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Tumor-dependent secretion of close homolog of L1 results in elevation of its circulating level in mouse model for human lung tumor

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ABSTRACT

Close homolog of L1 (CHL1) and its truncated form mainly play crucial roles in mouse brain development and neural functions. Herein, we newly identified that truncated form of CHL1 is produced and released from lung tumor tissue in a mouse model expressing human *EML4-ALK* fusion gene. Both western blot and direct ELISA analysis revealed that mouse CHL1 level in serum (including serum extracellular vesicles) was significantly elevated in *EML4-ALK* transgenic mice. The correlation between the tumor size and the amount of CHL1 secretion could be examined in this study, and showed a significant positive correlation in a tumor size-dependent manner. Considering these results, the measurement of circulating CHL1 level may contribute to assess a tumor progression in human lung tumor patients.

1. Introduction

A lot of molecules (genes) overexpressed in tumor tissues compared with normal tissues have been a concern in the oncology research, and often used as the targets for tumor diagnosis. Among them, secretory proteins, shedding forms of membrane proteins, and extracellular vesicles (EVs; *e.g.* exosomes) recently emerged [1] are secreted from tumor tissues into blood, and used for diagnosis as a serum tumor markers [2–4].

Close homolog of L1 (CHL1) is a type I transmembrane protein, and belongs to immunoglobulin superfamily [5,6], and is expressed in several mammalian species. The physiological function of CHL1 has been studied, and is mainly involved in the neural development and functions in mice [6–8]. On the other hand, CHL1 plays an important contribution in tumor cells such as breast cancer [9] and glioma [10]. Indeed, CHL1 is upregulated at the transcriptional level in several tumor cells [11]. There is no doubt that CHL1 is a remarkable molecule in tumor biology.

Despite a membrane protein, CHL1 has also been reported to suffer an ectodomain shedding by various proteases in brain tissues and nerve cells resulting in secretion outside a tissue (cell) as an its soluble form [12]. Particularly, ectodomain shedding of CHL1 in the brain has attracted attention, because the physiological functions of CHL1 have been studied in brain tissues and nerve cells. However, secretion of CHL1 molecule accompanying ectodomain shedding reaction of CHL1 in cancer tissue (cell) has not been studied. In particular, considering the serum tumor marker described above, there is no doubt it is an important finding.

Here we examined the secretion of mouse CHL1 (mCHL1) from tumor tissue (cell) by ectodomain shedding using an animal model, *EML4-ALK* transgenic mouse [13]. *EML4-ALK* transgenic mice carry human *EML4-ALK* fusion gene [14] allele in the lung, and is an early carcinogenic mouse model in which lung tumor is formed at 4 weeks of age. It is, therefore, useful not only for research on lung cancer development by the fusion gene but also for that on oncobiochemistry. In the previous study, we identified both gene expression and protein expression of CHL1 were elevated in the tumor tissue derived from *EML4-ALK* transgenic mouse (Kotani et al., *in preparation*). We measured the CHL1 protein levels secreted into the culture medium of primary cells established from the tumor tissue in *EML4-ALK* transgenic mouse and the circulating levels of secreted CHL1 protein in wild-type mouse and *EML4-ALK*

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transgenic mouse at each week of age.

2. Materials and methods

2.1. Animals

C57BL/6J mice were obtained by self-breeding of the pair mice purchased from Japan CLEA Co. (Tokyo, Japan), and were maintained under specific pathogen-free conditions at 20–25 °C in accordance with the Saitama Medical University Animal Experiment Committee (protocol number 2162). The *EML4-ALK* transgenic mouse [13] were maintained under specific pathogen-free conditions at 20–25 °C in accordance with the Saitama Medical University Animal Experiment Committee (protocol number 2158 and 2162) and the Saitama Medical University Safety Committee for Recombinant DNA Experiments (protocol number 1360). All animals were maintained under a 12-hr light/12-hr dark exposure and free access to food and water.

2.2. Collection of tumor tissue and serum samples from mice

Seven to forty-three weeks old mice (Supplementary Table 1) were deeply anesthetized with isoflurane and then sacrificed by cervical dislocation. After blood sampling from the heart, serum was prepared using a dedicated blood sampling tube (Fuchigami Co., Kyoto, Japan). Then, the tumor tissues of *EML4-ALK* transgenic mice were excised and weighed.

2.3. Preparation of primary cells from tumor tissue

The lung tumor tissues were minced and treated with collagenase (Worthington Biochemical, NJ) in sterile PBS solution at 37 °C for 30min. The large debris were removed from the mixture, and then washed 3 times with RPMI 1640 medium (Wako Chemicals, Osaka, Japan). The cells were seeded at collagen-coated 10 cm dishes (IWAKI glass, Shizuoka, Japan) with RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS; GIBCO, MA; Cat. No. 12483–020) at 37 °C under humidified air containing 5% CO_2 (approx. 200 μg of minced tissues per 10 cm dishes). The next day, medium was replaced with new medium followed by cultivation for 3 days. Subsequent medium change was performed, continued to culture for 2 days. The cultured medium was recovered as the conditioned medium. The cultured cells and conditioned medium were both collected respectively for the measurement of cellular and secreted mCHL1.

