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

Research Article

Tumor-dependent Secretion of Closed Homolog of L1 Results in Elevation of its Serum Level in Mouse Model for Human Lung Tumor

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Abstract

It has been pointed out that 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. The primary tumor cells derived from lung tumor tissues secreted CHL1 into culture medium. The secretion of truncated form of mouse CHL1 was also observed in HEK293 cells transiently expressed mouse CHL1. Both western blot and direct ELISA analysis revealed that serum levels of secreted mouse CHL1 were significantly elevated in *EML4-ALK* transgenic mice compared with that in wild-type mice. A part of secreted CHL1 into mouse serum appeared to be included in extracellular vesicles (E.V.). The correlation between the tumor mass and the amount of CHL1 secretion in 40 wild-type and 40 transgenic mice was examined, and showed a significant positive correlation. These findings demonstrated that the secretion of CHL1 occurred in not only the brain but also tumor cells resulting in the increase of circulating CHL1 levels in a tumor size-dependent manner. Considering that *EML4-ALK* transgenic mouse used in this study is a human lung cancer model, measurement of circulating CHL1 level may contribute to the diagnosis of human lung cancer.

Keywords: Closed homolog of L1, Lung cancer, *EML4-ALK* gene, tumor marker

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 (E.V.; *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 [6–11]. On the other hand, CHL1 plays an important contribution in tumor cells such as breast cancer [12] and glioma [13]. Indeed, CHL1 is upregulated at the transcriptional level in several tumor cells [14]. 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 a its soluble form [15]. This ectodomain shedding is catalyzed by several proteases, ADAM family proteases and BACE 1 [16,17], which involved in the production of A-beta peptide in Alzheimer's disease [18–22]. Particularly, ectodomain shedding of CHL1 by BACE1 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 molecule (mCHL1) from tumor tissue (cell) by ectodomain shedding using an animal model, *EML4-ALK* transgenic mouse [23]. *EML4-ALK* transgenic mice carry human *EML4-ALK* fusion gene [24] 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 onco-biochemistry. 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 serum levels of secreted CHL1 protein in wild-type mouse and *EML4-ALK* transgenic mouse at each week of age. Our findings revealed that CHL1 is secreted from the tumor tissue (cell), and is increased in the serum of tumor-bearing *EML4-ALK* transgenic mouse compared with that of wild-type mice.

Materials and Methods

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 [23] 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.

Collection of tumor tissue and serum samples from mice

Seven to forty-three weeks old mice (Supporting 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.

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.

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'-caaaacactcgagcaatagtgta-3' for "Xho I region"; forward 5'-tacactattgctcgagtgttttg-3' and reverse 5'-catctcaaaagcttctccatctt-3' for "Xho I-Hind III region"; forward 5'-aagatggagaagcttttgagatg-3' and reverse 5'-caccaatttatctggtaccctct-3' for "Hind III-Kpn I region"; forward 5'-agagggtaccagataaattggtg-3' and reverse 5'-cgcgaattctgcccggagtgggaaggtgg-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 (Fig. 1C; 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.

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 1,500 g for 15 min, serum component (without E.V.) was separated from the precipitates (including E.V.). The precipitates were resolved with 50 μl of PBS.

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 hr followed by goat TrueBlot®: anti-goat IgG HRP (Rockland, PA; 1:1000 dilution) at room temperature for 1 hr. 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 hr (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 hr followed by the treatment with HRP-conjugated anti-rabbit IgG (Promega; 1: 5000). The 2μl of serum samples and EV 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 hr. 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).

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 hr. After washing with 0.05 % Tween-PBS solution, each well was treated with 5 % skim milk solution at 37 °C for 1 hr 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 hr. After gentle washing, HRP substrate solution (SureBlue Reserve; SeraCare, MA) was added and developed at room temperature for 10 to 20 min. Twenty μl of 1N 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".

Statistical analysis

The statistical analyses and receiver operating characteristic (ROC) curve analysis were performed using R software (The R Foundation for Statistical Computing, Austria) and EZR (Saitama Medical Center, Jichi Medical University, Japan), which is a graphical user interface for R. Statistical significance test of relative index of serum CHL1 level between wild-type mice and *EML4-ALK* transgenic mice, and between male and female, were performed with Mann-Whitney test. We used a statistical significance level of 0.05 or smaller. Similarly, the correlation of age vs. serum CHL1 level and tumor mass vs. serum CHL1 level were indicated using Spearman's rank correlation coefficient.

Results

In vitro secretion of mCHL1 from tumor primary cells

Since tumor tissues of *EML4-ALK* transgenic mice infiltrated into the normal lung tissue, tumor tissue mass was carefully dissected from whole lung tissue. The primary lung tumor cells established from tumor tissue mass rapidly proliferated (Fig. 1A). By the western blot analysis, truncated form of mCHL1 was observed in the conditioned medium (Fig. 1B, lane "*S*"), demonstrating that mCHL1 was secreted extracellularly resulting from some processes. Several bands other than initially expected band were observed in the cellular mCHL1 (Fig. 1B, lane "*C*"). This phenomenon was also observed in the cells harvested within 24 hr after subsequent medium change (Data not shown).

Because the cellular mCHL1 seemed to be decomposed, we also confirmed whether systematic ectodomain shedding of mCHL1 occurred under our experimental condition. The cultivation of HEK293 cells expressed fluorescent protein-tagged mCHL1 (Fig. 1C and 1D) revealed that small molecular weight of mCHL1 as a truncated form mCHL1 was obviously detected in the supernatant without severe

degradation of cellular fluorescent protein-tagged mCHL1 (Fig. 1E). Also, we examined whether the shedding domain of mCHL1 existed in extracellular region of mCHL1 as previously reported [15]. Almost the fluorescent protein tag remained in the cellular component (Fig. 1F), suggesting that mCHL1 was disconnected in extracellular region. These observations were not affected by the type of fluorescent protein tag (Fig. 1D, 1E and 1F).

Detection of mCHL1 in serum from lung tumor-bearing mouse

In vitro experiment described above encouraged us to make the following measurement of circulating tumor-derived mCHL1 in *EML4-ALK* transgenic mice. The western blot analyses of serum samples indicated that although mCHL1 was detectable in both serum samples from wild-type and *EML4-ALK* transgenic mice, strong signal bands were observed in the serum samples from *EML4-ALK* transgenic mice compared with wild-type mice (Fig. 2A).

