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Proteome screening of pleural effusions identifies IL1A as a diagnostic biomarker for non-small cell lung cancer

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ABSTRACT

Non-small cell lung cancer (NSCLC) is a common malignant disease, and in ~10–20% of patients, pleural effusion is the first symptom. The pleural effusion proteome contains information on pulmonary disease that directly or indirectly reflects pathophysiological status. However, the proteome of pleural effusion in NSCLC patients is not well understood, nor is the variability in protein composition between malignant and benign pleural effusions. Here, we investigated the different proteins in pleural effusions from NSCLC and tuberculosis (TB) patients by using nano-scale liquid chromatography-tandem mass spectrometry (nLC-MS/MS) analysis. In total, 363 proteins were identified in the NSCLC pleural effusion proteome with a low false discovery rate (<1%), and 199 proteins were unique to NSCLC. The proteins in the NSCLC patients' pleural effusion were involved in cell adhesion, proteolysis, and cell migration. Furthermore, interleukin 1 alpha (IL1A), a protein that regulates tumor growth, angiogenesis, and metastasis, was significantly more abundant in the NSCLC group compared to the TB group, a finding that was validated with an ELISA assay.

1. Introduction

Lung cancer can be classified into two main types, small cell lung cancer (SCLC) and non-small cell cancer (NSCLC), with the latter constituting 80% of all lung cancer cases [1,2]. The 5-year survival rate for NSCLC is only 15%, with the high rate of mortality caused by the difficulty of early detection and the lack of adequate treatment strategies [2,3]. In the last decade, while a variety of clinical serum or pleural effusion markers have been identified for the diagnosis of NSCLC, they are yet to be incorporated into clinical practice because of their low sensitivity and specificity, especially at early stage of cancer [4]. Therefore, there remains an urgent demand for a definitive diagnostic marker for NSCLC.

In disease-specific biomarker discovery, proteomics analysis of specific body fluids is used clinically to investigate the development and progression of various diseases [5–7]. Exudative pleural effusion is a buildup of protein rich fluid in the cavity surrounding the lungs. It contains plasma proteins as well as other proteins released during lung inflammation or cancer [8]. In approximately 10–20% of NSCLC patients, malignant pleural effusion is the first symptom [9]. The pleural effusion proteome contains information on pulmonary disease that directly or indirectly reflects pathophysiological status [5–7]. The global protein composition of human malignant pleural effusion has been analyzed by using 2-dimensional differential in-gel analysis (2D-DIGE) [10,11]. However, the proteome of pleural effusion in NSCLC patients is still not well understood, nor is the variability in protein composition between malignant and benign pleural effusions.

In this study, we compared the proteomes of pleural effusion samples taken from patients with NSCLC and tuberculosis. In total, 363 proteins were identified in the NSCLC pleural effusion proteome with a low false discovery rate (<1%). Gene ontology enrichment analysis revealed signification overrepresentation of NSCLC pleural effusion proteins involved in "cell adhesion," "proteolysis," and "cell migration." To identify potential biomarkers for NSCLC, IL1A was validated with an ELISA assay in a cohort of NSCLC patients. Levels of IL1A were found to be significantly different in the NSCLC group compared to the TB group. Therefore, our results suggest that IL1A could be used as a potential diagnostic biomarker in effusion samples from NSCLC.

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2. Materials and methods

2.1. Sample collection

The study was conducted according to the guidelines of the Clinical Ethics Committee of Southwest Hospital. All patients in the study gave their informed consent. We recruited twenty patients with pleural effusion comprising ten NSCLC and ten tuberculosis patients. Each primary NSCLC diagnosis was verified by histopathological analysis. All enrolled TB patients were sputum smear-positive. Pleural fluid (10 mL) was collected in sterile tubes from the participants. After 10 min centrifugation at 1000×*g* and 4 °C to remove cells and cell debris, the supernatant was divided into 1 mL samples and immediately frozen at −80 °C. The samples were concentrated by vacuum freeze-drying, and then dissolved in PBS buffer. Total protein concentration was determined by using a BCA concentration measurement kit (Beyotime, Chongqing, China).

