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ORIGINAL ARTICLE

Enhanced metastatic growth after local tumor resection in the presence of synchronous metastasis in a mouse allograft model of neuroblastoma

Maho Inoue[1,2] · Yoshiki Tsuchiya[1] · Nobuya Koike[1] · Yasuhiro Umemura[1] · Hitoshi Inokawa[1] · Yuichi Togashi[2] · Junnosuke Maniwa[2] · Mayumi Higashi[2] · Shigehisa Fumino[2] · Tatsuro Tajiri[2] · Kazuhiro Yagita[1]

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Abstract

Purpose We investigated how local tumor resection affects metastatic lesions in neuroblastoma.

Methods MYCN Tg tumor-derived cells were injected subcutaneously into 129^{+Ter}/SvJcl wild-type mice. First, the frequency of metastasis-bearing mice was investigated immunohistochemically (metastatic ratio) at endpoint or post-injection day (PID) 90. Second, the threshold volume of local tumor in mice bearing microscopic lymph node metastasis (mLNM) was investigated at PID 30. Finally, local tumors were resected after exceeding the threshold. Mice were divided into local tumor resection (Resection) and observation (Observation) groups, and the metastatic ratio and volume of LNM were compared between the groups at endpoint or PID 74.

Results The metastatic ratio without local resection was 88% at PID 78–90. The threshold local tumor volume in the mice with mLNM was 745 mm^3 at PID 30, so local tumors were resected after exceeding 700 mm^3. The metastatic ratio and LNM volume were significantly greater in the Resection group ($n=16$) than in the Observation group ($n=16$) (94% vs. 38%, $p<0.001$; 2092 ± 2310 vs. 275 ± 218 mm^3, $p<0.01$; respectively) at PID 50–74.

Conclusion Local tumor resection might augment the growth of synchronous microscopic metastases. Our results provide insights into the appropriate timing of local resection for high-risk neuroblastoma.

Keywords Neuroblastoma · Metastasis · Local tumor resection · Concomitant tumor resistance · MYCN transgenic mouse

Introduction

Neuroblastoma is the most common solid extracranial childhood tumor. The prognosis of high-risk neuroblastoma (HR-NBL) patients with metastatic lesions at the time of the diagnosis remains poor despite advances in multimodal therapy [1–3]. The first recurrence is typically distant organ metastasis rather than locoregional recurrence [3]. Metastatic lesion growth is largely responsible for the loss of life in patients with HR-NBL.

Recently, evidence that surgery for local tumors might promote postoperative growth and extent of metastasis has been building in adult cancer cases [4, 5]. However, little is known about how local tumor resection affects metastatic lesions in NBL patients. While complete resection of local tumors is reported to have little effect on the overall survival of HR-NBL patients in some clinical cohorts [6], other studies have reported that complete resection improved the survival [7, 8]. Different characteristics of patients and intratumor heterogeneity may obscure the true effects of surgery on the overall survival in clinical studies [9]. To evaluate the postoperative progression of metastasis in a more reliable way, an animal model with a high prevalence of metastatic lesions and resectable local tumors would be useful.

The MYCN transgenic (Tg) mouse is a spontaneous NBL model in which human MYCN expression is driven by the rat tyrosine hydroxylase promoter in neural crest cells [10].

✉ Maho Inoue
maho-i@koto.kpu-m.ac.jp

[1] Department of Physiology and Systems Bioscience, Kyoto Prefectural University of Medicine, 465 Kawaramachi-Hirokoji, Kamigyo-ku, Kyoto 602-8566, Japan

[2] Department of Pediatric Surgery, Kyoto Prefectural University of Medicine, 465 Kawaramachi-Hirokoji, Kamigyo-ku, Kyoto 602-8566, Japan

Although MYCN Tg mice are widely used for research, distant metastasis in these mice is not as frequent as that observed in humans [11, 12]. Furthermore, complete resection of local abdominal tumors in MYCN Tg mice is difficult because of the high postoperative mortality due to local invasion to other organs and encasement of the aorta.

