitive impairment even in HIV-1 patients without any neurocognitive complaints. Thus, the potential for patients with no cognitive complaints to develop HAND should be kept in mind. The median age of subjects in this study was only 34 years of age, suggesting that HAND may be the predominant cause of cognitive dysfunction/dementia among young people. The prevalence of HAND in this study is similar to that in previous studies (*2,4,5*). This prevalence is lower than that in the study by Simioni *et al*. (*16*), possibly because of the differences in the screening methods used and the study samples. In addition, 118 eligible

patients declined to participate in the cognitive testing in the current study, and this inclusion bias might have affected the results.

Results of the current study indicate that being older, being female, having a lower level of education, a longer duration of HAART, and use of EFV ≥ 24 months in HAART regimens may contribute to an increased risk of developing HAND. In other studies, HAND is also reported to be more prevalent in patients who were older and who had lower levels of education (*17,18*). However, MoCA was not used to assess neurocognitive disorders in non-HIV infected controls matched by age and educational level in the current study. Thus, whether such HIV-independent changes potentially affect MoCA scores is unclear and requires further study. In contrast to the current results, a study by Joska *et al*. showed that being male rather than female was predictive of development of HIV-associated dementia (*18*). The disparity in findings can perhaps be explained by the fact that the female patients in the current study were significantly less educated than male patients. Surprisingly, a longer duration of HAART was found to be associated with a higher prevalence of HAND. HAART does not apparently prevent the development of HAND nor successfully treat HAND. This contradicts a recent study that noted a decrease in HAND proportional to the duration of HAART (*19*). However, the current results should not be taken to mean that HAART is not an effective treatment for HAND since most of the patients studied (72%, 170/236) were already on HAART before the study began and they were administered ART including EFV, which has side effects on the CNS including dizziness, impaired concentration, somnolence, abnormal dreams, and insomnia. These symptoms may result in patients having a poorer cognitive performance. This hypothesis is supported by a recent study reporting that efavirenz is associated with cognitive disorders in otherwise asymptomatic HIV-infected patients (*20*). Other previously reported risk factors for HAND include a decreased CD4 count, anemia, declining platelet count, high HIV-RNA viral load in the CNS, and hepatitis C coinfection (*20-22*).

The pathogenesis of HAND is not yet clear. A tentative hypothesis is that HIV-1 itself can cause dysfunction and damage in the CNS. Shortly after the primary infection, HIV-1 enters the brain in mononuclear cells and settles in perivascular macrophages and microglial cells. Replication of HIV-1 in these cells leads to immune activation and the production of viral and inflammatory proteins, which may be what eventually leads to various cognitive disorders. Studies have shown that antiretroviral drugs with a higher CPE are associated with a lower HIV-RNA load in CSF and better cognitive performance (*15,19*). The current study noted no significant differences in CPE in patients with normal or abnormal MoCA scores. This may be because there are limited anti-HIV drugs in China and HAART regimens for most patients consisted of the same drugs. In addition, HAND is not routinely screened for prior to commencing ART in order to give drugs with higher CPE scores to patients with HAND.

Having been validated in instances of mild cognitive impairment, MoCA could perhaps be used to screen for HAND, in combination with a neurocognitive complaint questionnaire, as was done in this study. Further study is needed to assess the ability of MoCA versus other methods of cognitive assessment to assess HIV-associated cognitive impairment. Combining MoCA with other cognitive and functional assessments may allow more accurate diagnosis of HIV-related cognitive impairments.

In conclusion, the current results show that neurocognitive disorders are frequent in HIV-infected patients with a baseline CD4 count ≤ 350 cells/μL in Shanghai, China. Being older, being female, having a low level of education, and a longer period of EFV use in HAART regimens may be associated with a greater risk of developing HAND. These data suggest that HAND needs to be screened for regularly in all newly diagnosed HIV patients, and perhaps in all patients on a routine schedule (especially in those patients with risk factors for HAND), in order to diagnose HAND and possibly intervene as early as possible.