2.2. 1D SDS–PAGE and MS analysis

Pleural effusion proteins (100 μg) were separated onto a 4–12% gradient gel (Invitrogen). After being stained with Coomassie brilliant blue, the gradient gel was cut into ten equal width bands for MS analysis. Each band was cut into 1 mm^3 blocks and destained with 50% acetonitrile (ACN) into 50 mM ammonium bicarbonate (NH_4HCO_3). These gel pieces were dehydrated with 100% ACN and digested overnight with 1 μg trypsin (Promega) in 50 mM NH_4HCO_3. The digested peptides were extracted with 5% formic acid in 50% ACN. Nano-scale liquid chromatography tandem mass spectrometry (nLC-MS/MS) was performed using a Bruker maXis 4G UHR-TOF mass spectrometer. Briefly, samples (10 μL) were loaded onto a 15 cm × 75 μm (ID) C18 column. The mobile phases were water with 1% formic acid and ACN with 1% FA. The details of the 90-min gradient are as follows: the initial concentration of ACN was 5%; from 5 to 65 min, ACN increased from 5% to 35%; from 65 to 75 min, ACN increased from 35 to 80% and was held at 80% for 5 min; finally, from 75 to 90 min, ACN returned to 5%.

High-resolution peptide fragment spectra were acquired from one MS scan followed by four MS/MS scans using a Bruker maXis 4G UHR-TOF mass spectrometer, and then these peptide spectra were searched against a target-decoy database of human tryptic peptides with the Mascot engine. The threshold of false discovery rate (FDR) analysis was set at <1% for protein identification. The data from all fractions of one pleural effusion sample were combined for comparative proteomic analysis.

2.3. Bioinformatics analysis

A list of the proteins' UniProt IDs was submitted to the DAVID online tools for Gene Ontology (GO) enrichment analysis (http://david.abcc.ncifcrf.gov/home.jsp). Functionally related proteins were clustered according to overrepresented biological annotations with a statistical "Enrichment Score". The protein spectra of pleural effusion were subject to hierarchical clustering with Cluster 3.0 and visualized with the TreeView software suite.

2.4. ELISA assay

CATSPER1, IL1A, and MYO1A in supernatants from the pleural effusions were measured by using an ELISA assay with the Human Cation channel sperm-associated protein 1 (CATSPER1) ELISA kit (MyBiosource), the Human IL-1 alpha/IL-1F1 Quantikine ELISA kit (R&D), and the Human Myosin-IA (MYO1A) ELISA kit (MyBiosource). Absorbance was measured at 405 nm using a microplate reader.

3. Results

3.1. The proteomics of pleural effusions in NSCLC and TB patients

We investigated the protein composition of pleural effusions from NSCLC and TB patients (Table 1) according to the schematic workflow of the proteomic screening protocol (Fig. 1A). Spectra from the maXis 4G UHR TOF mass spectrometer were matched against the UniProt database by using the MASCOT engine. To improve the confidence level of protein identification, we sorted the results by >99% probability and at least one unique peptide. A total of 229, 181, and 168 proteins were identified in the three individual TB patients. In the three individual NSCLC patients, 163, 232, and 195 proteins were identified (Fig. 1B and Table S1). Among these proteins, 131 (46.9%), 127 (47.0%), and 104 (42.4%) were identified in each pair of the three TB patients, respectively, while 102 (32%) proteins were identified in all three TB patients (Fig. 1B for specific combinations). In the three NSCLC patients, 107 (37.2%), 96 (36.6%), and 113 (37.2%) proteins were identified in each pair of patients, respectively, while 89 (24.5%) proteins were identified in all three patients (Fig. 1B for specific combinations). The heterogeneity of proteins in pleural effusion was significantly higher in NSCLC patients than in TB patients.

3.2. Functional analysis of the proteomes

We categorized the proteins by their GO annotation to determine enriched categories of annotation for each disease (Fig. 2). An overlap (164 proteins, 31.7%) was found in pleural effusion proteomes of NSCLC and TB patients (Fig. 1C). The biological process analysis also revealed several common over-represented biological process categories in NSCLC and TB pleural effusion, including serum albumin, globulin, apolipoprotein, and complement. The overlap between NSCLC and TB suggests that there are common features of exudative pleural effusion. In general, the over-represented biological process categories common to both NSCLC and TB were among the most enriched overall. Interestingly, significant differences were found among the over-represented processes of the two diseases. In NSCLC, we observed increased enrichment of processes in adhesion (enrichment *p*-value: CA ≈ 10^{-6}; TB ≈ 10^{-3}), proteolysis (enrichment *p*-value: CA ≈ 10^{-9}; TB ≈ 10^{-4}), and cell

Table 1
Clinical information of the non-small cell lung cancer (NSCLC) or tuberculosis (TB) patients.