2.4. Expression of fluorescent tag-conjugated CHL1 in HEK293 cells

The cDNA of mCHL1 was cloned by assembly of PCR fragments amplified using EmeraldAmp® PCR Master Mix (Takara Bio, Shiga, Japan) based on the mouse lung tumor cDNA library established using tumor tissues of *EML4-ALK* transgenic mice. The sequences of PCR primers used in the PCR cloning were as follows: forward 5′-cgcgcggccgcatgatggaattgccattatg-3′ and reverse 5′-caaacactcgagcaatagtgta-3′ for “Xho I region”; forward 5′-tacactattgctcgagtgttttg-3′ and reverse 5′-catctcaaagcttctccatctt-3′ for “Xho I-Hind III region”; forward 5′-aagatggagaagcttttgagatg-3′ and reverse 5′-caccaatttatctggtaccctct-3′ for “Hind III-Kpn I region”; forward 5′-agagggtaccagataattggtg-3′ and reverse 5′-cgcgaattctgcccggagtggaaggtgg-3′ for “Kpn I region”. The cDNA of both mCHL1 and fluorescent tag (EGFP, Azami green, or mCherry) were inserted into pcDNA 3.1 (+) expression vector (Supplementary Fig. 1A; Thermo Fisher Scientific, MA), then the resulting vector was transfected into HEK293 cells. The transfectant cells were cultured for 3 days with RPMI 1640 medium supplemented with 5% FBS at 37 °C under humidified air containing 5% CO_2. The observation of protein expression was performed using EVOS FLoid® Cell Imaging Station fluorescence microscopy (Thermo Fisher Scientific, MA) equipped with green and red fluorescence channels. The cultured cells and conditioned medium were both collected respectively.

2.5. Separation of extracellular vesicles from mouse serum

Twenty-five μl of each serum sample was mixed with 6.3 μl of ExoQuick exosome precipitation solution (System Biosciences, CA), and incubated at 4 °C for 15 min. After centrifugation at 1500 g for 15 min, serum component (without EVs) was separated from the precipitates (including EVs). The precipitates were resolved with 50 μl of PBS.

2.6. Western blot analysis

The conditioned medium obtained by primary lung tumor cell culture and mCHL1 transfectant HEK293 cells was concentrated by using Nanosep® Centrifugal Devices (30 kDa cut-off, Pall Co., NY). The concentrated residues and cell lysates were subjected to SDS-PAGE (6% gel), then transferred to an Immobilon®-P PVDF Membrane (Merck Millipore, Germany). After blocking with 5% skim milk solution, the membrane was treated with anti-mCHL1 antibody (AF2147; R&D systems, MN; 1 μg/ml) at room temperature for 1 h followed by goat TrueBlot®: anti-goat IgG HRP (Rockland, PA; 1:1000 dilution) at room temperature for 1 h. In the case of mCHL1-transfected HEK293 cells, the membrane was treated with HRP-conjugated anti-mCHL1 antibody, which was labeled by using peroxidase labeling kit-SH (Dojindo, Kumamoto, Japan), at room temperature for 1 h (1:3000 dilution). For the detection of fluorescent protein tag, the resulting membrane was treated with anti-GFP antibody or anti-Azami Green antibody (MBL life science, Nagoya, Japan; 1:1000 dilution) at room temperature for 1 h followed by the treatment with HRP-conjugated anti-rabbit IgG (Promega; 1: 5000). The 2 μl of serum samples and EV sample solutions described above were similarly subjected to 6% SDS-PAGE gel, then transferred to a PVDF Membrane. After blocking with 5% skim milk solution, the membrane was treated with HRP-conjugated anti-mCHL1 antibody (1:3000 dilution) at room temperature for 1 h. After antibody treatment of these membranes, the membrane was developed with an Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore, Germany). The membrane was exposed and analyzed using ChemiDoc MP image analyzer (BIO-RAD, CA).

2.7. Direct ELISA

The serum samples were therefore diluted 50-fold with PBS, subsequently applied to 96-well ELISA plates (CORNING, NY; No. 3369), and then incubated at 37 °C for 1 h. After washing with 0.05% Tween-PBS solution, each well was treated with 5% skim milk solution at 37 °C for 1 h for blocking. The 5% skim milk solution containing HRP-conjugated anti-mCHL1 antibody (1:3000 dilution) was added to each well, and incubated at 37 °C for 1 h. After gentle washing, HRP substrate solution (SureBlue Reserve; SeraCare, MA) was added and developed at room temperature for 10–20 min. Twenty μl of 1 M HCl solution was then added to each well followed by the measurement of O.D. 450 nm. In order to compare the data among each experimental plate, a representative sample was selected in each plate followed by the measurement of each representative sample in the same plate at a later date. To compensate the differences among ELISA plates, the relative values were determined based on the above results as “Relative index”.