In the present study, we established a mouse allograft model showing a high frequency of metastatic spread using a method for primary culture of MYCN Tg mouse-derived tumor cells that was reported recently [13]. Using this allograft model, we examined the effect of local tumor resection on metastatic growth. Our analyses will help further our understanding of the relationship between tumor resection and metastasis in advanced NBL.

Materials and methods

Ethics statement

All experiments reported here conformed to the guidelines for care and use of laboratory animals established by the University Committee on Use and Care of Animal at the Kyoto Prefectural University of Medicine.

Animals

MYCN Tg mice were created by Prof. William A. Weiss (University of California, San Francisco, CA, USA) [10]. The MYCN Tg mice on a 129^{+Ter}/SvJcl background were kindly gifted by Prof. Kenji Kadomatsu, Nagoya University [14], and maintained in our animal facility, where they were housed under the specific pathogen-free conditions.

Primary culture of tumorspheres from primary tumors of MYCN transgenic mice

We modified the procedure reported by Dongliang et al. [13] and Peng Huang et al. [15]. Tumors derived from MYCN Tg homozygote mice were dissected and mechanically dissociated with scissors, followed by enzymatic digestion using 0.25% trypsin (NACALAI TESQUE, INC. Kyoto, Japan) for 20 min with pipetting every 5 min. The digestion was stopped by adding the PrimNeuS medium reported previously*. After 25 µg/mL DNase Type I (Sigma-Aldrich, St. Louise, MO) was added, the solution was incubated for 3 min at 37 °C. The supernatant was collected into a new tube and centrifuged. To eliminate red blood cells, the pellet was treated with RBC lysis buffer (Biolegend, San Diego, USA) according to the manufacturer's instructions. Cells were cultured in a nontreated 6-cm petri dish in a tissue culture incubator at 37 °C, 5% CO_2. After tumorsphere formation was observed over the next 4 days of culture, tumorspheres were digested with Accumax (Innovative Cell Technologies, Inc. San Diego, USA) and dissociated into single cells by pipetting with a 1-mL tip, after which they were filtered through a 40-µm mesh, cultured in PrimNeuS medium, and passaged every 4 days.

*PrimNeuS (the culture condition for primary NBL-derived spheres) was reported by Dongliang et al. [13]. It consists of DMEM/Ham's F-12 (Sigma), 15% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), EGF (Peprotech, Rocky Hill, USA), 10 ng/mL, bFGF 15 ng/mL (Peprotech), 2% B27 supplement, 1% penicillin/streptomycin (NACALAI TESQUE, INC.), 1% non-essential amino acid (NACALAI TESQUE, INC.), 1% sodium pyruvate, and 55 µM β-mercaptoethanol.

Allograft mouse model

MYCN Tg tumor cells (1×10^5 or 5×10^5/50 µL) were mixed with 50 µL Matrigel (Corning Inc., Corning, NY, USA) and injected subcutaneously into 2-month-old wild-type mice which were obtained by crossing of MYCN Tg hemizygous male and female mice. The subcutaneous tumor volume was measured every 3–4 days using the following formula: $(A\times B^2)/2$, wherein A is the largest and B is the shortest dimension. A humane endpoint was used in this study, in which the mice were humanely euthanized when they showed food intake difficulty, moribund symptoms, bleeding, weight loss [20% of the body weight], marked increase in tumor size > 2.5 cm.

For the examination of the prevalence of metastases in allograft mice without local tumor resection, mice ($n=16$) were killed at post-injection day (PID) 90 or a humane endpoint to detect metastatic lesions by immunohistochemistry (IHC). For the investigation of correlation between local tumor volume and prevalence of microscopic lymph node metastasis (LNM), allograft mice ($n=28$) were killed at PID 30 to measure the volume of local tumor and surveyed metastatic lesions by IHC. The minimum volume of the local tumor in microscopic LNM-bearing mice was investigated to set the threshold volume for the subsequent experiment of local tumor resection described below. For the studying the effect of local tumor resection on metastatic growth, allograft mice ($n=32$) were randomly divided into two groups: the Resection group ($n=16$), in which the local tumors were completely excised under anesthesia on reaching the threshold volume set in the previous experiment; and the Observation group ($n=16$), in which the local tumors were left in situ. All animals in both groups were killed at PID 74 or a humane endpoint to measure the volume of axillary LNM and detect metastatic lesions by IHC. The size of LNM and locoregional recurrent tumors was measured every 3–4 days percutaneously after postoperative day (POD) 3. Local recurrence was defined as tumor recurrence on the

surgical scar by POD 30. The Resection group was further divided into two groups: Local recurrence group and No local recurrence group. The time for the size of the LNM to reach 5 mm in the major axis (subsequently referred to as "postoperative duration to evident metastasis") after local tumor resection was compared between the Local recurrence and the No local recurrence groups.

