Antitumor effect of polyphyllin D on liver metastases of neuroblastoma

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Abstract
Purpose We previously reported that polyphyllin D, a main component of the traditional Chinese medicinal herb Paris polyphylla, exhibited anticancer effects in vitro against human neuroblastoma cells. The aims of this investigation was to examine the presence or absence of in vivo anti-metastasis effects of polyphyllin D were to establish a liver metastasis model of neuroblastoma and to evaluate the anti-metastasis effects of polyphyllin D.

Methods Subcutaneous and intraperitoneal tumors, and metastasis models were established in immune-deficient BALB/c nude and BALB/c Rag-2/Jak3 double-deficient (BRJ) mice using the human neuroblastoma cell lines IMR-32, LA–N-2, or NB-69. For evaluating polyphyllin D activity, we used a mouse model of liver metastasis with the IMR-32 cells line injected through the tail vein. We analyzed the livers number and area of liver tumors in of the phosphate buffer solution- and polyphyllin D-treated groups.

Results Liver metastasis and intraperitoneal dissemination models were successfully established in immune-deficient BRJ mice using the three human neuroblastoma cell lines. In the liver metastasis, the model of IMR-32 cells, we found that polyphyllin D suppressed both the number and total area of metastatic foci the average number of metastatic foci, average focus areas, and number of cleaved caspase-3-positive cells were significantly lower in the polyphyllin D group (p = 0.016, 0.020, 0.043, respectively).

Conclusions We developed a mouse models of neuroblastoma metastasis and demonstrated for the first time that polyphyllin D has an antitumor effect on neuroblastoma liver metastases.

Keywords Polyphyllin D · Neuroblastoma · Immune deficiency mice · Liver metastasis · Antitumor effect

Introduction
Neuroblastoma is the most common refractory solid childhood malignancy. It has been reported that the 5 year overall survival rate of high-risk neuroblastoma is less than 50% [1]. The main clinical problem in treating neuroblastoma is metastasis to the liver, bone marrow, bone, lymph nodes, and/or cranium [2]. Furthermore, metastatic lesions are often resistant to the current therapies. To overcome this issue, it is critical to develop new therapeutic agents and animal models for the evaluation of these drugs. For neuroblastoma studies, subcutaneous models [3, 4] and a few metastatic and orthotopic models have been developed [5–8].

Nude, severe combined immunodeficiency (SCID), and non-obese diabetic (NOD)/SCID mice that carry T- and/or B-cell deficiency are frequently used as tumor-bearing
models. NOD/SCID/interleukin-2 receptor gamma null (NSG) mice have an additional defect in natural killer (NK) cells [6]. In our experiments, we used BALB/c Rag-2/Jak3 double-deficient (BRJ) mice, which lack T, B, and NK cells and are an attractive model for human solid tumor engraftment [9].

Polyphyllin D is the main component of the traditional Chinese medicinal herb Paris polyphylla, which belongs to the lily family. It has been reported that polyphyllin D showed antitumor effects against liver, bladder, pancreatic, breast, and lung cancers [10–13]. We previously reported that polyphyllin D suppressed the proliferation of human neuroblastoma using IMR-32, LA-N-2, and NB-69 cells [14]. Among these lines, IMR-32 and LA-N-2 carry the MYCN gene, whereas NB-69 does not. Interestingly, upon polyphyllin D treatment, necroptosis was induced in IMR-32 and LA-N-2 cells, whereas apoptosis was observed in NB-69 cells.

In this study, we established mouse models using nude and BRJ mice with human neuroblastoma cell lines (IMR-32, LA-N-2, and NB-69), and examined whether polyphyllin D has an antitumor effect in vivo.

Materials and methods

Polyphyllin D

Polyphyllin D, diosgenyl-alpha-L-rhamnopyranosyl-(1→2)-[alpha-L-arabinofuranosyl-(1→4)-beta-D-glucopyranoside [15] (Fig. 1), was purchased from Funakoshi Co. Ltd. (Tokyo, Japan), dissolved in dimethyl sulfoxide (100 mg/mL), and stocked at −80 °C for up to 2 weeks. The stock solution was further diluted with phosphate-buffered saline (PBS) to adjust the working concentration before it was administered to mice.