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Commentary

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From SARS to H7N9: The mechanism of responding to emerging communicable diseases has made great progress in China

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Summary **The outbreak of severe acute respiratory syndrome (SARS) in 2003 indicated that China's existing former mechanism for emergency management was very vulnerable. The Chinese Government has since established a new mechanism for responding to emerging communicable diseases. This paper examined the current status of and developments in China's response to emerging communicable diseases from the outbreak of SARS in 2003 to the outbreak of H7N9 virus infection in 2013. Results indicated that the current mechanism for emergency responses to emerging communicable diseases in China has made great achievements in terms of command and decision-making, organization and collaboration, monitoring and early warning systems, protection, and international communication and cooperation. This mechanism for responding to emerging communicable diseases allowed China to successfully deal with outbreaks of the H5N1 bird flu, H1N1 flu, and H7N9 bird flu. However, a better coordination system, a more complete Office of Responses to Public Health Emergencies, administrative responsibility and error correction, better personnel training, and government responsibility may help to improve the response to emerging communicable diseases. Such improvements are eagerly anticipated.**

Keywords: Emerging communicable diseases, department coordination system, Office of Responses to Public Health Emergencies, field epidemiology training program

1. Introduction

In the spring of 2013, humans were infected with the H7N9 virus, and the number of cases increased rapidly. From March 31 to May 31, 2013, there were 132 cases of humans infected with the H7N9 avian influenza virus in China, 39 of which resulted in death (*1,2*). Afterwards, 5 new cases of infection with the H7N9 virus were reported prior to October 31st, according to the National Health and Family Planning Commission (NHFPC) of China (*3*). Most recently, 2 new cases of H7N9 infection were confirmed in Hong Kong and 1 case was confirmed in Zhejiang. The Chinese Government and Ministry of Health tried their best to control infection with the H7N9 virus, and they are still doing so. However, global concerns about public health in China have been sparked and there is a doubt as to whether China's response to emerging communicable diseases has made improvements since the last outbreak of an emerging communicable disease, severe acute respiratory syndrome (SARS), in 2003.

After the outbreak of SARS in 2003, the Chinese Government found its existing mechanism for emergency management to be very vulnerable, and the Government started to consider creating a new mechanism for responding to emerging communicable diseases. In order to promptly and effectively deal with various emerging communicable diseases, a new mechanism for responding to public health emergencies with a unified command, ready reaction, and coherent, ordered, and effective operation had to be established. The establishment of a mechanism for emergency responses to emerging communicable diseases is a key aspect of the response to a public health emergency. China established such a mechanism for emergency

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responses to emerging communicable diseases and has made some progress since the outbreak of SARS in 2003. With the help of this new mechanism for emergency management, China successfully handled the outbreak of H5N1 and H1N1 avian influenza in 2005 and 2009. Moreover, evidence has shown that China's public health response to the H7N9 virus infection was faster and more effective in terms of transparency in reporting, surveillance, screening, and stockpiling of antimicrobials (*4,5*). This is a result of China's establishment of a mechanism for responding to emerging communicable diseases after the outbreak of SARS in 2003.

2. China's establishment of a new mechanism for responding to emerging communicable diseases

Advanced medicine literally means to practice After the SARS outbreak in 2003, China established a mechanism for emergency responses to emerging communicable diseases, and has made rapid advances. The government and society have focused on facilities for disease control and prevention that deal with emergency responses to emerging communicable diseases. China has essentially reached its goal of establishing a system of disease control and prevention. Monitoring, early warning, and emergency responses to emerging communicable diseases are key responsibilities and missions of facilities for disease control and prevention.

2.1. *Command and decision-making*

Since 2003, the NHFPC has sought to create an information network and platform for command in public health emergencies and shift decision-making with regard to emerging communicable diseases from central to local authorities. Therefore, a public health information network was envisioned to cover the entire country and connect every region of the country. In accordance with this vision, the Chinese Government has sought to establish an emergency command system that is unified, effective, ready to respond, and accurate. Although the existing system is not as perfect as that envisioned, obvious improvements have been made, and the quality and efficiency of emergency command and decision-making has improved. This new command and decision-making helped to prevent and control the outbreak of the H7N9 virus infection in 2013.