Immunohistochemistry

The procedure was performed as previously described [14]. In brief, allograft tumors and mouse organs were 4% paraformaldehyde-fixed at room temperature overnight. The bone specimens were decalcified in Kalkitox (Wako Chemicals, Osaka, Japan) after fixation for 1 week. Tumors and organs were embedded in paraffin, and 4-μm tissue sections on slide glasses using a microtome. The paraffin sections were deparaffinized and rehydrated. Antigens were retrieved by the boiling slides in 10 mM sodium citrate buffer (pH 6.0) for 10 min, and endogenous peroxidase activity was quenched in 3% H_2O_2 for 10 min. The sections were blocked with blocking solution (Blocking One, NACALAI TESQUE, INC.) and subsequently incubated overnight with rabbit anti-TH antibody (#2792, 1:300; CST, Danvers, MA, USA) or rabbit anti-Phox2B monoclonal antibody (ab183741; 1:1000; Abcam, Cambridge, UK) in blocking solution at 4 °C. The sections were then incubated with secondary biotin-labeled goat anti-rabbit IgG antibody (Boost IHC Detection Reagent#8114, 1:100; CST) and incubated at room temperature for 30 min. DAB chromogen was developed in DAB solution (K3468, Dako) for 2 min. The nuclei were counterstained by hematoxylin (8656, Sakura Finetek Japan).

Statistical analyses

Statistical analyses were performed with the R version 3.5.0 software program. Differences were deemed statistically significant when the p value was less than 0.05. The frequency of metastasis was evaluated by Fisher's exact test. The volume of tumor and LNM and postoperative duration to evident metastasis from the day of local tumor resection was evaluated by the Wilcoxon–Mann–Whitney test.

Results

Establishment of MYCN Tg tumor cell allograft model showing the high prevalence of metastasis

To evaluate the prevalence of metastasis in the MYCN Tg tumor allograft model, NBL cells were prepared from primary tumors of MYCN Tg mice, cultured with repeated passaging, and injected subcutaneously into wild-type mice. Tumor growth was observed in 13 out of 16 allograft mice. Five mice exhibited significantly faster tumor growth than others and were dead before organ harvesting, so they were excluded from the analysis. Eight mice were killed at PID 78-90 and investigated for metastasis by IHC with Phox2B and TH, representative markers for NBL. As a result, 88% of mice ($n = 7/8$) showed distant metastasis. Local subcutaneous tumors at PID 78 showed extensive Phox2B-positive and moderate TH-positive cells (Fig. 1a). This result suggests the undifferentiated nature of the tumor.

Macroscopic metastases were observed in the axillary lymph node and the ovary (Fig. 1b). Metastases were detected in various organs, including axillary lymph nodes (63%; $n = 5/8$), lung (63%; $n = 5/8$), ovary (25%; $n = 2/8$), bone marrow (6%; $n = 1/8$), and submandibular lymph node (image not shown) (6%; $n = 1/8$) (Fig. 1d). These results indicate that this allograft model using MYCN Tg mouse-derived NBL cells has highly metastatic characteristics.