Cell culture

The human neuroblastoma cell lines IMR-32, LA-N-2, and NB-69 were obtained from Sumitomo Dainippon Pharma (SD Pharma, Osaka, Japan). IMR-32 cells were cultured in Eagle’s minimal essential medium (EMEM; SD Pharma) supplemented with 1% non-essential amino acids (SD Pharma), 2 mM glutamine, and 10% fetal bovine serum (FBS). LA-N-2 cells were cultured in a 1:1 mixture of Ham’s F-12 (SD Pharma) and EMEM supplemented with 2 mM glutamine and 10% FBS. NB-69 cells were cultured in RPMI 1640 supplemented with 2 mM glutamine and 15% FBS. All cells were incubated at 37 °C in 5% CO2 and were passaged using 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) (Life Technologies, Carlsbad, CA, USA) at 70–80% confluency. All cell lines were mycoplasma-free.

Establishment of a neuroblastoma liver metastasis model animal experiments

Four-week-old male BALB/c nude mice were purchased from Charles River Laboratories Japan INC (Kanagawa, Japan). 6 week-old male BRJ mice [9] were raised in a specific pathogen-free barrier facility at the Education and Research Facility of Animal Models for Human Diseases at Fujita Health University. Before the experiments, the mice were allowed to acclimatize for at least 1 week. The animals were fed sterile laboratory food and water. All research and animal care procedures were approved by our institutional committee.

Each BALB/c nude mouse was subcutaneously injected with 3×10^6 cells in 100 μL of FBS-free medium through the left hind leg using a 26-gage needle. The same number of cells were injected intraperitoneally in BALB/c nude and BRJ mice. BALB/c nude and BRJ mice were used for the metastasis model. A total of 1×10^6 cells in 100 μL of FBS-free medium were injected through the tail vein of each mouse using a 27-gage needle.

In the liver metastasis model, 200 μL of PBS or polyphyllin D (2 mg/kg) were intraperitoneally administered daily, 1 week after the cell injections. The number of mice in the PBS and polyphyllin D groups was five and seven, respectively. 4 weeks later, all the mice were sacrificed, perfusion-fixed, and used for analysis.

Fig. 1 Chemical structural formula of polyphyllin D. The molecular weight is 855.02 and the molecular formula is C_{44}H_{70}O_{16}
Evaluation of the anti-metastasis effects of polyphyllin D histopathology

For evaluating the anti-metastasis effects of polyphyllin D, we used the BRJ mouse model of liver metastasis with IMR-32 cells and analyzed the liver weight and liver tumors number and area. To evaluate microscopic metastasis, harvested organs were fixed in a 4% paraformaldehyde solution at 4 °C for 48 h, embedded in paraffin, and sectioned into 3 µm-thickness at the maximum cut surface. After staining with hematoxylin and eosin (H&E), the average number of metastatic foci and nuclear divisions in each of 20 randomly selected high-power microscopic fields were determined at 400× magnification.

Paraffin-embedded liver neuroblastoma blocks were used for immunohistochemical analysis. The samples were deparaffinized, heated in citrate buffer solution (pH 6.0) or EDTA (pH 8.0) to unmask antigens, and washed with PBS. The primary and secondary antibodies used were anti-Ki-67 (1:100, Gene Tex, North America, USA) or cleaved caspase-3 (1:100, Cell Signaling Technology, Danvers, USA), and Simple Stain MAX PO (1:100, Nichirei Biosciences Inc., Tokyo, Japan), respectively. After slide pictures were taken using a TOCO virtual slide scanner (PATH IMAGING Inc., Tokyo, Japan), they were assessed with the help of Image J (National Institutes of Health, Bethesda, Maryland, USA) [16, 17]. The tumor number and metastatic foci per liver area were counted using Ki-67 staining. Ki-67-positive cells per tumor area were also evaluated in the 16–20 foci. The apoptotic cell number was determined by cleaved caspase-3 positivity per tumor area in the 16–20 foci.

Statistical analysis

All statistical analyses were performed using EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a modified version of R commander (version 1.6–3) designed to add statistical functions frequently used in biostatistics [18]. Clinical and histopathological data were analyzed using the t test or Kruskal–Wallis test. p values of 0.05 or less were considered statistically significant.