2.2. *Organization and collaboration*

Responding to emerging communicable diseases requires cooperation between multiple departments such as finance, agriculture, education, public security, urban management, and forestry. Therefore, good organization and collaboration is needed. Organization of and collaboration in responses to emerging communicable diseases includes organization and collaboration between central and local authorities, between localities, and between government departments and the Health Ministry. To accomplish these tasks, the Chinese Government established a department coordination system and an Office of Responses to Public Health Emergencies.

Department coordination system NHFPC established a system to coordinate between different departments. The Chinese Government established this system to coordinate among 31 relevant departments to respond to emerging communicable diseases. The department coordination system has effectively enhanced communication of information on emerging communicable diseases among different departments. The system also incorporates prevention of disease emergencies, drills and training, and personnel supervision and testing, representing a considerable achievement of the organization of and collaboration among different departments.

Office of Responses to Public Health Emergencies Organization and collaboration also includes cooperation between different health departments. To better integrate resources, NHFPC established an Office of Responses to Public Health Emergencies, which also serves as the Headquarters for Responses to Public Health Emergencies. Since then, a preliminary system for organization of and collaboration in emergency responses to emerging communicable diseases has been initially established in China. When public health emergencies occur or emerging communicable diseases appear, all types of public health facilities can be adequately coordinated by the Headquarters for Responses to Public Health Emergencies, and an investigation of the cause and determination of a treatment can be promptly performed.

In light of the organization of and collaboration in the response to H7N9, the department coordination system and Office of Responses to Public Health Emergencies helped to improve cooperation between different departments in the face of an emerging communicable disease.

2.3. *Monitoring and early warning system*

After the outbreak of SARS in 2003, the Chinese Government established a new mechanism of monitoring, forecasting, and providing early warnings of emerging communicable diseases. With the new monitoring and early warning system, NHFPC and provincial health departments can publish information warning of an infectious disease based on results of their analysis in order to facilitate a rapid response. China has made great progress in the establishment of an early warning system for emerging communicable diseases. In particular, *i*) a reporting system covering the entire country has been established, the timeliness

of reporting has been greatly improved; *ii*) information about results of analyses and forecasts of emerging communicable diseases is better shared among different departments; *iii*) advanced early warnings of infectious diseases and emerging communicable diseases have been discussed, and remote sensing and geographic information systems have been introduced in the field of public health.

Advances in the new monitoring and early warning system have helped to improve China's response to emerging diseases. As an example, the new H7N9 virus infection was detected and reported by the monitoring system just before the infection occurred in the spring of 2013.

2.4. *Protection*

In terms of protection from emerging communicable diseases, the Chinese Government has taken action in 3 areas.

Complete regulation Scientific responses and laws, regulations, and plans for emergency responses to emerging communicable diseases that are tailored to China have been initially established. These policies have helped to create a standardized response to emerging communicable diseases.

Field Epidemiology Training Program A public health system has gradually been established in China. Trained medical personnel, public health workers, and experts in responding to emerging communicable diseases, programs for types of emergency responses, and new techniques and methods for emergency responses can ensure that personnel have the technical proficiency to conduct emergency responses to emerging communicable diseases. In more specific terms, a field epidemiology training program (FETP) is now being emphasized in China. This is the advanced form of training proposed by the World Health Organization. This training enhances monitoring by an emergency response system by high-level training of personnel in field epidemiology to meet the challenge of a disease outbreak or other public health emergency. FETP in China started in 2001 (*6*), sponsored by NHFPC and the Chinese Center for Disease Control and Prevention (CDC). This is a preferred method of training teams to deal with emerging communicable diseases and can enhance the proficiency of emergency response personnel. China's FETP has recruited 244 students in thirteen terms over the past twelve years. Of these, 184 students in eleven terms have graduated and are now national or provincial level experts in emergency responses to emerging communicable diseases. There are now several provinces with their own FETP, including Zhejiang, Guangdong, Tianjin, and Shandong and Shanghai. Students who have graduated are now playing an important role in responding to emerging communicable diseases. For example, Zhejiang's FETP (*7*) recruited 244 students in fifteen terms from 99% of the province's counties. Of these students, 201 have graduated and are all members of provincial teams for emergency responses to emerging communicable diseases and other health event. The FETP graduates have greatly helped to prevent and control the H7N9 virus infection.