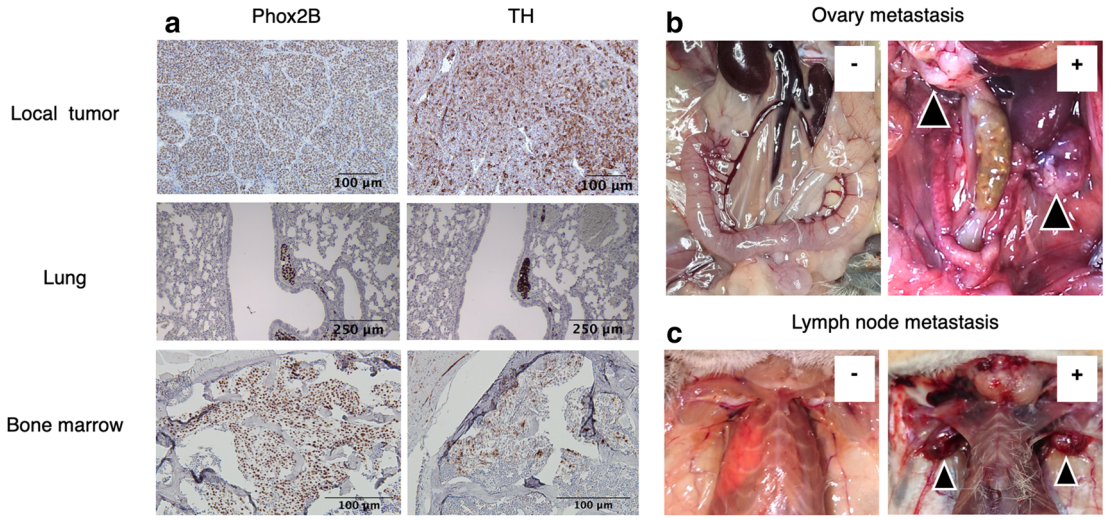
The association between local tumor volume and occurrence of microscopic LNM

We next investigated the relationship between the size of the subcutaneous local tumor and the occurrence of microscopic metastasis. We conducted the second round of the subcutaneous injection of NBL cells. As a result, all 28 allograft mice had tumor growth. These mice were killed at PID 30 and surveyed for microscopic metastasis in the axillary lymph nodes. Among them, only six mice had microscopic LNM. No macroscopic metastasis larger than 5 mm in the major axis were observed. Lymph nodes with microscopic metastasis were slightly edematous macroscopically (Fig. 2a) and partially invaded by Phox2B-positive tumor cells (Fig. 2b). There were no other distant organ metastases detected microscopically.

Local tumor volume was significantly larger in microscopic LNM-positive mice than in LNM-negative mice: 2453 ± 943 mm^3 ($n = 6$) vs. 651 ± 1158 mm^3 ($n = 22$), $p < 0.01$ (Fig. 2c). This suggests the positive correlation between the local tumor volume at PID 30 and the occurrence of LNM. As the minimum local tumor volume in the microscopic LNM-bearing mice was 745 mm^3, we set 700 mm^3 as the threshold volume for the subsequent local tumor resection experiment that aimed to evaluate the effect of local tumor resection on metastatic lesions.

An accelerated growth of distant metastasis after local tumor resection

To examine the effect of local tumor resection on distant metastatic lesions, NBL cells were injected subcutaneously, and the 32 tumor-bearing mice were divided in two



d

overall prevalence	prevalence in respective organs				
any organ	axillary lymph node	lung	ovary	bone marrow	submandibular lymph nodes
88%	63%	63%	25%	6%	6%
n = 7/8	n = 5/8	n = 5/8	n = 2/8	n = 1/8	n = 1/8

Fig. 1 High prevalence of metastasis in the MYCN Tg allograft model. Allograft mice ($n=8$) were killed at PID 78-90 and investigated for metastasis. **a** IHC with Phox2B and TH. Local subcutaneous tumor showed extensive Phox2B-positive and moderate TH-positive cells. Lung and bone marrow metastases were detected by Phox2B and TH at PID 78. **b**, **c** Macroscopic metastasis in the ovary (arrowheads to the right of **b**) and axillary lymph nodes (arrowheads to the right of **c**). Shown are representative images of mice with (+) or without (−) macroscopic metastases. **d** Prevalence of distant metastatic lesions in various tissues