Results

Creation of mice models

We previously demonstrated that polyphyllin D exerted antitumor effects against human neuroblastoma cell lines in vitro [14]. To determine the antitumor effect of polyphyllin D in vivo, we constructed mouse models of neuroblastoma tumors.

Initially, we started experiments using BALB/c nude mice in which IMR-32 and LA-N-2 cells formed subcutaneous tumors, but NB-69 did not (Table 1). We also tried using intraperitoneal or tail vein injection models. However, neither supported tumor formation.

In contrast, in BRJ mice, which lack T, B, and NK cell functions, metastatic liver tumors were efficiently generated through tail vein injection of IMR-32, LA-N-2, or NB-69 cells (Table 1, Fig. 2A, B). Similarly, mice injected intraperitoneally developed disseminated tumors (Table 1). In the pilot study, metastasis foci were analyzed when abdominal distension became obvious, which was 7–8 weeks after the cell injection. Metastatic foci were found in the lungs, kidneys, or pancreas, but not in the heart, brain, spleen, bone marrow, or bones (Fig. 2C). Among these models, the tail vein injection model using IMR-32 cells was chosen for the evaluation of polyphyllin D activity. With this model, we evaluated the mice 5 weeks after the cell injection, because at this time point, metastatic foci became reasonably large, were not fused with each other, and were easily countable.

Suppression of liver metastasis by polyphyllin D

We intraperitoneally administered polyphyllin D a week after IMR-32 cells were injected and evaluated liver tumors. Regarding liver weights, we observed a significant difference between the PBS and polyphyllin D groups, but no

### Table 1 Neuroblastoma mouse models

| cell line | BALB/c nude mice | BRJ mice |
|-----------|------------------|----------|
|           | subcutaneous     | intraperitoneal | tail vein | intraperitoneal | tail vein |
| IMR-32    | 3/4              | 0/3       | 0/3       | 2/2             | 4/4       |
| LA–N-2    | 3/4              | 0/3       | 0/3       | 2/2             | 4/4       |
| NB-69     | 0/4              | 0/3       | 0/3       | 2/2             | 2/2       |

BRJ BALB/c Rag-2/Jak3 double-deficient. Neuroblastoma mouse models with IMR-32, LA-N-2, and NB-69 cells. “Subcutaneous,” “intraperitoneal,” and “tail vein” denote the sites of cell injection. The numbers of treated mice and those with successful tumor formation are indicated.
There were no obvious adverse effects or poor physical conditions observed in the polyphyllin D group. Nevertheless, the average number of metastatic foci visualized and identified by Ki-67 staining (Fig. 3A) was 70.7 and 33.3/cm² in the PBS and polyphyllin D groups, respectively (2.1-fold lower in the polyphyllin D group) (Fig. 3B) p value, 0.016. Similarly, average focus areas were twofold lower in the polyphyllin D group with 4.62 and 2.27% in the PBS and polyphyllin D groups, respectively (Fig. 3C) p value, 0.020. In contrast, there was no significant difference in the number of nuclear divisions between the two groups (Fig. 4A, B) or in comparison with that of Ki-67-positive cells (Fig. 5A, B).

We also found a significant difference in the number of cleaved caspase-3-positive cells per tumor area, with 171.6 and 94.5 cells/mm² in the PBS and polyphyllin D groups, respectively (p value, 0.043, Fig. 6A, B).

### Discussion

In this study, we established reliable mouse models for evaluating the anticancer effects of drugs using BRJ mice and three independent neuroblastoma cell lines (Table 1). In these models, intraperitoneal and liver metastatic tumors grew in BRJ mice, but only some subcutaneous tumors were observed in BALB/c nude mice. This difference may be attributed to the difference in the extent of immune deficiency between the two strains, BRJ mice do not have NK cells, unlike BALB/c nude mice.