Financial support More government funds were allocated for establishment of a system to respond to emerging communicable diseases and actual emergency responses. Thanks to these policies, emergency response facilities have received financial compensation and emergency response funds, enhancing their ability to conduct emergency responses to emerging communicable diseases.

2.5. *International communication and cooperation*

China has improved its international communication and cooperation with international organizations and countries in terms of responding to public health emergencies. With the help of international communication and cooperation, China can now share the latest information on emerging communicable diseases and easily receive support from or supply support to other regions. For example, China, Hong Kong, and Macau signed a cooperative agreement on responding to public health emergencies in 2005. This agreement provided a basis for cooperation and communication of information on emerging communicable diseases among these regions. To some degree, this agreement has helped to enhance cooperation and communication among these three regions and has led to a more effective response to emerging communicable diseases.

International communication and cooperation allows the world to conduct emergency responses before a pandemic occurs. When the outbreak of H7N9 occurred in 2013, the response by different countries and regions at the same time helped to reduce the impact of the disease.

3. Challenges of and prospects for the response to emerging communicable diseases in China

Although the establishment of a mechanism for emergency responses to emerging communicable diseases in China has made great achievements and helped to control the H7N9 virus in a short period of time, some challenges still remain.

Better coordination system Organization of and collaboration in responses is a key factor for prevention and control of emerging communicable diseases. The system of coordination among different departments and specialized facilities at the local level is still weak and may need to be enhanced.

More complete Office of Responses to Public Health

Emergencies Responses to emerging communicable diseases require support from different areas, so administration of responses to emerging communicable diseases requires comprehensive decision-making ability. Therefore, a more complete Office of Responses to Public Health Emergencies may prove useful.

Administrative responsibility and error correction The key to prevention and control of emerging communicable diseases is to allocate responsibility. Mechanism of administrative responsibility and fault rectifications such as responsibility system of "government - health administration department - medical institution - medical staff" may provide a solution.

Better personnel training A shortage of trained personnel and insufficiently trained personnel are major problems hampering the development of facilities for disease control and prevention (*7-9*). One potential solution is for administrators of responses to public health emergencies and experts to regularly conduct training and emergency drills to deal with emerging communicable diseases.

Government responsibility The Government should be responsible for preventing and controlling emerging communicable diseases. Therefore, more studies are needed to determine what types of action the Government should take during an outbreak of an emerging communicable disease.

In conclusion, China's response to emerging communicable diseases improved significantly after the outbreak of SARS in 2003, with improvements in command and decision-making, organization and collaboration, monitoring and early warning, protection, and international communication and cooperation. Thus, China successfully dealt with outbreaks of the H5N1 bird flu, H1N1 flu, and H7N9 bird flu. This was especially evident in the spring of 2013, when China successfully detected and rapidly controlled new cases of H7N9 bird flu (*10*) and Zhejiang dealt with its first case of H7N9 this autumn. However, a better coordination system, a more complete Office of Responses to Public Health Emergencies, administrative responsibility and error correction, better personnel training, and government responsibility may help to improve China's response to emerging communicable diseases. Such improvements are eagerly anticipated.

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Letter

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Call for action for setting up an infectious disease control action plan for disaster area activities: Learning from the experience of checking suffering volunteers in the field after the Great East Japan Earthquake

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Keywords: Earthquake, measles, transmission, screening

Summary: *After the Great East Japan Earthquake on March 11th, 2011, a journalist visited the disaster area with febrile symptoms and was diagnosed with measles of the D genotype, which is not indigenous to Japan. After continuing activities in disaster areas and Tokyo, 11 measles cases were reported, some of which were identified as genotype D. Meanwhile non-profit activities directed towards volunteers were offered including interviews to screen for subjective symptoms, check body temperature and advise volunteers to refrain from working in shelter areas during the period of sickness. As a consequence, disease transmission was controlled among volunteers. In disaster areas, anyone can be an infection vector. In order to prevent transmission of infectious diseases, a field action plan, which includes body temperature checks and standard precautions should be formulated and put into place. If the action plans are shared among local governments and non-governmental organizations (NGOs), they can become a norm and be expected to control infectious disease transmission.*

Almost two and a half years have passed since the earthquake and tsunami hit the Pacific coast of Tohoku on March 11th, 2011. As of July 1st, 2013, the death toll is reported to be approximately 15,883 with 2,654 persons still listed as missing (*1*).