Sample	Age (years)	Gender	Protein (g/dL)	ADA (<15U/L)	CEA (<10 ng/mL)	TNM stage	Diagnosis
NSCLC1	60	M	52.5	5.5	33.5	IIB	NSCLC
NSCLC2	59	M	41.9	1.2	8.1	IIIA	NSCLC
NSCLC3	66	F	46.7	2.3	9.2	IIB	NSCLC
TB1	64	M	51.4	39.2	4.4	-	Tuberculosis
TB2	42	M	49.3	23.7	3.5	-	Tuberculosis
TB3	40	M	65.4	8.5	5.1	-	Tuberculosis

ADA: adenosine deaminase; CEA: carcinoembryonic antigen; TNM: Tumor Node Metastasis.

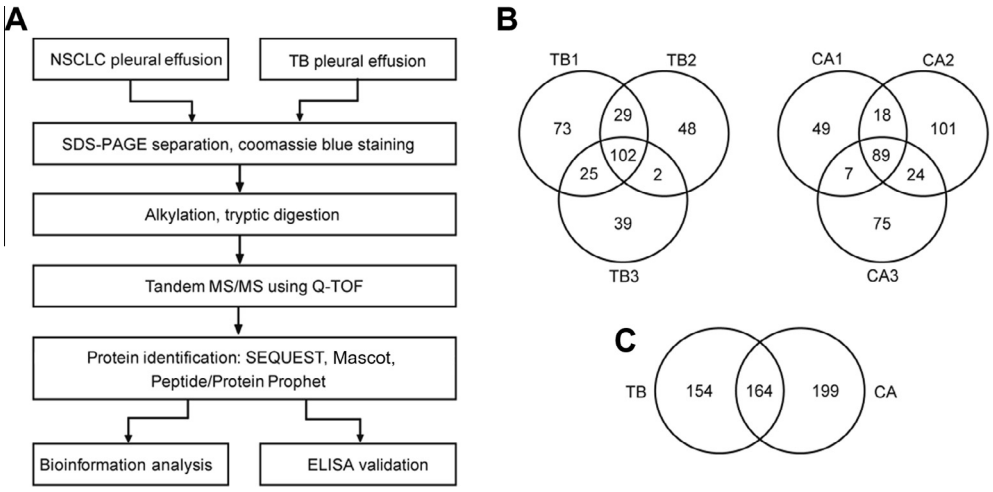

Fig. 1. Schematic illustration of the major steps in the parallel proteomics platform and Venn diagrams of the identified proteins. (A) Schematic overview of the proteomic analysis of pleural effusions from non-small cell lung cancer (NSCLC) and tuberculosis (TB) patients. (B–C) Venn diagram showing the number of proteins and the overlapping proteins found in both NSCLC (CA) and TB patients.