In addition, it was suggested based on the experiments using HEK293 expression system that both truncated form mCHL1 and very faint full length mCHL1 were also contained in serum. Therefore, the serum E.V including full length mCHL1, which was isolated from each serum samples by polymer-based precipitation method, and remaining serum components were analyzed, respectively. It was found that mCHL1 was present not only in serum component but also in exosome, and its abundance ratios between E.V. fraction and remaining serum component were different from each other (Fig. 2B).

Measurement of mCHL1serum levels by direct ELISA

For accurate and sensitive measurement of mCHL1 in serum samples, direct ELISA was performed using anti-mCHL1 antibody. The optimal dilution ratio was determined by preliminary experiment (Supporting Fig. 1). It was found that the reactivity was improved by using the diluted serum to some extent. As a result of measurement using 50-fold dilution sample of serum in this assay system, the relative indexes in serum samples of 40 wild-type mice were indicated as approximately 0.05 or less (Fig. 3A). In contrast to this, a lot of relative index of 40 *EML4-ALK* transgenic mouse was more than 0.05, with some samples exceeding 0.1 (Fig. 3B). The mean value of the relative index in wild-type mice was found to be 0.0187 (S. D. = 0.0105), and that in *EML4-ALK* transgenic mice was found to be 0.0633 (S. D. = 0.0586). Since the *p* value was small (1.801×10^{-9}), there was a significant difference in serum mCHL1 level between wild-type and *EML4-ALK* transgenic mice (Fig. 3C). Moreover, serum mCHL1 levels were compared between males and female of *EML4-ALK* transgenic mice, indicating that female mice had significantly higher mCHL1level than that in male mice (Fig. 3D; *TG*). On the other hand, in wild-type mice, the significant difference between male and female was not observed (Fig. 3D; *WT*). Based on the ROC curve of each serum level of mCHL1, area under the curve (AUC) and cut-off value were calculated as 0.865, and 0.0293, respectively (Fig. 3E).

Correlation between tumor mass and serum mCHL1 level

By Spearman's rank correlation analysis, a significant correlation was observed

between serum mCHL1 level and tumor mass (Fig. 4A), suggesting that the mCHL1 secretion and the elevation of its serum level occurred in a tumor-dependent manner. Interestingly, a significant correlation between serum mCHL1 level and age at week was observed in wild-type mice (Fig. 4B; *Upper column*), but no correlation was observed in *EML4-ALK* transgenic mice (Fig. 4B; *Lower column*).

4. Discussion

The typical lung cancer model mice frequently used are transplantation models in nude mice inoculated with other lung tumor cells. Unlike these typical model mice, there are advantages in *EML4-ALK* transgenic mice that can examine, for instance, the tumor growth and serum protein markers in young age. Here we first established the primary cells that can be reproducibly generated from the tumor tissues derived from *EML4-ALK* transgenic mice. The primary cell has a high proliferative ability, and is considered to represent well the state of lung tumor cells initiated by *EML4-ALK* fusion gene. There has been no report on the event that CHL1 is highly expressed in human *EML4-ALK*-positive lung tumor and is secreted from tumor cells (tissue) until now, whereas we first showed at least its truncated form is secreted extracellularly from the

primary cells. On the other hand, the gene expression omnibus (GEO) profile of the human lung cancer cell line in the NCBI database showed that some human lung cancer cell lines (*e.g.* LK2 cells) moderately expressed CHL1 molecule (data not shown). From these facts, it is suggested that modulate expression of CHL1 may be observed in lung tumor in general rather than in specific lung tumor.

Unlike the secreted form, cellular mCHL1 was detected as various fragments (Fig. 1B), implying that there are many proteases in this tumor primary cells (*e.g.* ADAM family proteases [15,22]), which are capable of cleaving mCHL1 at several domain. It was not clear whether BACE1 contributed to the cleavage of mCHL1 observed in primary lung tumor cells established in this study. According to the gene expression profile of *EML4-ALK* transgenic mice tumor tissue (Kotani *et al*., *in preparation*), the gene expression of BACE1 was not significantly different from that of normal lung tissue (Data not shown).

A truncated form mCHL1 shorter than the full length mCHL1 was detected in the culture supernatant of the primary cell. Simultaneously, in HEK293 expression experiments, almost fluorescent tag was detected in intracellular mCHL1 but not little in secreted form mCHL1 (Fig. 1E and 1F). These indicated that most secreted mCHL1 does not contain cytoplasmic region due to cleavage at the extracellular domain of mCHL1 as described in previous research [15]. On the other hand, the faint signals of full-length mCHL1 including fluorescent protein tag were detected (Fig. 1E and 1F). It was postulated that full-length mCHL1 was included in the recently noted extracellular vesicles (*e.g.* exosome), which was secreted from the tumor cell (tissue).

Based on the results of *in vitro* experiments described above, we speculated that mCHL1 secreted from tumor cells was released into the blood stream resulting in circulating whole body. By the western blot analyses of mCHL1 in sampling serum, mCHL1 was detected both in wild-type and *EML4-ALK* transgenic mouse serum (Fig. 2A). In wild-type mice, these have been considered to secrete from normal tissues such as brain tissues as reported previously [6]. By the isolation of E.V. from serum samples, it was also interesting that the mCHL1 in E.V. was strongly detected in a part of serum samples of *EML4-ALK* transgenic mouse (Fig. 2B). In general, the E.V. secreted from tumor tissue has been said to show special features compared with that from normal tissues [25], however, the functions of CHL1-expressing E.V. from lung tumor tissue detected in this study is unknown.

