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Corrigendum to “Chlorambucil-conjugated PI-polyamide (Chb-M’), a transcription inhibitor of the RUNX family, has anti-tumor activity against SHH-type medulloblastoma with p53 mutation” [Biochem. Biophys. Res. Commun. 620 (10 September 2022) 150–157]

Yasuzumi Matsui^{a, b}, Yohei Mineharu^{a, c}, Yuki Noguchi^b, Etsuko Yamamoto Hattori^{a, b}, Hirohito Kubota^d, Masahiro Hirata^e, Susumu Miyamoto^a, Hiroshi Sugiyama^{f, *}, Yoshiki Arakawa^{a, **}, Yasuhiko Kamikubo^{b, ***}

^a Department of Neurosurgery, Graduate School of Medicine, Kyoto University, Kyoto, 606-8507, Japan

^b Department of Human Health Sciences, Graduate School of Medicine, Kyoto University, Kyoto, 606-8507, Japan

^c Department of Artificial Intelligence in Healthcare and Medicine, Kyoto University, Kyoto, 606-8507, Japan

^d Department of Pediatrics, Graduate School of Medicine, Kyoto University, 606-8507, Kyoto, Japan

^e Department of Diagnostic Pathology, Kyoto University Hospital, Kyoto City, Kyoto, 606-8507, Japan

^f Department of Chemistry, Graduate School of Science, Kyoto University, Kyoto, 606-8502, Japan

The authors would like to apologize for any inconvenience caused.

1. A correction in Abbreviations.

Chb-M’: Chlorambucil-conjugated PI-polyamide.

2. A correction in Figure 1 legend.

(A) Clustering analysis of transcriptional levels of RUNX families indicated that the expression pattern was different among SHH, Group 3 and Group 4. (B) All the RUNX family members, i.e., RUNX1, RUNX2 and RUNX3 were upregulated in SHH-type medulloblastoma.

3. Corrections in the main text “3.2. Chb-M’ has an anti-tumor activity against medulloblastoma cell lines”.

Chb-M’ inhibited the tumor growth of Daoy cells with an IC50 of 0.812 μ M, whereas control reagents, Chb-S or Chb did not inhibit the cell growth. Chb-M’ also inhibited the growth of ONS76 cells as compared with Chb-S or Chb but the efficacy was low with an IC50 of 81.4 μ M.

4. A correction in the Figure 2.

Chb was mistakenly described as DMSO. As Chb was used as a control, the results of the experiments remain unchanged.

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* Corresponding author. Department of Chemistry, Graduate School of Science, Kyoto University, Sakyo-ku, Kyoto, 606-8502, Japan.

** Corresponding author. 54 Kawaharacho, Shogoin, Sakyo-ku, Kyoto City, Kyoto, 606-8507, Japan.

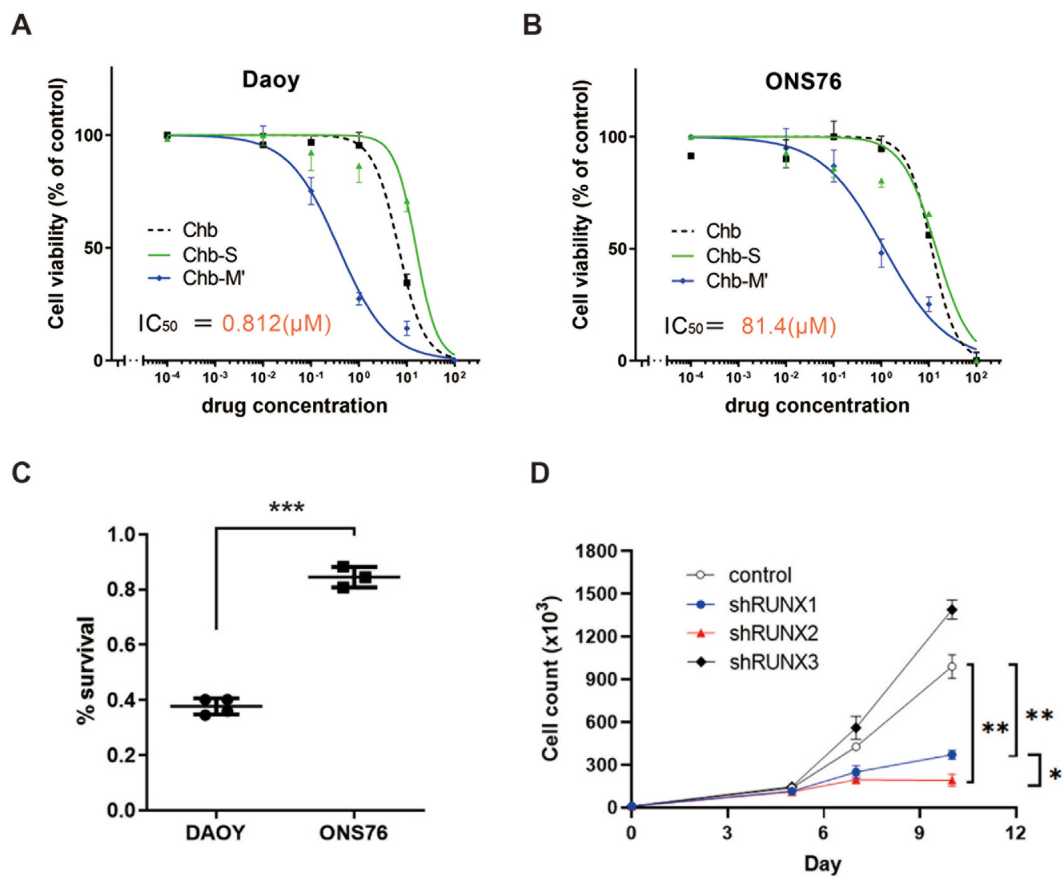
*** Corresponding author. Human Health Sciences, Graduate School of Medicine, Kyoto University, 53 Kawahara-cho, Syogoin, Sakyo-ku, Kyoto, 606-8507, Japan.