Studies on a previous model showed that after an incubation period of 12 weeks post cell injection, metastases were found in the liver, adrenal glands, bones, and lungs [7]. In our model, we analyzed liver metastasis 5 weeks after tail vein injection of IMR-32 cells. This incubation period was used to count the liver foci. Liver tumors harvested 7 or

### Table 2 Effect of polyphyllin D on liver and mouse weights

|                  | PBS (n=5) | Polyphyllin D (n=7) | p value |
|------------------|-----------|---------------------|---------|
| liver weight (g) | 1.91 ± 0.14 | 1.61 ± 0.20         | 0.019*  |
| mouse weight (g) | 25.92 ± 1.81 | 25.24 ± 1.03        | 0.43    |

Data are shown as mean ± standard deviation values. Quantitative analysis with t test *p < 0.05
8 weeks after the injection became enlarged and fused with each other, which made them very difficult to count; thus, we did not analyze the organs at the time point of 12 weeks. Nevertheless, we observed metastasis in the lung, kidney, and pancreas, supporting the notion that the current model, at least in part, mimics the metastatic features of neuroblastoma.

In our previous in vitro experiment, we reported that polyphyllin D induces necroptosis rather than apoptosis in IMR-32 cells [14]. Consistently, the number of cleaved caspase-3-positive cells in the metastatic foci was significantly lower in the polyphyllin D group than in the PBS group (Fig. 6). These results indicate that polyphyllin D might have caused necroptosis rather than apoptosis in IMR-32 liver tumors in vivo, as observed in vitro.

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**Fig. 3** Evaluation of IMR-32 liver tumors in the PBS (n=5) and polyphyllin D (n=7) groups. A Liver tumors visualized by Ki-67 staining in the PBS and polyphyllin D groups (scale bar=5 mm). B Number of liver metastatic foci per liver area in the PBS and polyphyllin D groups. C Tumor area of liver metastatic foci per liver area in the PBS and polyphyllin D groups. Quantitative analysis with t test. *p < 0.05

**Fig. 4** IMR-32 liver tumors with H&E staining in the PBS and polyphyllin D groups. A Typical nuclear divisions in both groups are indicated by arrows (scale bar=50 µm). B Number of nuclear divisions in the PBS and polyphyllin D groups. Quantitative analysis with t test.
We also found significant differences in liver weight, the number of liver metastasis foci, and the total foci areas between the PBS- and polyphyllin D-treated groups for the first time, demonstrating that polyphyllin D has an antitumor effect against liver metastases of neuroblastoma (Table 2, Fig. 3B, C). Additionally, these data suggest that polyphyllin D suppresses two independent steps of metastasis: the residing and subsequent proliferation phases in the liver.

Our experiments have several limitations. First, we evaluated liver metastases with a single cell line IMR-32 and did not investigate whether or not polyphyllin D is effective for other metastases and improves cumulative survival in other cell lines. Second, the study was performed in mice carrying human cell, therefore, the actual effect in humans needs to be studied in future experiments.

Fig. 5 IMR-32 liver tumors with Ki-67-staining in the PBS and polyphyllin D groups. A Liver tumors with Ki-67 stain in both groups (scale bar = 200 µm). B Ki-67-positive cells per liver tumor area in both groups. Quantitative analysis with t test

Fig. 6 IMR-32 liver tumors with cleaved caspase-3-staining in the PBS and polyphyllin D groups. A Liver tumors with cleaved caspase-3 stain in both groups (scale bar = 200 µm). B Cleaved caspase-3-positive cells per liver tumor area in both groups. Quantitative analysis with t test. *p < 0.05

Conclusions

Creating animal models for rare diseases such as pediatric solid tumors is of utmost importance. Here, we developed subcutaneous tumor, intraperitoneal tumor, and liver metastasis mouse models for neuroblastoma and showed for the first time that polyphyllin D has an antitumor effect on liver metastases of neuroblastoma. Further experiments are required to elucidate the precise mechanisms of in vivo tumor suppression.

Author contributions All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by YK, SW, AN, TT, AN, MS, NA, SO, TT, MM, TY, ML, and TS. The first draft of the manuscript was written by YK and all...
authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Availability of data and material The data that support the findings of this study are available from the corresponding author, Yasuhiro Kondo, upon reasonable request.

Declarations

Conflict of interest All authors declare that they have no conflict of interest.

Ethical approval This study was approved by our institutional review board (AP19142, DP19019).

Informed consent Informed consent is not required for this study.

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