In order to quantitatively examine a serum level of mCHL1, a direct ELISA was carried out due to simple manipulation and the difficulty to obtain an appropriate mCHL1 capture antibody used in a sandwich ELISA. The variability observed in the data was thought to be influenced by the dependence on the size of lung tumor in *EML4-ALK* transgenic mouse (Fig. 4A). The correlation between tumor mass and mCHL1 serum levels also indicated that the increase serum level of mCHL1 was tumor-dependent phenomenon. Similar report mentioned that the correlation between the elevation of cleavage protein and tumor volume in breast cancer model mouse [26]. In addition to this, the correlation between age and serum mCHL1 level observed in wild-type mice (Fig. 4B, *Upper column*) was canceled in *EML4-ALK* transgenic mouse (Fig. 4B, *Lower column*), suggesting that the age-dependent increase in serum mCHL1 level

observed in wild-type mice could be disturbed by tumor formation. Interestingly, it was found that there was a gender difference in the extent of increase of mCHL1 serum level through lung tumor formation by unknown mechanism (Fig. 3D). There was no significant difference in the tumor mass between male and female *EML4-ALK* transgenic mice (data not shown). Considering these results, it was speculated that female tumor tissue secretes more mCHL1 molecules compared to male tumor tissues.

Previous studies have tried to find the tumor markers using mouse tumor model [26–28]. As revealed in this study, mouse lung tumor transformed by the human *EML4-ALK* fusion gene secretes mCHL1 molecules resulting in the elevation of serum mCHL1 level. Also, ROC curve analysis for *EML4-ALK* transgenic mouse revealed serum mCHL1 level was an excellent indicator for lung tumor. However, it remains unclear that the phenomenon in this study can actually be observed in human lung cancer patients. First, the tumor tissues in *EML4-ALK* transgenic mouse used in this study seems to be relatively larger than that in typical lung tumor-bearing patient, therefore, it is unknown whether an elevation of serum mCHL1 level observed in *EML4-ALK* transgenic mouse is also reproduced in human lung tumor patients. In practice, young mice (7 weeks old) with small tumors (mouse No. 38, 39, 40) showed low levels of serum mCHL1 (Fig. 3B). Similarly, it is necessary to consider that mCHL1 elevation is in the whole lung tumor patient, or is a phenomenon specific to *EML4-ALK* lung cancer patient, which is about 5 % of whole lung cancer patients. By these confirmations, we can assess the usefulness of measurement of human serum mCHL1 level as an indicator in lung tumor.

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Figure Legends

Fig. 1 Ectodomain shedding of mouse CHL1

(*A*) A representative image of primary tumor cells established from lung tumor tissue in *EML4-ALK* transgenic mouse. Bar; 100 μm. (*B*) Western blot analysis of truncated form of mCHL1 secreted into culture supernatant. Mouse CHL1 (mCHL1) was detected by using anti-mCHL1 antibody as described in "Materials and Methods". White arrows indicate the putative mCHL1 fragments. Black arrow indicates the truncated mCHL1 molecule. *C*; Cell lysate, *S*; Culture supernatant. (*C*) Expression vector of fluorescent tag-conjugated mCHL1. The fluorescent protein tags (EGFP, Azami Green, and mCherry) were inserted into the C-terminal position of mCHL1. (*D*) Expression of fluorescent tag-conjugated mCHL1 in HEK293 cells detected by fluorescent microscopy (*Upper column*; fluorescein, *Lower column*; phase contrast). EGFP (*Left*), Azami Green (AG; *Middle*), and mCherry (*Right*)-conjugated mCHL1 were expressed by above expression vector. White bar located in lower right; 100 μm. (*E*) Western blot analysis of fluorescent tag-conjugated mCHL1. Each fluorescent tag-conjugated mCHL1 expressed in the cells and secreted in culture supernatant was subjected to Western blot analysis using anti-mCHL1-HRP antibody. (*F*) Western blot analysis of EGFP or Azami Green-conjugated mCHL1. Each fluorescent tag-conjugated mCHL1 expressed in the cells and secreted in culture supernatant was subjected to Western blot analysis using anti-EGFP or anti-Azami Green antibody. *C*; Cell lysate, *S*; Culture supernatant.

Fig. 2 Detection of serum mCHL1 in wild-type and lung tumor-bearing mouse

(*A*) Western blot analysis of mCHL1 in mouse serum obtained from wild-type (WT) and *EML4-ALK* transgenic (TG) mouse. Each mouse serum (2 μl) was subjected to Western blot analysis using anti-mCHL1-HRP antibody. (*B*) Detection of mCHL1 in

extracellular vesicles (E.V.) including wild-type (WT) and *EML4-ALK* transgenic (TG) mouse serum. Each serum was treated with ExoQuick exosome precipitation solution described in "*Materials and Methods*". The precipitated E.V. fractions (*Upper column*; E.V.) and remaining serum component (*Lower column*; Serum without E.V.) were subjected to Western blot analysis using anti-mCHL1-HRP antibody. Arrows indicate the bands of mCHL1.

Fig. 3 Increase of serum mCHL1 level in lung tumor-bearing mouse

(*A*, *B*) Direct ELISA analysis of serum mCHL1. The serum collected from 40 mice of wild-type (*A*) and *EML4-ALK* transgenic mice (*B*) were applied to the direct ELISA analysis for the calculation of "Relative Index" as described in "*Materials and Methods*". (*C*) Comparison of serum mCHL1 levels between wild-type (WT) and *EML4-ALK* transgenic (TG) mouse. The serum mCHL1 level of *EML4-ALK* transgenic mouse was significantly higher than that of wild-type mouse (p=1.80x10^{-9}). (*D*) Gender differences of serum mCHL1 levels observed in *EML4-ALK* transgenic mouse. There was no significant difference of serum mCHL1 level between male and female in wild-type mouse (n.d.), in contrast, significant difference was observed in *EML4-ALK* transgenic mouse (p=9.70x10^{-4}). (*E*) ROC curve for serum mCHL1 level of wild-type and lung tumor-bearing *EML4-ALK* transgenic mouse. AUC = 0.865.

Fig. 4 Correlation between serum mCHL1 level and lung tumor mass

(*A*) Scatter plot of tumor mass versus relative index. The spearman's rank correlation coefficient was 0.692 (p = 7.97x10^{-6}). (*B*) Scatter plot of mouse age versus relative

index in wild-type (*Upper* column) and *EML4-ALK* transgenic mouse (*Lower* column). The spearman's rank correlation coefficient was 0.47 (p = 2.23 x10^{-3}) in wild-type mouse, and was 0.172 (p = 0.290) in *EML4-ALK* transgenic mouse.