E-mail addresses: hs@kuchem.kyoto-u.ac.jp (H. Sugiyama), arakawa@kuhp.kyoto-u.ac.jp (Y. Arakawa), kamikubo.yasuhiko.7u@kyoto-u.ac.jp (Y. Kamikubo).

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Chlorambucil-conjugated PI-polyamides (Chb-M'), a transcription inhibitor of RUNX family, has an anti-tumor activity against SHH-type medulloblastoma with p53 mutation

Yasuzumi Matsui ^{a, b}, Yohei Mineharu ^{a, c}, Yuki Noguchi ^b, Etsuko Yamamoto Hattori ^{a, b}, Hirohito Kubota ^d, Masahiro Hirata ^e, Susumu Miyamoto ^a, Hiroshi Sugiyama ^{f, *}, Yoshiki Arakawa ^{a, **}, Yasuhiko Kamikubo ^{b, ***}

^a Department of Neurosurgery, Graduate School of Medicine, Kyoto University, Kyoto, 606-8507, Japan

^b Department of Human Health Sciences, Graduate School of Medicine, Kyoto University, Kyoto, 606-8507, Japan

^c Department of Artificial Intelligence in Healthcare and Medicine, Kyoto University, Kyoto, 606-8507, Japan

^d Department of Pediatrics, Graduate School of Medicine, Kyoto University, 606-8507, Kyoto, Japan

^e Department of Diagnostic Pathology, Kyoto University Hospital, Kyoto City, Kyoto, 606-8507, Japan

^f Department of Chemistry, Graduate School of Science, Kyoto University, Kyoto, 606-8502, Japan

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ABSTRACT

Malignancy of medulloblastoma depends on its molecular classification. Sonic Hedgehog (SHH)-type medulloblastoma with p53 mutation was recognized as one of the most aggressive types of tumors. We developed a novel drug, chlorambucil-conjugated PI-polyamides (Chb-M'), which was designed to compete with the RUNX consensus DNA-binding site. Chb-M' specifically recognizes this consensus sequence and alkylates it to inhibit the RUNX transcriptional activity. In-silico analysis showed all the RUNX families were upregulated in the SHH-type medulloblastoma. Thus, we tested the anti-tumor effects of Chb-M' *in vitro* and *in vivo* using Daoy cell lines, which belong to SHH with p53 mutation. Chb-M' inhibited tumor growth of Daoy cells by inducing apoptosis. The same inhibitory effect was also observed by knocking down of RUNX1 or RUNX2, but not RUNX3. Apoptosis array analysis showed that Chb-M' treatment induced phosphorylation of p53 serine 15 residues. In a subcutaneous tumor model, intratumoral injection of Chb-M' induced tumor growth retardation. Chb-M' mediated inhibition of RUNX1 and RUNX2 can be a novel therapeutic strategy for SHH-type medulloblastoma with p53 mutation.

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1. Introduction

Medulloblastoma is a pediatric malignant tumor in the cerebellum, primarily affecting infants and young children. Based on the molecular classification, it is subdivided into four major classes, including WNT, Sonic Hedgehog (SHH), Group 3, and Group 4 [1]. TP53 mutation in SHH and Myc amplification in Group 3 are well

known to have a worse prognosis [2], while whole chromosomal aberration signature (at least two of chromosome 7 gain, chromosome 8 loss, and chromosome 11 loss) predicts favorable prognosis [3]. Despite advancement of the molecular diagnosis, molecular targeted therapy has not sufficiently been developed for clinical usages [2]. Recently, some potential approaches have been reported, such as Smoothened inhibitors and Bromodomain and extraterminal bromodomain inhibitors [4,5].

Runt-related transcription factor (RUNX) regulates tumorigenesis in various types of malignancy [6]. The RUNX family consisted of RUNX1, RUNX2, and RUNX3, and they regulate the transcription of downstream genes by recognizing and targeting a core consensus DNA-binding sequence 5'-TGTGGT-3' [7]. We have developed a novel alkylating agent-conjugated low molecular weight polyamides, Chb-M' that can suppress the function of RUNX families [8,9]. Chb-M' binds specifically to the DNA binding

* Corresponding author. Department of Chemistry, Graduate School of Science, Kyoto University, Sakyo-ku, Kyoto, 606-8502, Japan.