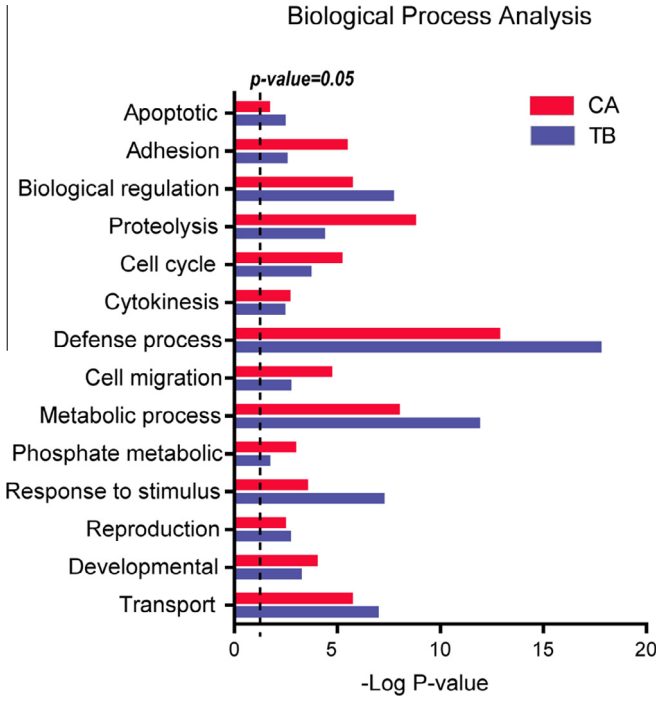

Fig. 2. Biological process analysis of the proteins identified in non-small cell lung cancer and tuberculosis pleural effusions. Enrichment *p*-values represent the one-tailed Fisher exact probability.

migration (enrichment p-value: CA $\approx 10^{-4}$; TB $\approx 10^{-2}$). The pathway categories that were highly enriched in NSCLC were closely related to the biological functions of tumor cells [13,14]. In contrast, pathways involved in the defense process, the metabolic process, and the response to stimulus were significantly more enriched in TB than in NSCLC.

3.3. Comparing protein abundance in plural effusions from NSCLC and TB patients to identify the optimum discriminatory biomarkers in NSCLC

A hierarchical two-dimensional cluster with the number of protein spectra showed that NSCLC or TB pleural effusion proteomes represented similar protein expression profiles (Fig. 3A and Fig. S1A). The pleural effusion proteomes were clustered into three groups: the common proteins, the TB-specific proteins, and the NSCLC-specific proteins (Fig. 3A). Expression of proteins in the common group was stable, regardless of disease. Surprisingly, several NSCLC markers in the common group were previously reported in the literature, including alpha-2-glycoprotein 1, zinc-binding (AZGP); apolipoprotein A-I (APOA1); apolipoprotein D (APOD); alpha-2-HS-glycoprotein (AHSG); clusterin (CLU); complement component 3 (C3); serpin peptidase inhibitor, clade F, member 1 (SERPINF1); serpin peptidase inhibitor, clade A, member 1 (SERPINA1); transthyretin (TTR); and gelsolin (GSN) [4]. However, we did not find a substantial difference in protein abundance between NSCLC and TB (Fig. S1B). TB-specific proteins in pleural effusions were related to infection, including complement-related and TB infection-associated proteins. NSCLC specific proteins were related to the occurrence of cancer, including CATSPER1, EFR3B, LRRC47, MYO1A, HRG, IL1A, BCAN, DSCAM, HAVCR2, PZP, ACAA1, FBLN1, PGLYRP2, and LUM (Table 2) (Fig. 3B and C). To evaluate potential diagnostic biomarkers, CATSPER1, IL1A, and MYO1A were tested in a cohort of patients with commercial ELISA assays (Fig. 4). IL1A in the NSCLC group was found to be significantly different from that in the TB group ($p < 0.05$). Our data suggest that patients with high levels of IL1A in their pleural effusion were most likely to be suffering from NSCLC.

4. Discussion

The pleural effusion proteome contains a wealth of information that directly reflects lung pathophysiological processes and provides potential value for the diagnosis of lung disease. However, detection of malignant pleural effusion is often delayed in clinical practice [9]. Therefore, identifying diagnostic biomarkers in pleural effusion constitutes vitally important research for pulmonary diseases such as NSCLC. Proteomic analysis complements genetic analysis as an integral tool for investigation of tumor biology [12,13]. Here, we applied a global proteomic approach to analyze and compare the pleural effusions of NSCLC and TB patients with the aim of identifying new clinical biomarker proteins for NSCLC.