Supporting Figure Legends

Supporting Fig. 1 Preliminary experiment for determining optimal serum dilution

The serum samples from wild-type (WT) and *EML4-ALK* transgenic (TG) mouse were diluted stepwise. The diluted samples were applied to ELISA plate, and then subsequent process was performed according to "*Materials and Methods*".

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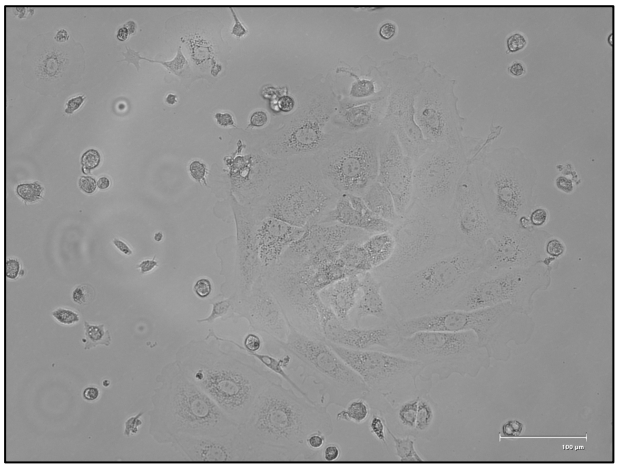
A



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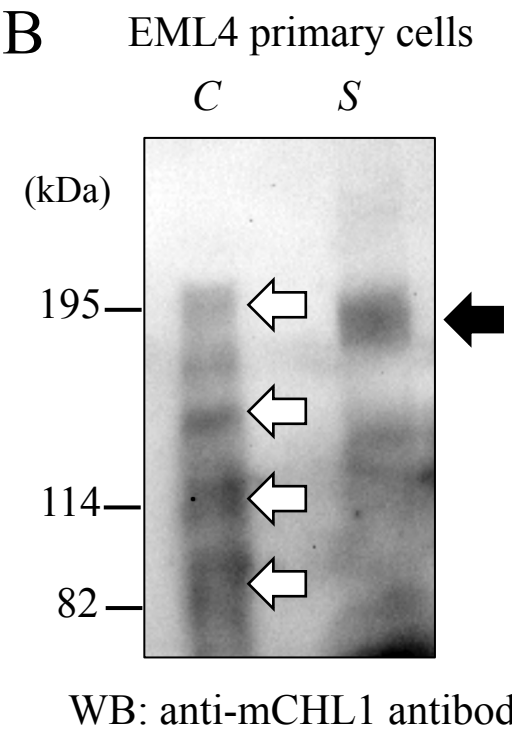

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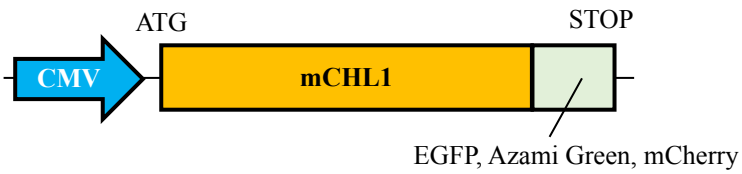

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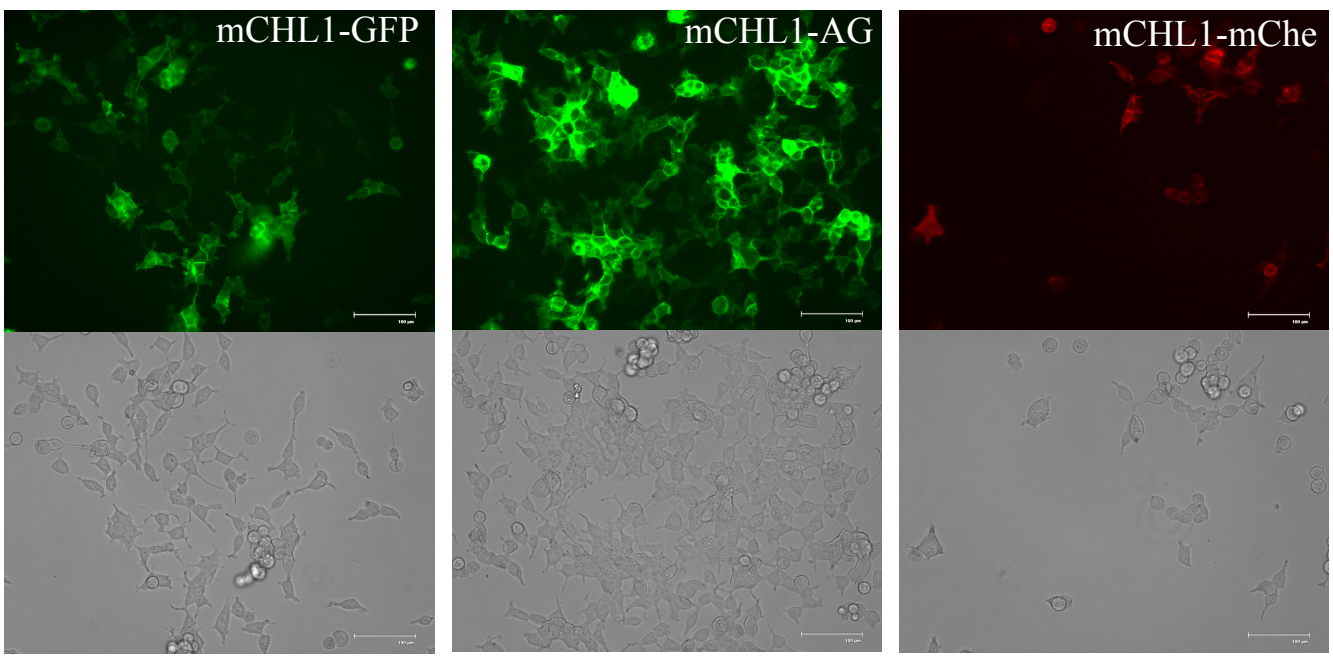
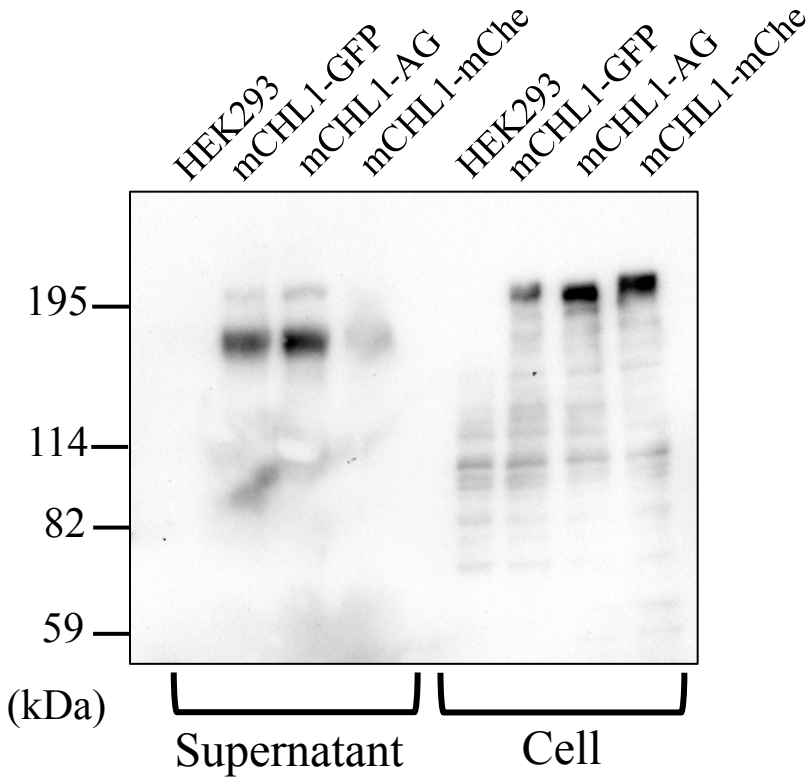