groups: the Resection group ($n=16$) and the Observation group ($n=16$). Local tumor resection was performed when the local tumor exceeded 700 mm^3 in size, based on the results of the previous experiment. Mice were killed at a humane endpoint or PID 74 and analyzed for metastases. As a result, the frequency of mice with macroscopic LNM and/or distant metastasis was significantly higher in the Resection group than in the Observation group: 94% ($n=16$) vs. 38% ($n=16$), $p<0.001$ (Fig. 3a). Specifically, macroscopic LNM and lung metastasis were significantly more common in the Resection group than in the Observation group: Macroscopic LNM, 81% vs. 31%, $p<0.05$; lung metastasis, 63% vs. 6%, $p<0.05$ (Fig. 3b). Neither group had metastatic lesions in the liver or brain. The mean volume of LNM was significantly greater in the Resection group than in the Observation group: $2092\pm2{,}310$ mm^3 vs. 275 ± 218 mm^3, $p<0.01$ (Fig. 4a, b). The time for the local tumor volume to reach 700 mm^3 was not significantly different between the Resection and Observation groups (38 ± 12 days vs. 45 ± 15 days, $p=0.13$), suggesting that the growth rates of the local tumor before reaching 700 mm^3 were not significantly different between the groups. These results suggest that local tumor resection may augment the growth of distant metastasis.

Suppressive effect exerted by the residual local tumor on metastatic growth

The macroscopic LNM-bearing mice in the Resection group ($n=13$) were analyzed further to elucidate the correlation between the existence of a local tumor and metastatic growth. Six mice had postoperative local tumor recurrence, while seven mice had no local tumor recurrence. We compared the duration to evident metastasis after local tumor resection between the Local recurrence and No local recurrence groups. Our findings demonstrated that the postoperative duration to evident metastasis was significantly longer in the Local recurrence group than in the No local recurrence group 27 ± 9 days vs. 14 ± 5 days, $p<0.05$) (Fig. 5a, b). The volume of local tumor at the point of resection was not significantly different between the two groups: 1121 ± 563 mm^3 vs. 1148 ± 354 mm^3, $p=0.92$ (Fig. 5b). The time from injection to local tumor resection (PID at local resection) tended to be shorter in the Local recurrence group than in the No local recurrence group (32 ± 5 days vs. 46 ± 15 days, $p=0.05$), suggesting that the growth rate of the local tumor before resection might be higher in the Local recurrence group than in the No local recurrence group, or at least not significantly different. Despite relatively fast local tumor growth before resection, the Local recurrence