** Corresponding author. 54 Kawaharacho, Shogoin, Sakyo-ku, Kyoto City, Kyoto, 606-8507, Japan.

*** Corresponding author. Human Health Sciences, Graduate School of Medicine, Kyoto University, 53 Kawahara-cho, Syogoin, Sakyo-ku, Kyoto, 606-8507, Japan.

E-mail addresses: hs@kuchem.kyoto-u.ac.jp (H. Sugiyama), arakawa@kuhp.kyoto-u.ac.jp (Y. Arakawa), kamikubo.yasuhiko.7u@kyoto-u.ac.jp (Y. Kamikubo).

Abbreviations

Chb	Chlorambucil
Chb-M'	Chlorambucil-conjugated Propidium Iodide polyamide
Chb-S	Chlorambucil-conjugated Scramble
DMSO	Dimethyl sulfoxide
FBS	Fetal Bovine Serum
HE	Hematoxylin-eosin
IC ₅₀	Half maximum inhibitory concentrations
PBS	Phosphate-buffered saline
PI	Propidium Iodide
PS	Penicillin-Streptomycin
SHH	Sonic hedgehog
WNT	Wingless-Type MMTV Integration Site Family

sequence of *RUNX* and reduces the *RUNX* transcriptional activity. We previously showed that Chb-M' could suppress acute myeloid leukemia and solid tumors such as gastric cancer or rhabdoid tumors [10–12].

RUNX2 was reported to be upregulated in medulloblastoma in WNT-type medulloblastoma [13]. However, *RUNX* expressions in other types of medulloblastoma remain not well studied, and no study tested the therapeutic efficacy of *RUNX* suppression in medulloblastoma. Together with the finding that *RUNX* collaborates with mutant p53 to suppress tumor cell death [14,15], we hypothesized that switching off the *RUNX* transcriptional functions can be a novel treatment strategy for medulloblastoma with p53 mutation. In this study, we tested the therapeutic efficacy of Chb-M' for this aggressive tumor with a dismal prognosis.

2. Methods

2.1. Ethics statement

All animal studies were properly conducted following the Regulation on Animal Experimentation at Kyoto University, based on International Guiding Principles for Biomedical Research Involving Animals. All procedures employed in this study were approved by Kyoto University Animal Experimentation Committee (permit number: Med Kyo 18293). All procedures in the study were performed following the approved guidelines and regulations.

2.2. Cell lines

The human medulloblastoma cell lines Daoy, ONS76 and D286 were obtained from ATCC. Daoy cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% Penicillin-Streptomycin (PS). ONS76 cells were maintained in RPMI1640 medium supplemented with 10% heat-inactivated FBS and 1% PS. HEK293T cells were purchased from RIKEN BRC, Japan and maintained in DMEM with 10% FBS and 1% PS. All cells were cultured at 37 °C, 5% CO₂.

2.3. Chb-M' synthesis

We have developed a drug, Chb-M', consisting of a PI polyamide in which the sequence recognizes the *RUNX*-binding consensus site (5'-TGTGGT-3' and 5'-TGCGGT-3'), conjugated to the alkylating agent chlorambucil (Chb). Chb-M' was synthesized as described previously [11]. Chb-Scramble (Chb-S), is also consisting of PI

polyamides targeting 5'-WGGCCW-3' sequence.

2.4. Measurement of the half maximal inhibitory concentration (IC₅₀)

For cell survival assay, cells were seeded at a density of 3×10^3 cells/well in 96-well plates. Cells were treated with the indicated concentrations of each compound in dimethyl sulfoxide (DMSO) and then incubated for 72 h. Cell viability was examined by Cell Count Reagent SF (Nacalai tesque, Japan) and Infinite® 200 PRO multimode reader (TECAN, Switzerland) according to the manufacturer's instructions. Percent inhibition curves were drawn and IC₅₀ of the indicated compounds was calculated based on the median-effect method.

2.5. Cell fertility assay

To evaluate cell proliferation, we used 1×10^4 cells/well of medulloblastoma cells were seeded in 6-well plates. For the tetracycline-inducible gene or shRNA expression, doxycycline was added to the culture at a final concentration of 3 μM. Trypan blue dye exclusion assays were performed every other day.

2.6. shRNA gene silencing

Specific shRNAs targeting human *RUNX1*, *RUNX2*, and *RUNX3* were designed and subcloned into pENTR4-H1tetOx1, CS-RfA-ETBsd, CS-RfA-ETV, and CS-RfA-ETR vectors (RIKEN BioResource Center, Japan). Nontargeting control shRNA was designed against luciferase (*sh_Luc*). The target sequences are provided in [Supplementary Table 1](#).

2.7. Apoptosis assay

Apoptosis induction was detected by Western blotting of cleaved caspase 3 and PARP. The degree of apoptosis was determined by flow cytometry analysis using APC Annexin V Apoptosis Detection Kit with Propidium Iodide (PI) (BioLegend, USA). In brief, approximately 1×10^5 cells of the indicated control and experimental groups were washed in phosphate-buffered saline (PBS), suspended in annexin-binding buffer, and then mixed with 5 μL of annexin V and 10 μL of PI Solution. The reaction mixtures were incubated for 15 min. After incubation, cells were processed for flow cytometric analysis.