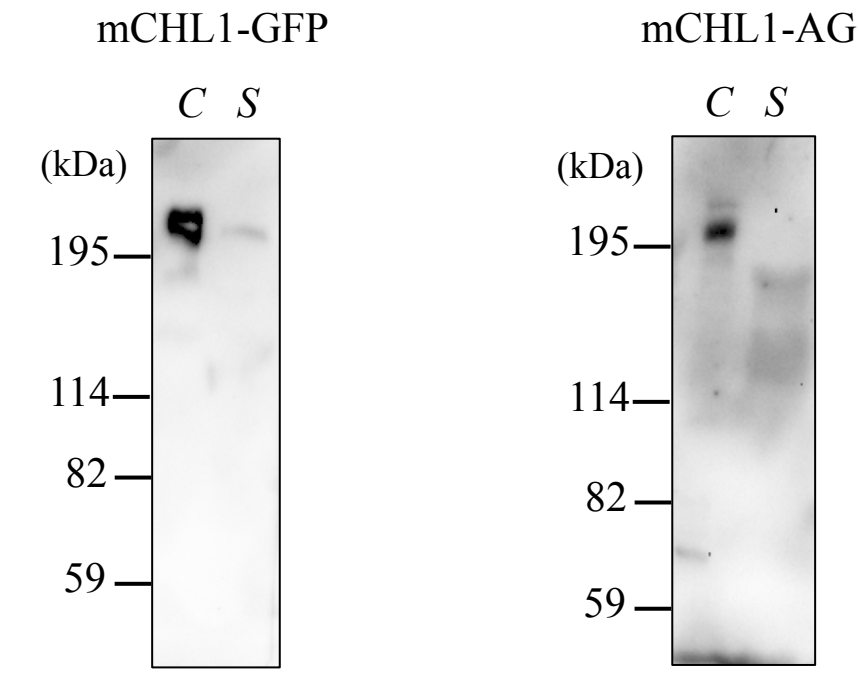
Fig. 1 Kotani N. et al.

E



WB: anti-mCHL1-HRP antibody

F



WB: anti-GFP antibody

WB: anti-AG antibody

Fig. 1 Kotani N. et al.