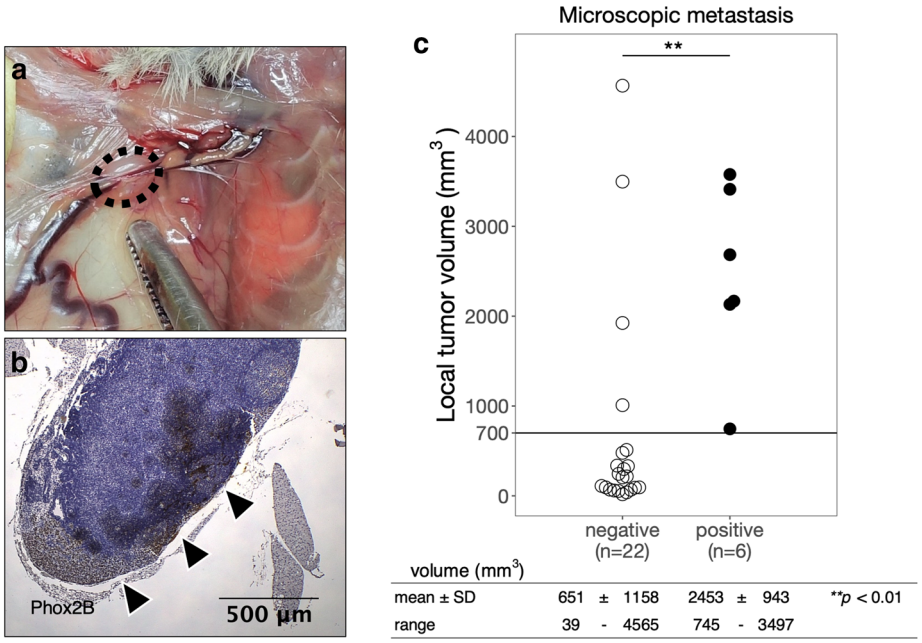


Fig. 2 The association between local tumor volume and occurrence of microscopic lymph node metastasis. Allograft mice ($n=28$) were killed at PID 30 and surveyed for microscopic metastasis in axillary lymph nodes. **a** A lymph node with microscopic metastasis was slightly edematous macroscopically. The lymph node is indicated with a dotted circle. **b** Phox2B and Hematoxylin staining of a lymph node with microscopic metastasis. The lymph node was partially invaded by Phox2B-positive tumor cells (arrowheads). **c** The comparison of the local tumor volume between microscopic LNM-positive and LNM-negative groups at PID 30. The minimum local tumor volume in the microscopic LNM-positive mice was 745 mm^3. 700 mm^3 was set as the threshold volume for subsequent experiment of local tumor resection (black line). The local tumor volume was significantly larger in microscopic LNM-positive mice than in LNM-negative mice: 2453 ± 943 mm^3 vs. 651 ± 1158 mm^3 (mean ± SD, $*p < 0.01$)

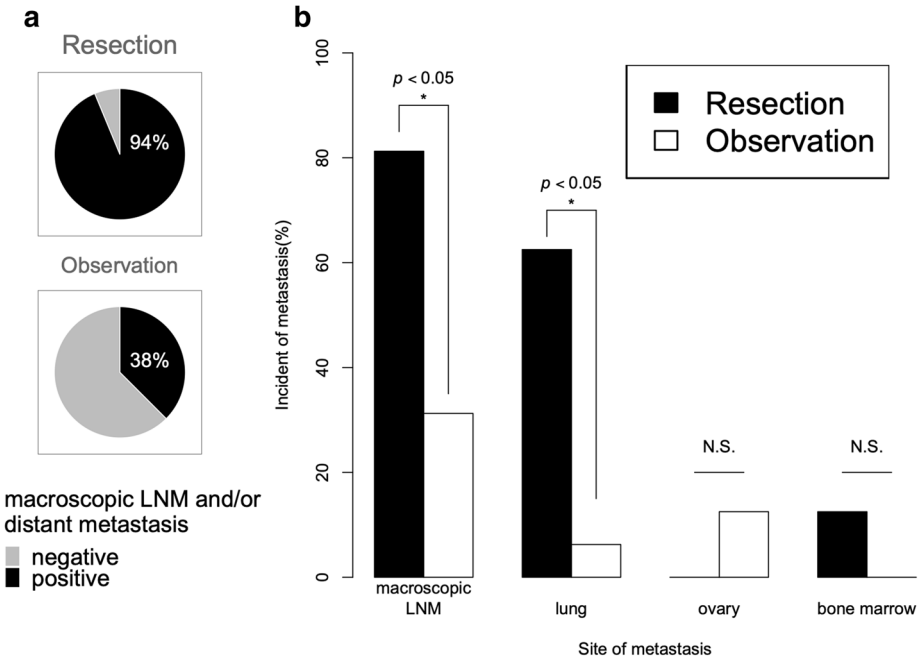


Fig. 3 The accelerated growth of distant metastasis after local tumor resection. **a** The frequency of mice with macroscopic LNM and/or distant metastasis was significantly higher in the Resection group than in the Observation group: 94% vs. 38%, $p < 0.001$. Macroscopic LNM is defined as lymph node metastasis larger than 5 mm in the major axis. **b** The comparison of the frequency of mice bearing metastases in respective organs. Macroscopic LNM and lung metastasis were significantly more common in the Resection group than in the Observation group: lymph node, 81% vs. 31%, $*p < 0.05$; lung, 63% vs. 6%, $*p < 0.05$