2.8. Human apoptosis array

The human apoptosis array was performed using Proteome Profiler Human Apoptosis Array Kit (ARY009, R&D Systems, USA) according to the manufacturer's instructions. In brief, cells were solubilized in Lysis Buffer 17 prepared with protease inhibitors. After blocking with Array Buffer 1 for 1 h, the arrays were incubated with the protein lysate at 4 °C for overnight, and then washed and incubated with Streptavidin-HRP Detection Antibody in Array Buffer 2/3 for 1 h at room temperature. Detection was by Chemi Reagent Mix and signals were captured using ChemiDoc™ XRS positive Imager (Bio-Rad Laboratories, USA) according to the manufacturer's recommendations.

2.9. Xenograft mouse model

All animal studies were properly conducted following the Regulation on Animal Experimentation at Kyoto University, based on International Guiding Principles for Biomedical Research Involving Animals. Tumor cells (Daoy, 1×10^7 cells/200 μL) were

injected subcutaneously into female immunodeficient nude mice (BALBc/slc-nu/nu, 6–12 weeks of age, 20 g). These mice transplanted with the tumor were treated on day 7 with intratumoral injections of Chb-M' (injection of 1600 µg/kg body weight twice weekly) or an equal dose of DMSO as a control. The effect was assessed by measuring the tumor volume twice a week with a caliper, where the volume was approximated as $\pi/4 \times \text{length} \times \text{width} \times \text{height}$ and the volume ratio was calculated as compared with the volume at the treatment initiation.

2.10. Histopathological analysis

The xenograft mouse tumor models were sacrificed 63 days after tumor implantation, and the tumors were sectioned and stained using hematoxylin-eosin (HE).

2.11. Gene expression analysis

The gene expression data for 285 human medulloblastoma samples was downloaded from the Gene Expression Omnibus (GEO; GSE37382) and extracted from the GSE37382 Series Matrix file. The expression data was log2 transformed using R, followed by z-score normalization, and the heatmap was generated using the heatmap R package. The significance of differences in gene expression levels among three groups was estimated using Kruskal-Wallis test followed by Dunn's post hoc test for multiple comparisons. The gene expression data of the four medulloblastoma cell lines were downloaded from Cancer Cell Line Encyclopedia (CCLE) website (<https://data.broadinstitute.org/ccle/>) [16]. The data file used was RNA-seq expression data normalized to transcripts per million (TPM).

2.12. Statistical analysis

The results were represented as the average $\pm$ SEM values obtained from three independent experiments. Differences between experimental and control groups were assessed by an unpaired Student's *t*-test, and comparison among three groups were analyzed by Kruskal-Wallis test followed by Dunn's post hoc test. The *p* value at <0.05 was considered to be statistically significant. All the statistical analyses were performed using the Graphpad Prism 9 software (GraphPad Software, USA) and R.

3. Results

3.1. Expression analysis of RUNX families in medulloblastoma

To check the expression levels of RUNX families among different medulloblastoma subtypes, we analyzed GSE37382, the largest microarray data set of medulloblastoma. All the RUNX genes including RUNX1, RUNX2, and RUNX3 were significantly highly expressed in SHH-type medulloblastoma as compared with Group 3 ($p < 0.001$) or Group 4 ($p < 0.001$) medulloblastoma (Fig. 1A and B). The same trend was confirmed in medulloblastoma cell lines using the CCLE database. Daoy cells, ONS76 cells showed higher expressions of RUNX genes as compared with D283 cells (Group 3) or D341 cells (Group 4) (Fig. 1C). Among SHH-type medulloblastoma, a p53 mutant cell line (Daoy, p53 C242F) showed higher expressions of RUNX2 and RUNX3 than a p53 wild-type cell line (ONS76). These results indicated that suppression of RUNX genes may be a therapeutic target for SHH medulloblastoma, especially for those with p53 mutation.

3.2. Chb-M' has an anti-tumor activity against medulloblastoma cell lines

We then tested the anti-tumor effects of Chb-M', which is designed to repress the RUNXs-target gene expression, in SHH-type medulloblastoma cell lines. The half-maximum inhibitory concentrations (IC₅₀) of Chb-M' in two different medulloblastoma cell lines (Daoy and ONS76) are measured (Fig. 2A and B). Chb-M' inhibited the tumor growth of Daoy cells with an IC₅₀ of 0.812 µM, whereas control reagents, Chb-S or DMSO did not inhibit the cell growth. Chb-M' also inhibited the growth of ONS76 cells as compared with Chb-S or DMSO but the efficacy was low with an IC₅₀ of 81.4 µM. The percent survival of cells treated by Chb-M' relative to Chb-S at 1.0 µM concentration was significantly lower in Daoy than in ONS76 (Fig. 2C). These results suggested that the efficacy of Chb-M' may be higher in p53 mutant cells than p53 wild-type cells.

3.3. RUNX1 and RUNX2 dual inhibition retards proliferation of Daoy medulloblastoma cells

To confirm whether the anti-tumor effects of Chb-M' are derived from the direct inhibition of RUNX family transcriptional activity, we next tested the efficacy of knocking down RUNX genes by shRNA. Daoy cells were treated with shRNAs against RUNX1, RUNX2, RUNX3. Compared with control shRNA (sh_Luc), RUNX1 and RUNX2 suppressions inhibited the tumor growth, whereas RUNX3 suppression did not inhibit the cell growth (Fig. 2D). Suppression of tumor growth was significantly stronger in RUNX2 than RUNX1 ($p < 0.01$).