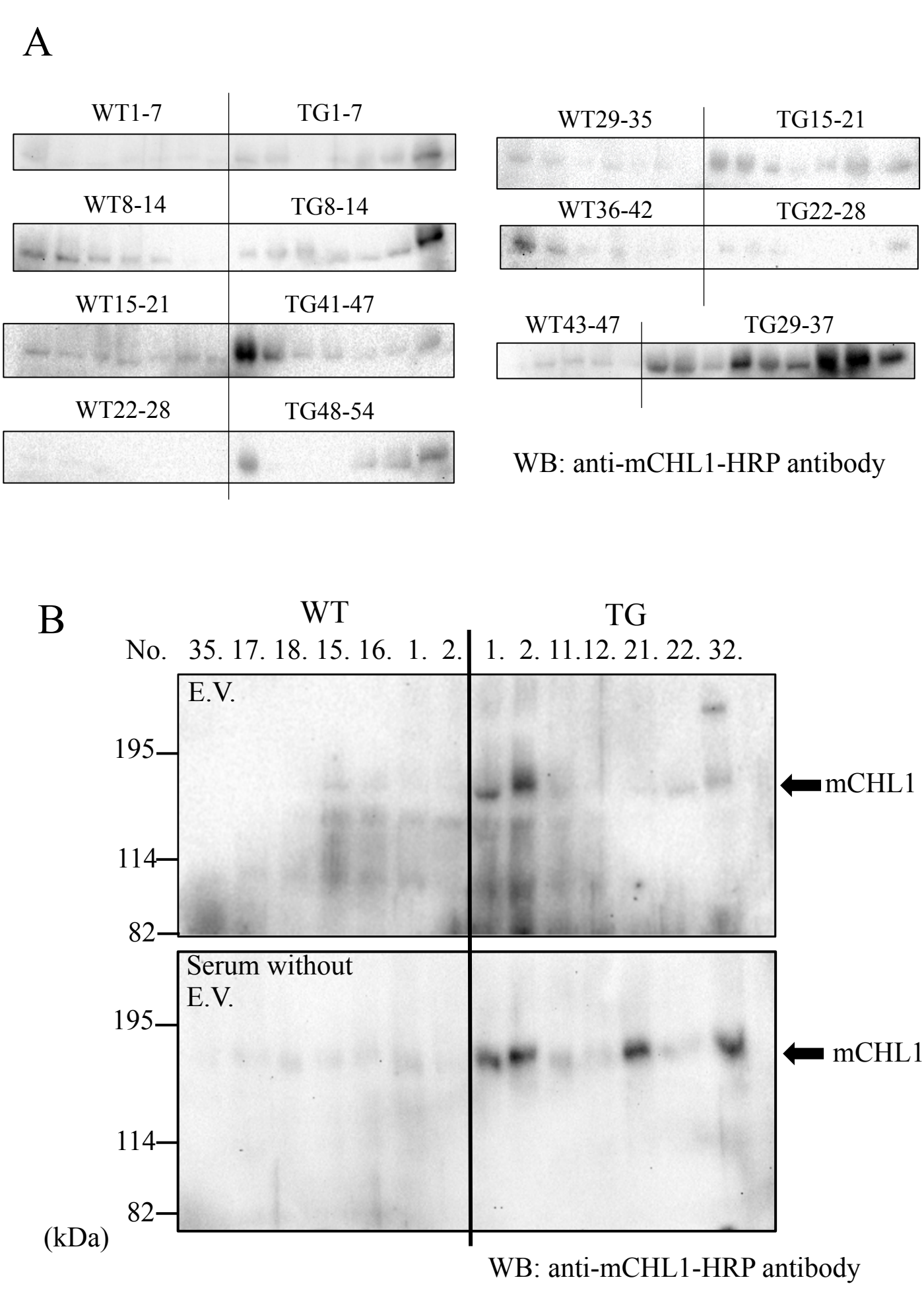


Fig. 2 Kotani N. et al.

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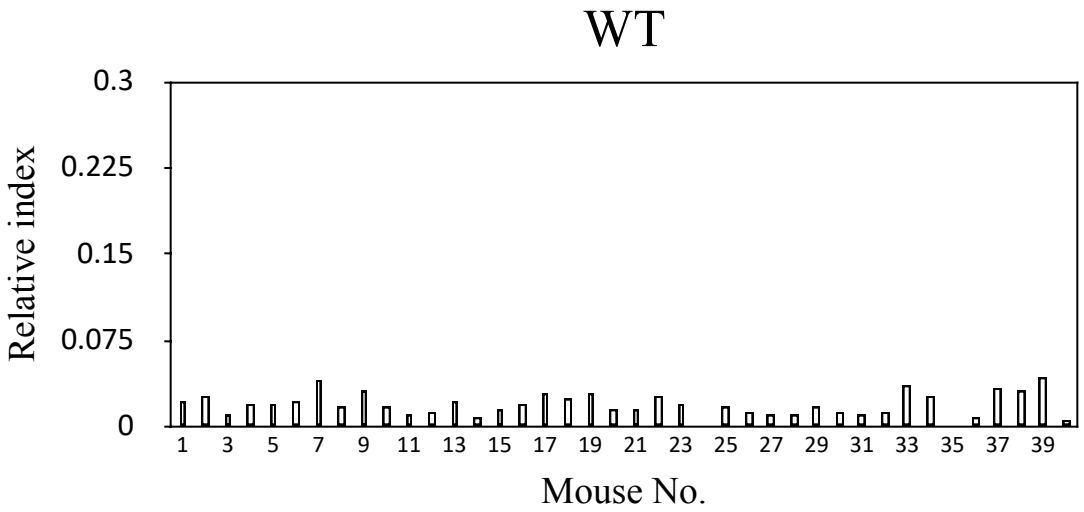

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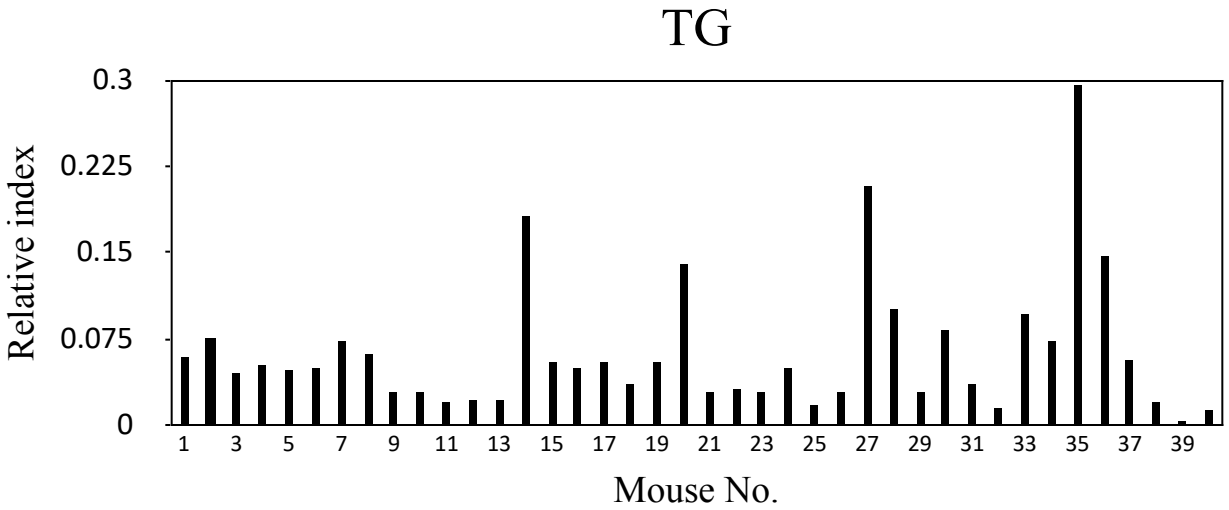

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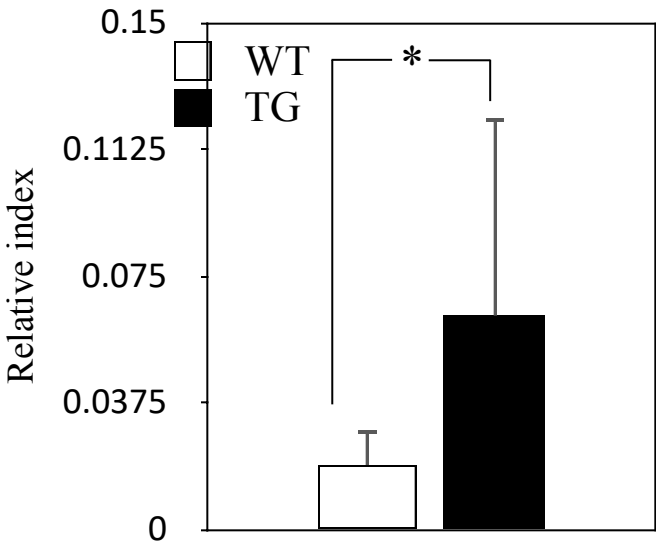

*P=1.80x10^{-9}

Fig. 3 Kotani N. et al.