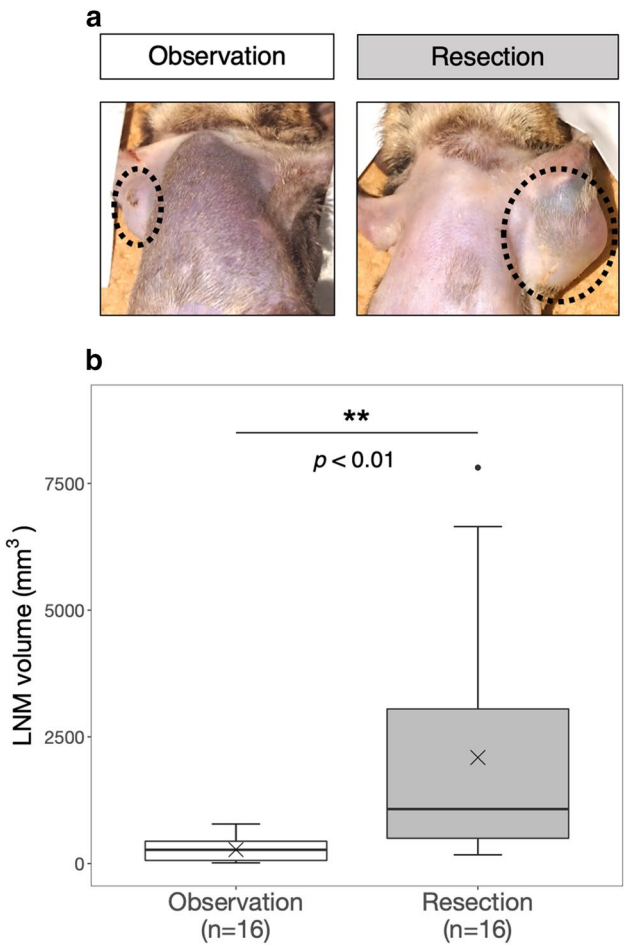


Fig. 4 An enhanced growth of lymph node metastasis after local tumor resection. **a** Representative images of macroscopic axillary LNM indicated with dotted circle at PID 60 in the respective groups. **b** The volume of LNM at PID 50–74. In the box plots, the boundary of the box closest to zero indicates the 25 th percentile, a black line within the box marks the median, a cross mark within the box marks the mean, and the boundary of the box farthest from zero indicates the 75th percentile. Whiskers above and below the box indicate the 10th and 90th percentiles. Point above the whisker indicates outlier outside the 10th and 90th percentiles. The mean LNM volume was significantly greater in the Resection group than in the Observation group: 2092 ± 2310 mm^3 vs. 275 ± 218 mm^3 (mean ± SD, $^{**}p < 0.01$)

group showed a longer postoperative duration to evident metastasis. These observations strongly suggest that LNM is suppressed by a latent residual local tumor.

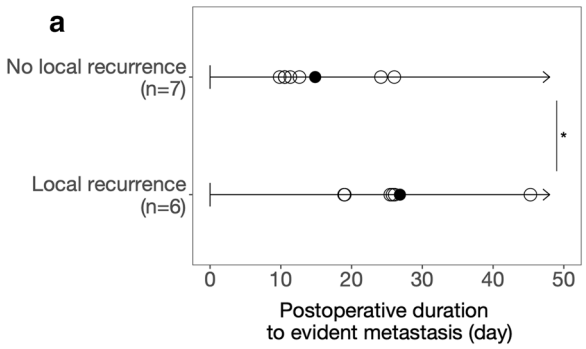
Discussion

In this study, we established MYCN Tg allograft model showing macroscopic metastases that are hardly observed in original MYCN Tg mice [10, 11]. This allograft model also showed a higher prevalence of metastases and a wider variety of metastatic organs than other transgenic mouse models previously reported [12, 16]. By utilizing this model, we simulated a common clinical condition of HR-NBL in which microscopic metastasis exists at the time of surgery, demonstrating enhanced metastatic growth after local tumor resection.

In several types of cancers, the survival outcome of local tumor resection in patients with synchronous metastases is of interest and still being investigated. For instance in colorectal cancers, complete resection of both the primary tumor and metastatic legions has survival benefits, even in patients with multiple metastases, and the effects of primary tumor resection on patients with unresectable metastases is under investigation [17, 18]. In contrast, in breast cancers, local tumor resection for stage IV breast cancer with distant metastases provides little survival advantage [19].