3.4. Chb-M' treatment induces p53 apoptotic pathway

We examined the mechanism of cell death in Daoy cells by Chb-M'. The Apoptotic level was evaluated by flow cytometry analysis using Annexin V and PI. Apoptotic induction was significantly higher in Daoy cells treated with Chb-M' (5 µM) after 72hrs. than those treated with DMSO (Fig. 3A). We next exploited the human apoptosis array to determine the main components of apoptosis induced by Chb-M' in Daoy cells. As a result, phosphorylated p53 at Ser15 and cleaved caspase-3 were highly expressed, while survivin was downregulated (Fig. 3B). These results suggested that Chb-M' derived RUNX suppression induced apoptosis via phosphorylation of p53 at Ser15.

3.5. Chb-M' has anti-tumor effect against medulloblastoma in a mouse xenograft model

We further investigated *in vivo* therapeutic efficacy of the Chb-M' by subcutaneously injecting Daoy cells into a mouse xenograft model. We monitored the size of tumors up to 45 days after engraftment and observed that Chb-M' suppressed the growth of medulloblastoma (Fig. 4A). Histopathological analysis showed that the tumor size was decreased (Fig. 4B).

4. Discussion

In this study, we evaluated the expression difference of RUNX among other medulloblastoma subtypes and found that RUNX1, RUNX2, RUNX3 expression was significantly higher in the SHH-type medulloblastoma, especially for p53 mutant SHH subtype, as compared with Group 3 and Group 4. Considering the hypothesis that RUNX family members have compensatory mechanism [17], a pan-RUNX inhibitor could be reasonable for a better therapeutic effect. Thereby, we investigated the efficacy of our original drug, Chb-M', which can switch off all the RUNX family members and