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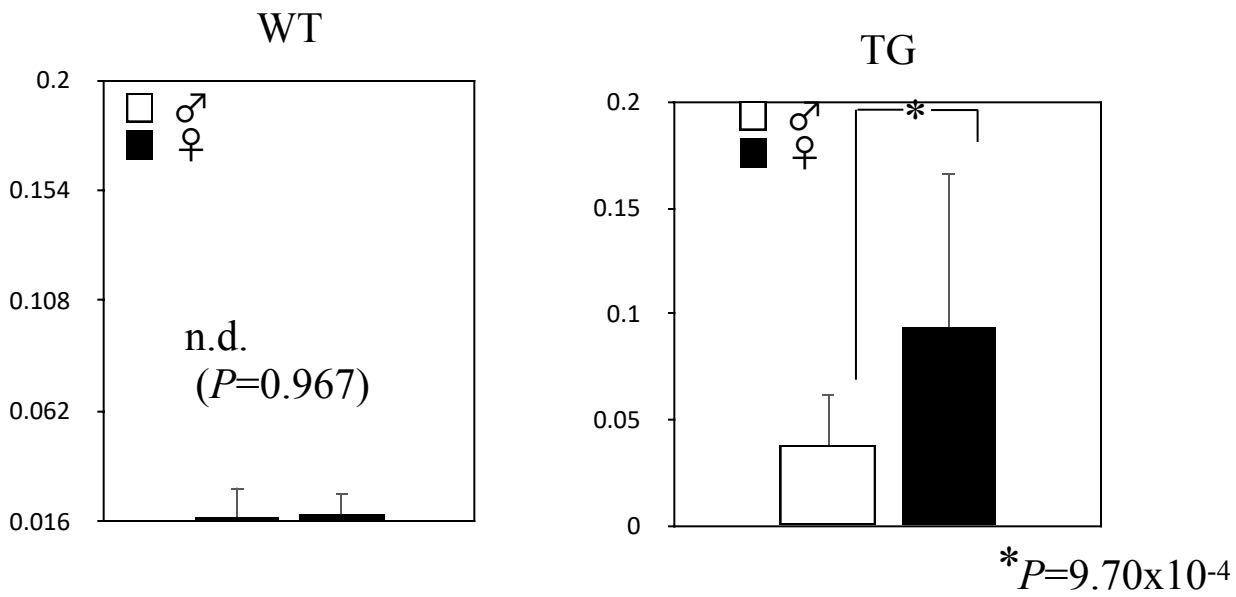

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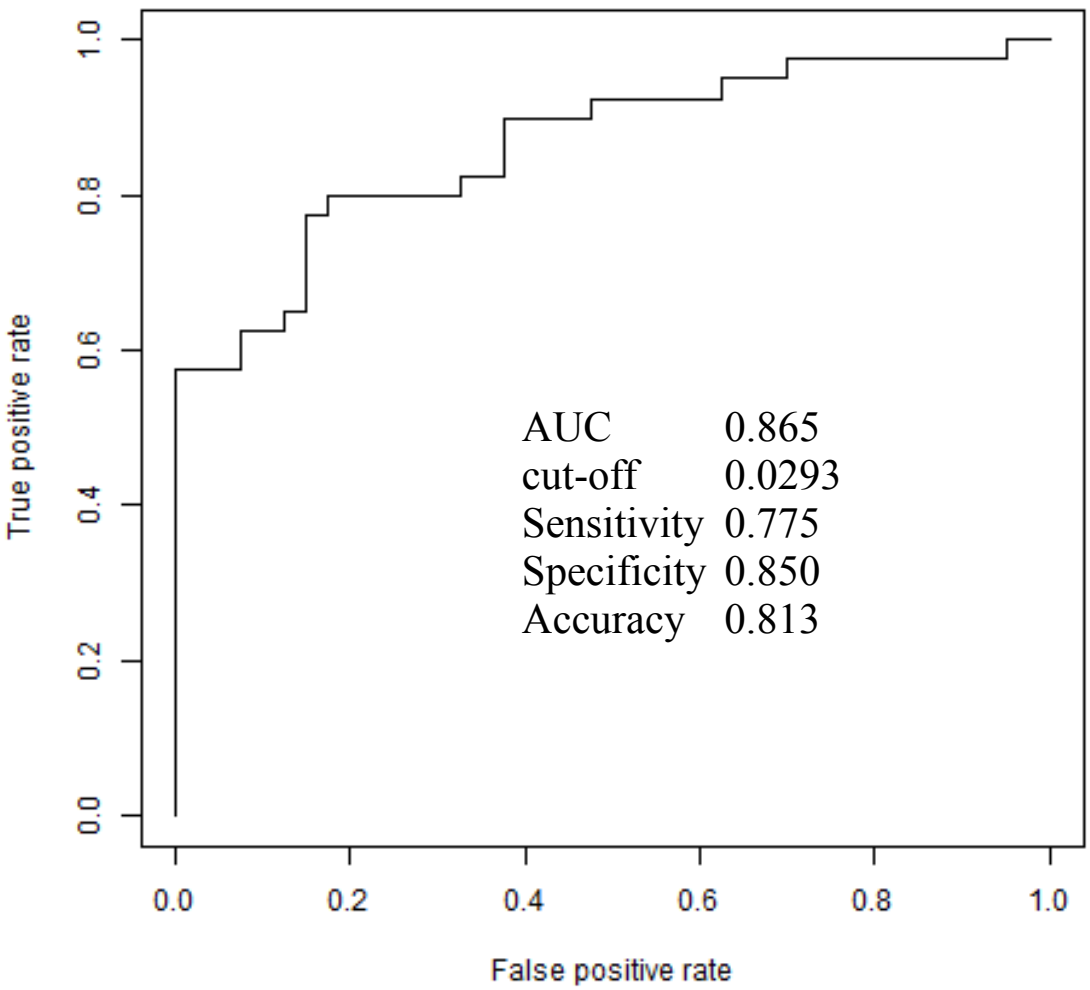
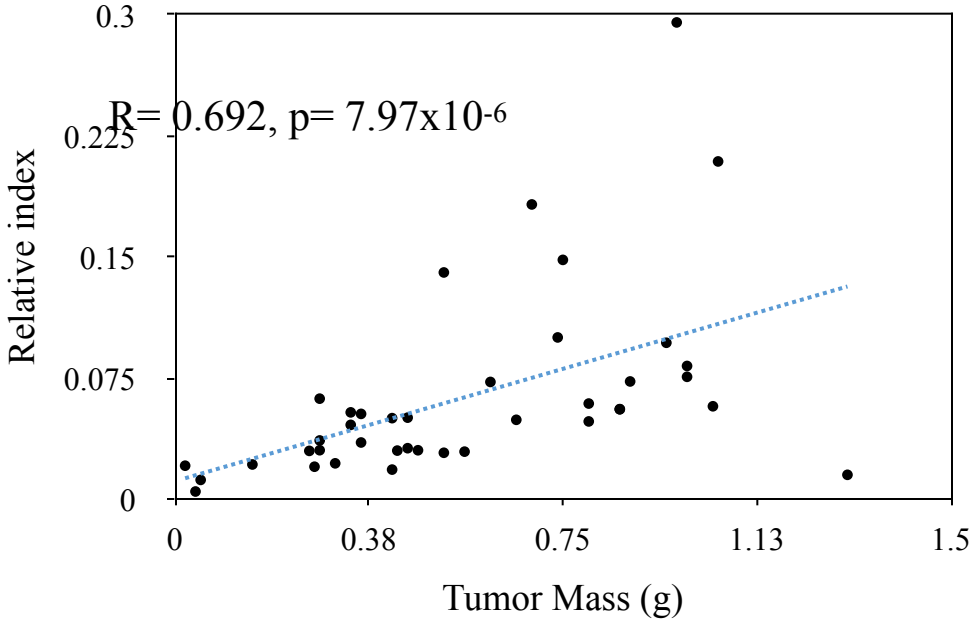