A disaster affects everything, the control function of local governments may be lost and misrule may emerge because officers of local governments can themselves be victims. The Japanese experience this time is nothing short of misrule. Under such circumstances, it is noteworthy that many volunteers participated in aiding shelter residents.

Lessons for health should be learned and shared from all disasters in order to benefit future preparedness. As such, this disaster offered us an important lesson for when there is an influx of people into areas that have been affected by a disaster.

In this article, we review a case of measles importation in the disaster period and a response from a company in which non-profit activities for controlling infectious disease transmission were directed at volunteers. We conclude that simple activity guidelines in disaster areas, including precautions, are useful and should be shared internationally.

A case of measles importation into a disaster area

In April, 2011, news hit the Tohoku disaster area that a foreign journalist had visited with febrile symptoms (*2*), and had continued his/her activities in the disaster area and Tokyo, before finally being diagnosed with measles. The measles virus genotype of the journalist was D4, which is not indigenous to Japan. Fortunately, no measles outbreak was observed in the disaster area. However, 11 measles cases were observed in Japan thereafter, some of which were identified as the D4 genotype (*3,4*).

Neither the journalist's attitude to precautions nor his/her vaccine history were officially disclosed, so the relationship between his/her activities and measles virus dissemination is unclear. What is clear is that, since measles is contagious during the febrile period (*5*),

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the activities of the journalist should have been limited in order to prevent the dissemination of the pathogen.

A good practice for controlling infectious diseases

Lessons can be drawn from the non-profit activities offered by the company Carepro, which usually offers health check services. The objective of the company's activities in the affected area was to check the health status of volunteers and control infectious disease epidemics within the disaster area. When they started activities 17 days after the disaster, they found that among the more than 100 volunteers working there, some were suffering from influenza-like illnesses or enterocolitis. The staff from Carepro advised volunteers to check for subjective symptoms and take their body temperature each day. If volunteers felt ill, they were advised to refrain from working in shelters. Besides alleviating volunteers' fatigue, their activities contributed to controlling the incidence of infectious diseases among volunteers including influenza/enterocolitis (from 14/100 to 1/100) (*6*) and halted the transmission of common infectious diseases to those affected by the disaster.

While other factors including improvement of the shelter conditions may have influenced the dramatic reduction in disease incidence, their experience in these focused activities is considered good practice for controlling infectious disease transmission.

Considering feasible means of disease prevention

When a disaster occurs, a range of different people tend to enter the affected area, including professional people such as police, rescue teams, medical teams, journalists and volunteers, any of whom can be a vector of infectious diseases (*7*). Any pathogen, including those that are vaccine preventable, can easily pass national borders.

In addition, in disaster areas, where resources are limited, it is difficult to both handle sophisticated medical techniques including detailed diagnosis and to force people coming from outside the disaster area to receive vaccinations and/or intensive medical checks. Existing guidelines are too complicated for local government officers in devastated regions to implement in the event of a disaster, because they are written in a problem-oriented, list-wise manner. Officers may be too exhausted to understand detailed guidelines and reorganize their plans while coping with difficult situations. (*8,9*).

Thus the simple method provided by Carepro, which includes checking body temperature and physical condition every day before starting volunteer work, is useful for public health activities. Learning from this lesson, more feasible means of preparation for coming disasters should be considered, in particular, with sharing experiences and lessons of troubles faced by local government officers. The formulation of simple action plans for field activities in which standard, reasonable and applicable precaution methods, which may be assumed even when the general population is suffering the effects of a disaster, should be included. If such action plans are circulated among local governments and non-governmental organizations (NGOs) which provide disaster-relief activities and training for the plan is repeated, then they may become a norm for safeguarding activities in disaster areas, which can be shared internationally.

In conclusion, every influx of population into disaster areas can be a vector for the transmission of infectious diseases. For preventing shelter residents from acquiring infectious diseases, it is recommended that action plans, in which basic health checks are included, are established and drilled.

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