For localized NBL without metastasis, local tumor resection is prognostically beneficial [20, 21]. In our study as well, there were no metastases observed before local tumors exceeded 700 mm^3 in size. In addition, when the local tumor was resected before reaching 300 mm^3 in size, there were no metastases even after POD 120 (data not shown). These results suggest that complete local tumor resection before the onset of metastasis leads to complete remission, which is consistent with the clinical evidence. As for advanced NBL with metastases, whether or not local tumor resection improves the overall survival is still uncertain [7–9]. As metastatic lesion growth is the main prognostic indicator for patients with HR-NBL, it is critically important to elucidate how local tumor resection affects metastatic growth. In our study with a mouse allograft model, local tumor resection led to metastatic growth.

To study this effect further, we focused on local tumor recurrence. On analyzing the correlation between local recurrence and metastatic growth, we speculated that the "existence of a local tumor" was more important than "surgical invasiveness". As for "surgical invasiveness", consequences of surgical intervention—including inflammation, suppression of local immunity, and seeding of tumor burden—were assumed to cause metastatic spread [5]. Regarding "existence of a local tumor", concomitant tumor resistance (CR) is suspected to correlate with the inhibitory effect provided by the primary tumor on its metastases [22–24]. CR is the phenomenon according to which a tumor-bearing host inhibits the growth of secondary tumor implants. CR was first described by Ehrlich in 1906 [25], and clinical evidence of the correlation between CR and metastatic growth after primary tumor removal has been shown in several types of cancers [26, 27]. Ruggiero et al. identified tyrosine isomers as suppressive agents of secondary tumor growth, revealing their antitumor effects by the early inhibition of the mitogen-activated protein/extracellular signal-regulated kinase pathway and inactivation of STAT3 [24]. Another mechanistic explanation for CR is that the immune response



b

	Postoperative duration to evident metastasis (days)	Local tumor volume at resection (mm^3)	PID at local resection (day)
No local recurrence	14 ± 5	1148 ± 354	46 ± 15
Local recurrence	27 ± 9	1121 ± 563	32 ± 5
	$^*p < 0.05$	$p = 0.92$	$p = 0.05$

Fig. 5 Suppressive effect exerted by the residual local tumor on metastatic growth. **a** The comparison of the duration to evident metastasis between the Local recurrence and the No local recurrence groups. The duration to evident metastasis was defined as the postoperative time until the local tumor reached 5 mm in the major axis. Solid circles indicate the means of the time in respective groups, and transparent circles indicate individual values. The duration to evident metastasis was significantly longer in the Local recurrence group than in the No local recurrence group: 27 ± 9 vs. 14 ± 5 days (mean ± SD, $^*p < 0.05$). **b** Local tumor volumes and PID at local resection were comparable between the Local recurrence and the No local recurrence groups. Shown as the mean ± SD

generated by the primary tumor inhibits secondary tumor growth, which was originally proposed by Bashford [28]. This hypothesis, known as "concomitant immunity" is supported by other studies using immunogenic mouse models, although this immunologic hypothesis does not explain why non-immunogenic tumors also show CR [23]. In the present study, the volume of local tumor at the day of resection was not significantly different between the Local recurrence and the No local recurrence groups, suggesting that the degree of surgical invasiveness was comparable between the two groups. Therefore, the accelerated metastatic growth after local tumor resection was assumed to be brought by decreased CR rather than surgical invasiveness. To clarify the genuine effects of the existence of a local tumor on metastatic lesions, further studies including comparisons between local tumor resection and sham operation are needed.

For the treatment of advanced NBL patients with metastatic lesions, multimodal therapy including the combination of local tumor resection, high-dose chemotherapy (HDC), radiotherapy, autologous stem cell transplant, differentiation therapy and immunotherapy is considered to be standard [1–3]. However, how to integrate these therapies for the optimal treatment strategy remains unclear, especially concerning the most appropriate timing and indications for local tumor resection. At present, a prospective clinical trial is being conducted by Japan Neuroblastoma Study Group (JNBSG), wherein radical primary tumor resection is intentionally delayed until the completion of all chemotherapy courses, including HDC, with the aim of shortening the interval between each chemotherapy course and subsequently strengthening the time-dose intensity of the entire treatment [3]. This strategy of delayed surgery after systemic treatment is expected to provide more intense suppression on metastatic lesions before local tumor resection. Our results provide insight into the potential superiority of delayed surgery after systemic treatment to control metastases for advanced NBL with distant metastasis.

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Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest in association with the present study.

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