Fig. 3 Kotani N. et al.

A



B

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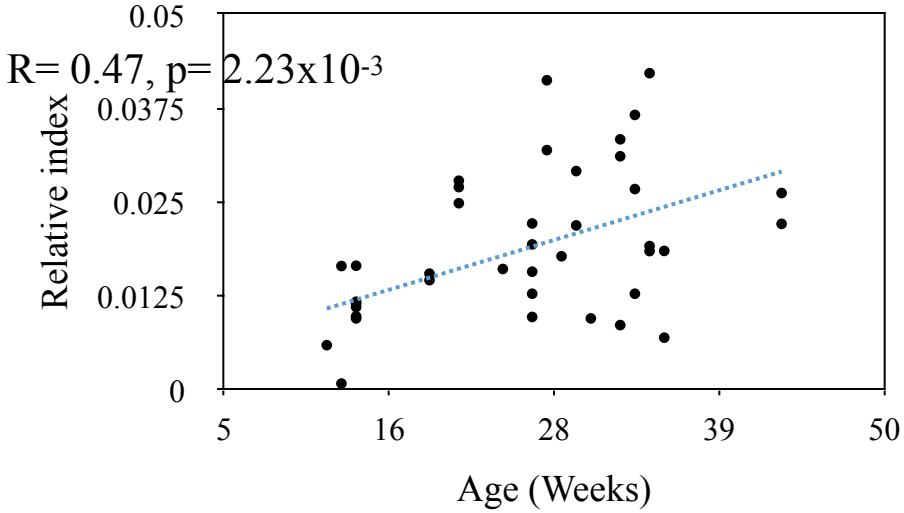

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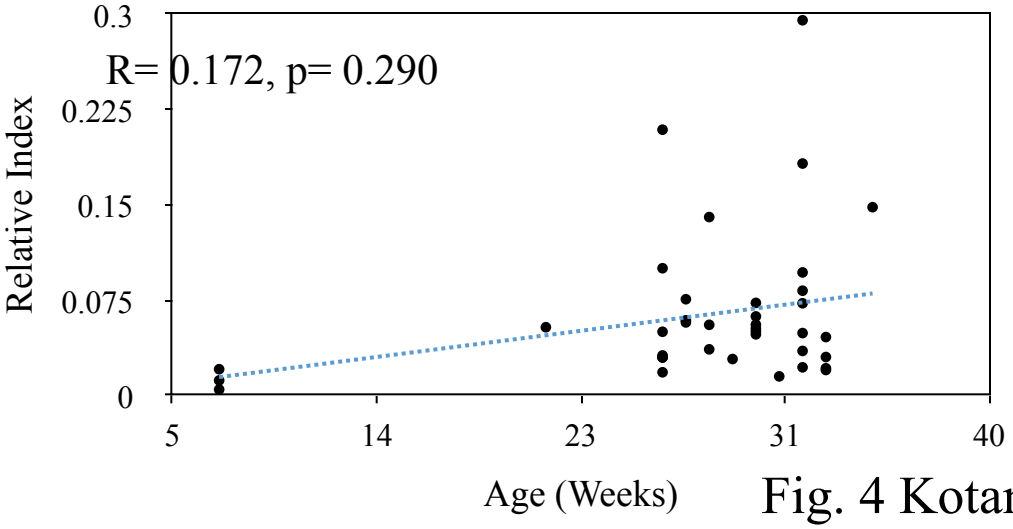

Fig. 4 Kotani N. et al.