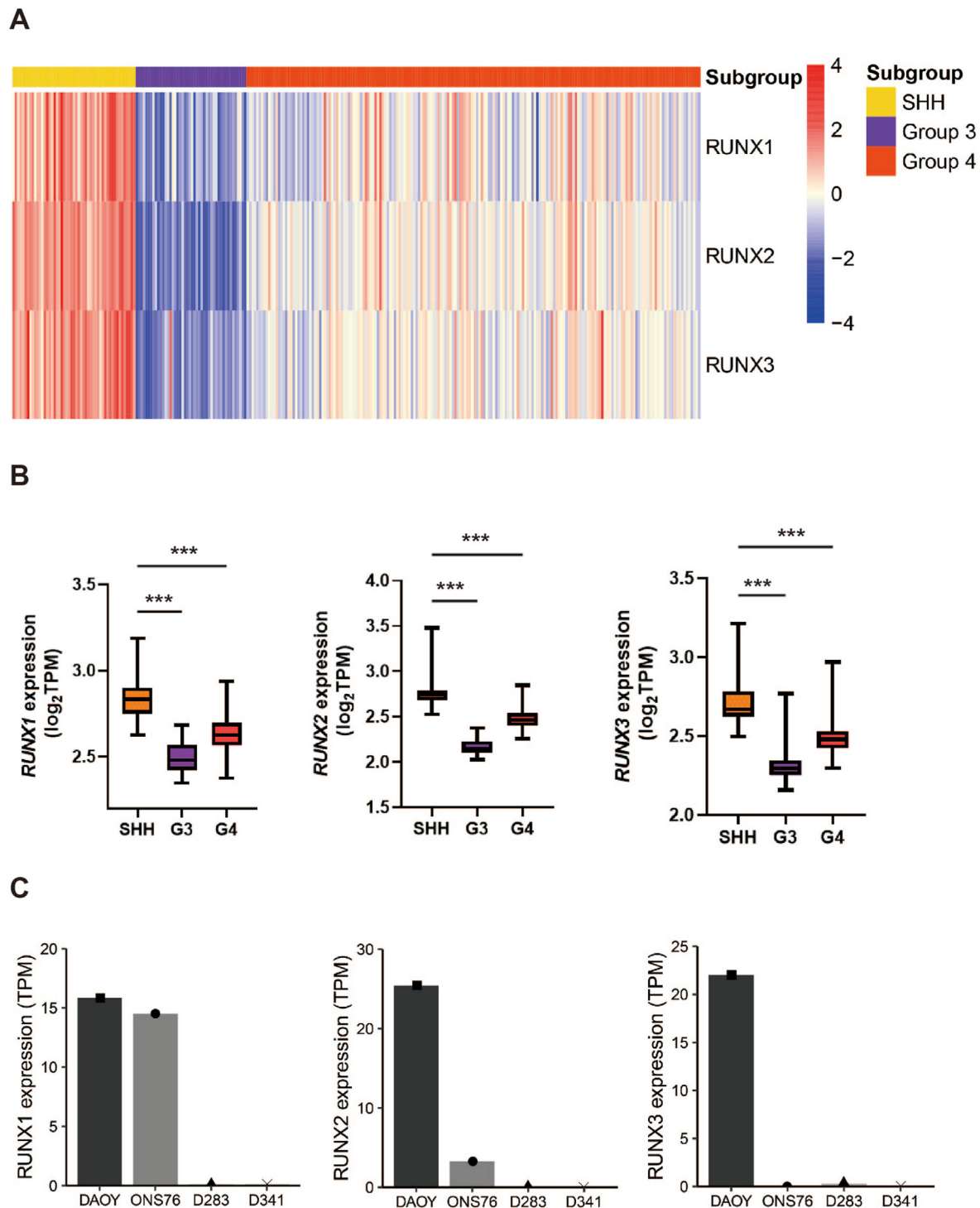


Fig. 1. Upregulation of RUNX families in SHH type medulloblastoma. (A) All the RUNX family members, i.e., RUNX1, RUNX2 and RUNX3 were upregulated in SHH-type medulloblastoma. (B) Clustering analysis of transcriptional levels of RUNX families indicate that the expression pattern is different among SHH, Group 3 and Group 4. (C) Similar pattern of expression was confirmed in human medulloblastoma cell lines. SHH type showed significantly higher expression of RUNX families than group 3 and group 4 tumors, and the expressions in SHH tumors were higher in p53 mutant (Daoy) than in wild type (ONS76). ***: $p < 0.001$ by Kruskal-Wallis test followed by Dunn's post hoc test.

control the expressions of RUNX-regulated genes. Excitingly, Chb-M' suppressed the growth of human medulloblastoma cell lines with an IC₅₀ less than 1 μ M as well as the tumor growth in mouse xenograft model.

We found that Chb-M' treatment induced apoptosis in medulloblastoma by cleaved caspase 3 increase, the significant increase of Annexin V positive cells, and an increase of p53 phosphorylation at

Ser15. This is in line with the evidence that knocking down of RUNX1 mediated stimulation of p53-dependent cell death pathway in acute myeloid leukemia [11]. RUNX1-depletion caused a remarkable induction of p53 and protein expressions of its target genes including p21, BAX, PUMA, and proteolytic cleavage of caspase-3 and its substrate PARP [11]. The prolonged half-life of p53 in RUNX1-depleted cells relative to control ones indicated that

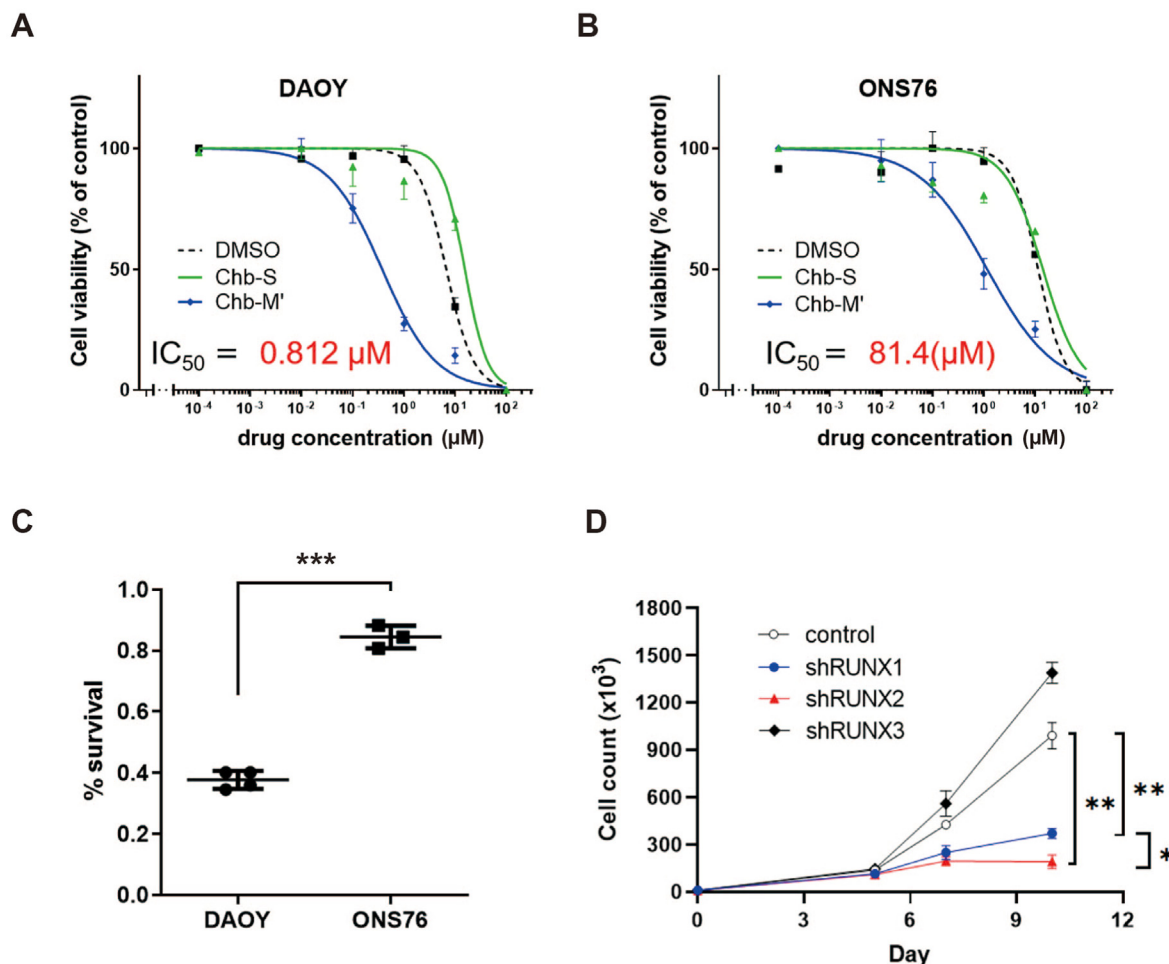


Fig. 2. Chb-M' is more effective in SHH medulloblastoma with p53 mutation than that with wild-type p53 and *RUNX1/2* inhibition prohibits proliferation of medulloblastoma cells.