Proteins in the NSCLC effusions were highly enriched with annotations for biological processes related to “cell adhesion,” “proteolysis,” and “cell migration.” Conversely, the proteins

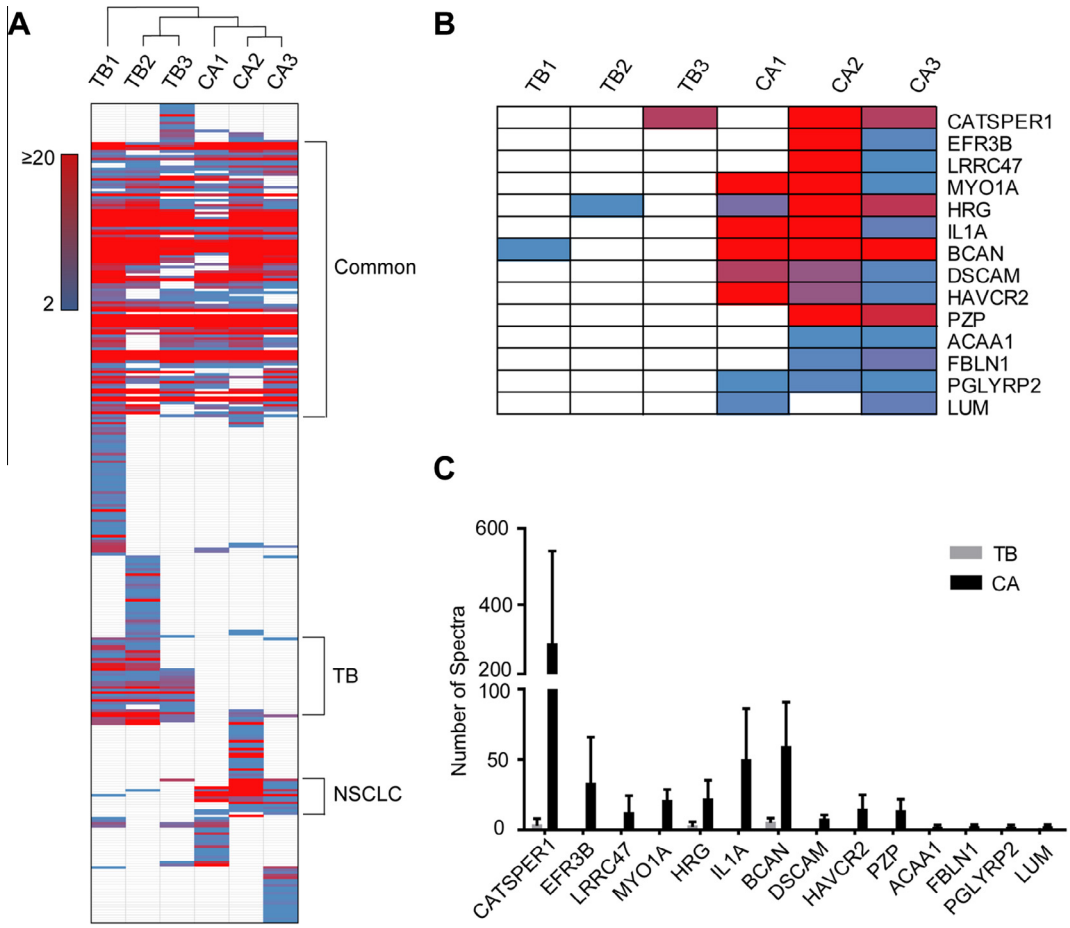

Fig. 3. Identifying potential candidate biomarkers in pleural effusion sampled from non-small cell lung cancer (NSCLC) patients by using cluster analysis. (A) Hierarchical two-dimensional clustering of NSCLC (CA1, 2, and 3) and tuberculosis (TB1, 2, and 3) pleural effusion proteomes according to the number of protein spectra (where spectra $\geqslant 2$). Each row represents an individual proteomic signal and each column an individual sample. (B) The candidate biomarkers for NSCLC pleural effusions, based on hierarchical two-dimensional clustering analysis. (C) Spectra analysis of the candidate biomarkers in the three NSCLC patients.

Table 2
Identification of the candidate biomarkers in non-small cell lung cancer (NSCLC) patients.