To test the efficacy of Chb-M' for SHH medulloblastoma cell lines, IC₅₀ was calculated by measuring cell viability at various drug concentration. (A) Chb-M' was effective for Daoy, a p53 mutant cell line, with the IC₅₀ of 0.812 μmol/L. (B) Chb-M' was not so effective for ONS76, a p53 wild type cell line. (C) Percent survival of cells treated by Chb-M' relative to Chb-S at 1.0 μM was compared between Daoy and ONS76. (D) To test the growth suppression of Daoy by knocking down the *RUNX*s, tumor proliferation was compared between cells treated by sh-*RUNX* and those treated by controls. Both sh-*RUNX1* and sh-*RUNX2* significantly suppressed tumor cell proliferation and the effect was larger in sh-*RUNX2* than sh-*RUNX1*. On the other hand, sh-*RUNX3* did not suppress tumor cell growth. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ by Student's *t*-test.

RUNX1 negatively regulates p53 protein in acute myeloid leukemia [11]. These findings suppose that the mechanism of Chb-M' induced apoptosis in medulloblastoma is similar to those observed in acute myeloid leukemia.

Mutant p53 acts as a strong dominant-negative inhibitor against p53 family members and this is one of the mechanisms of poor prognosis in p53 mutant tumors [18]. *RUNX2* inhibits pro-apoptotic p63 in collaboration with mutant p53 in pancreatic cancer Panc-1 cells exposed to Gemcitabine, and *RUNX2* depletion up-regulates p73 and/or p63, which increases in Gemcitabine sensitivity of p53-mutated pancreatic cancer cells [15]. Thus, *RUNX* inhibition is a reasonable strategy for p53 mutated malignant cancers because it can break the inhibitory effect of mutated p53 on proapoptotic p63 or p73, and in fact, Chb-M' was effective against SHH medulloblastoma with mutant p53 rather than that with wild-type p53. In our previous report, Chb-M' was more effective for acute myeloid leukemia with wild-type p53 than that with mutant p53 [11]. This might because Chb-M' exhibited the therapeutic effect via *RUNX1* inhibition for acute myeloid leukemia [11].

There are several limitations in this study. First, we did not confirm direct involvement of apoptosis pathway including p53 as a therapeutic target of Chb-M'. Although we found elevated

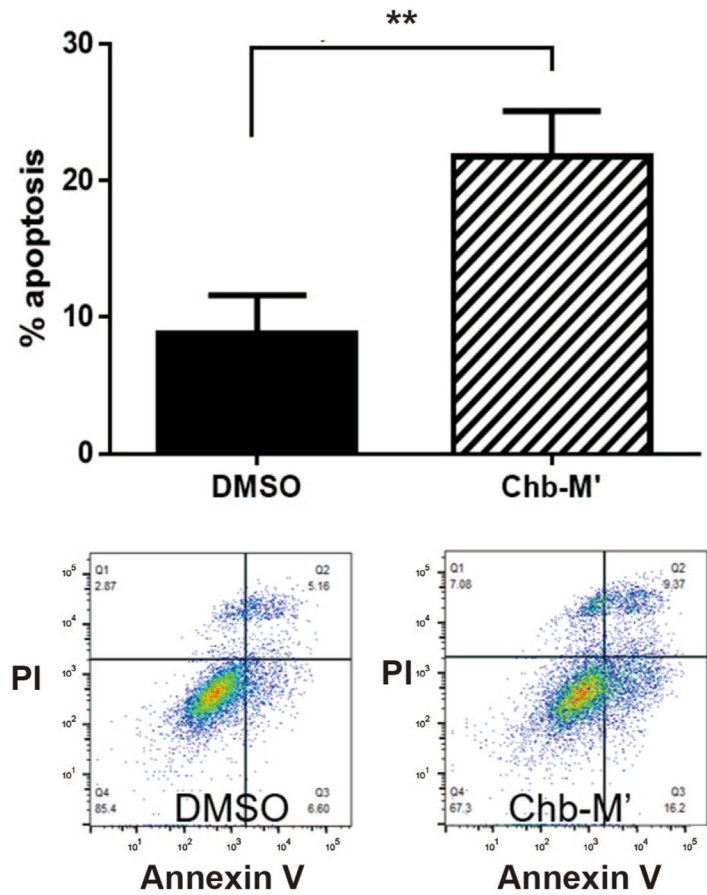
phosphorylation of p53 at Ser15 and downregulation of *survivin* in Daoy cells treated with Chb-M' in apoptosis array, validation by Western blotting or rescue experiments are lacking. We did not confirm whether apoptosis inhibition diminishes the anti-tumor effects of Chb-M'. Further study is needed to clarify specific molecules that is critical for anti-tumor effects induced by Chb-M' in medulloblastoma. Second, we did not test the anti-tumor effects of Chb-M' for WNT (*RUNX* expression is higher than SHH) or Group 4 medulloblastoma. In this study, we focused on developing a new treatment for the most aggressive type of medulloblastoma, SHH with p53 mutation, it remains unknown whether the same treatment may also be useful to other subtypes.