Gene name	Description	Function	Related to cancer
CATSPER1	Cation channel sperm-associated protein 1	Ion channel	–
EFR3B	Protein EFR3 homolog B	Metabolism	–
LRRC47	Leucine rich repeat containing 47	Binding	–
MYO1A	Myosin IA	Cell motion	Intestine [28], Stomach [29]
HRG	Histidine-rich glycoprotein	Angiogenesis	Brain [30], Breast [31], Kidney [32], Ovarian [33], Pancreatic [34]
IL1A	Interleukin 1, alpha	Immune response	Stomach [35], Ovarian [36], Prostate [37], Lung [38]
BCAN	Brevican	Migration	Gliomas [39,40], Breast [41]
DSCAM	Down syndrome cell adhesion molecule	Cell adhesion	Lung [42]
HAVCR2	Hepatitis A virus cellular receptor 2	Immune response	Leukemia [43], Lung [44], Liver [45]
PZP	Pregnancy-zone protein	Proteinase inhibitors	–
ACAA1	Acetyl-CoA acyltransferase 1	Metabolism	–
FBLN1	Fibulin-1	Migration	Breast [46], Colon [47], Prostate [48], Stomach [49]
PGLYRP2	Peptidoglycan recognition protein 2	Inflammatory	-
LUM	Lumican	Metastasis	Melanoma [50], Prostate [51], Breast [52]

specific to TB pleural effusions were closely related to infection, including complement-related protein C1QA [14] and CD2-associated protein (CD2AP) [15] (Fig. S1C). Unexpectedly, several proteins that had been considered as diagnostic molecular markers for NSCLC were found in the plural effusions of both NSCLC and TB patients. For example, gelsolin as a Ca^{2+}-regulated actin filament severing protein has previously been described as a specific NSCLC biomarker [4,16–18]. However, gelsolin was identified in the NSCLC and TB groups. Additionally, SAA1 (serum amyloid A1) [19–21] and CST3 (cystatin C) [4], which have been considered as unique biomarkers for NSCLC in previous studies, were identified not or only once in three NSCLC samples.

By hierarchical cluster analysis, we identified 14 candidate diagnostic biomarkers for NSCLC. CATSPER1, IL1A, and MYO1A were validated with an ELISA assay. IL1A was identified as the most appropriate diagnostic biomarker for NSCLC. IL1A belongs to the IL1 family, which includes IL1A, IL1B, IL-1RN, and IL-18, and is a pluripotent cytokine that plays a key role in normal physiological

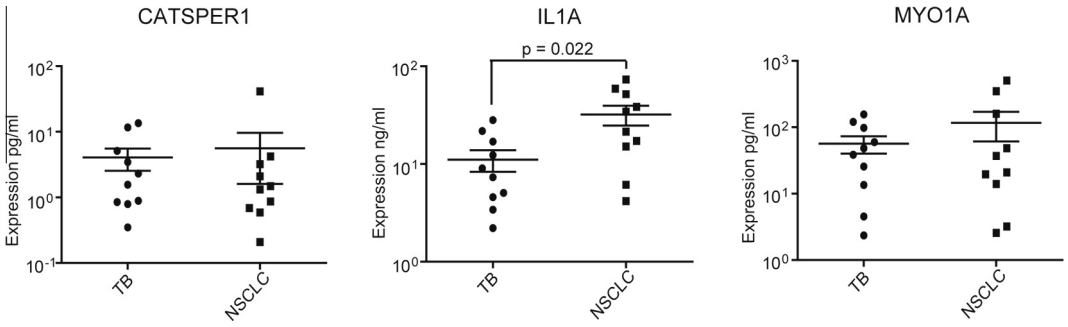

Fig. 4. Validation of selected candidate biomarkers for non-small cell lung cancer by ELISA detection. The *p*-value shown for IL1A was determined by using the Wilcoxon rank sum test.

and pathological responses [22,23]. IL1A exists in nearly all cells and is up-regulated during cell damage. When the body is injured, IL1A is released as an early warning molecule and mediates inflammatory recruitment to the site of the injury [23]. In addition, IL1A participates in the regulation of transcription during the inflammatory process [24]. In a recent study, two forms of IL1A (the membrane and secretable forms) were found to play different roles in the tumor process. The membrane form of IL1A activates immune-mediated anti-tumor mechanisms and inhibits tumor cell proliferation [23]. In the tumor microenvironment, the secretable form of ILA is a highly pro-inflammatory cytokine that increases tumor angiogenesis and invasiveness [25–27]. Therefore, the high levels of IL1A in the pleural effusions suggest an important role for the protein in invasion of NSCLC cells. In future studies, it would be worthwhile investigating the expression of IL1A in other malignant body fluids of cancer patients.

Transparency Document

The Transparency document associated with this article can be found in the online version.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.12.083.

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