In conclusion, our data showed that the expressions of *RUNX*s were higher in SHH medulloblastoma especially with p53 mutation and a novel treatment strategy switching off the *RUNX*s by Chb-M' was effective for this subtype of medulloblastoma.

Declaration of competing interest

Yoshiki Arakawa reports a relationship with Philips, Otsuka, Chugai, Nihon Medi-Physics, Daiichi Sankyo, Stryker, Eisai, Japan Blood Products Organization, Ono Pharmaceutical, Taiho Pharma,

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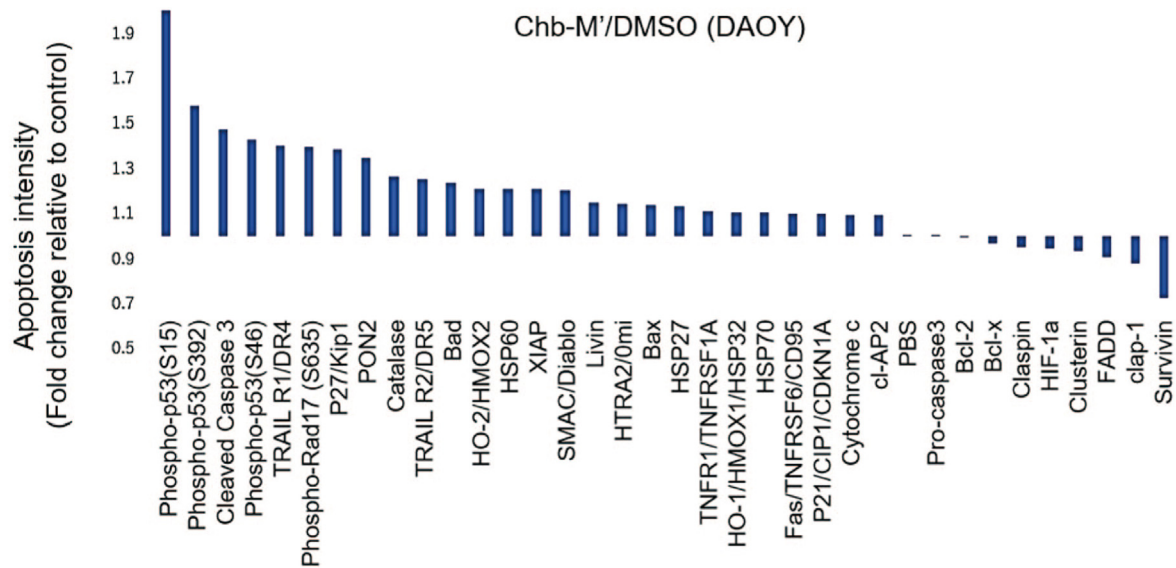


Fig. 3. Chb-M' induces apoptosis for medulloblastoma by induction of phosphorylation of p53 at Ser15. (A) Apoptosis was evaluated by flow cytometry using PI and Annexin V. Chb-M' treatment significantly increased apoptosis in Daoy. Representative dot plot was shown in the lower panel. (B) Human apoptosis array showed increased expression of phosphorylated p53 at Ser15 and cleaved caspase 3, while survivin was downregulated. **: $p < 0.01$ by Student's t -test.

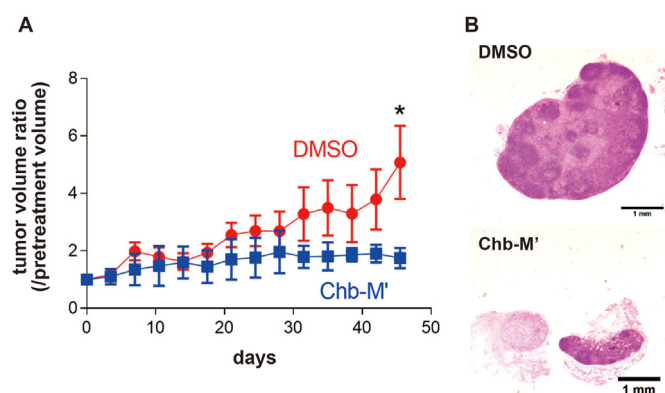


Fig. 4. Anti-tumor effect of Chb-M' in a mouse xenograft model.

(A) Human medulloblastoma xenograft model in female nude mice transplanted with Daoy cells followed by treatment with DMSO (n = 6) or with Chb-M' (n = 5). Tumor growth was significantly suppressed by Chb-M'. *: $p < 0.05$ by Student's *t*-test. (B) Histological analysis indicates a reduced tumor volume. Scale bars: 50 μ m.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbrc.2022.06.090>.

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