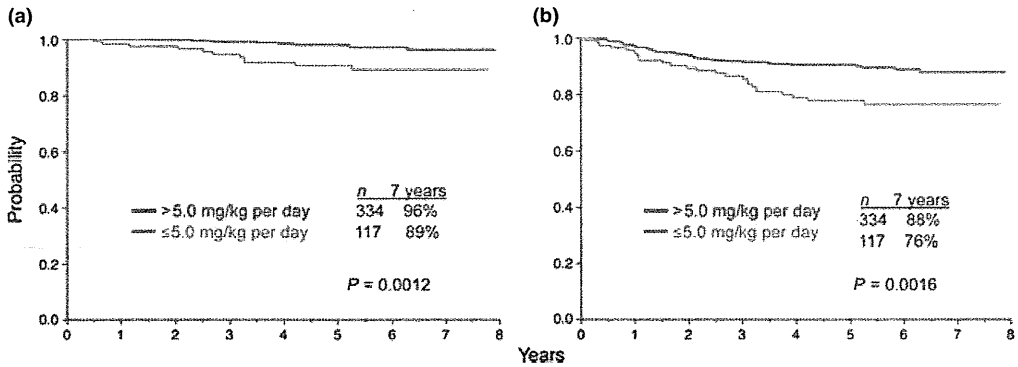

Fig. 4. (a) Overall and (b) event-free survival according to the mean daily dose during the first 24 months per body weight. The cut-off value was set at >5.0 mg/day/kg (e.g. if a patient whose body weight was <60 kg received imatinib at a mean daily dose of 300 mg).

Table 5. Number of patients and survival according to the mean daily dose of imatinib during the first 24 months per body weight

	Mean daily dose/body weight (mg/day/kg)				
	>5.0†		≤5.0		*P*-value
	Actual bodyweight (kg)	No. patients	Actual bodyweight (kg)	No. patients	
Imatinib daily dose group‡					
400 mg	<80	266	≥80	28	
300 mg	<60	63	≥60	27	
200 mg	<40	5	≥40	62	
Estimated 7-year OS	96%		89%		0.0012
Estimated 7-year EFS	88%		76%		0.0016

†The cut-off value was set at >5.0 mg/day/kg (e.g. the mean daily dose of imatinib during the first 24 months (300 mg) divided by body weight [<60 kg]). ‡Mean daily doses in the 400-, 300-, and 200-mg groups were ≥360, 270–359, and <270 mg imatinib, respectively. Patients who discontinued imatinib were not included in the analysis. EFS, event-free survival; OS, overall survival.

to the mean daily dose during the first 6, 12, and 24 months of treatment. The rate of achieving CCyR or MMR differed significantly between the 300- and 400-mg groups during the first 24 months. Even so, there were no significant differences in OS, PFS, and EFS between the 300- and 400-mg groups during the first 6, 12, or 24 months of treatment. Conversely, the 200-mg group showed markedly inferior cytogenetic and/or molecular responses, as well as inferior survival, compared with the 300- and 400-mg groups. We also analyzed outcomes according to the mean daily dosage during the first 24 months per BW, with the results suggesting that patients who had relatively high daily dosage per BW were likely to have better OS and EFS even though the actual daily dose had been lower than 400 mg imatinib. The OS and EFS in the 300-mg group in the present study were not inferior compared with rates reported in the IRIS study (85% at 7 years vs. 83% at 6 years), which suggests that a considerable number of Japanese patients who received doses lower than 400 mg demonstrated an adequate response. A prospective comparative study would be necessary to confirm this observation.

Two recent studies showed a correlation between the plasma trough levels (C_{min}) and response, suggesting that maintaining C_{min} above approximately 1000 ng/mL was associated with improved outcomes.[22,23] In the present study, the mean daily dose was 331 ± 108 mg during the first 24 months and the relatively high dosage of imatinib per BW was associated with better OS and EFS, whereas in the IRIS study the mean daily dose among the patients who continued receiving imatinib was 382 ± 50 mg.[1] On the basis of our results, we assume that the relatively small body size of Japanese patients compared with their Western counterparts may have affected C_{min}, although differences in the metabolism of imatinib because of ethnicity cannot be ruled out either. Therefore, we measured the C_{min} of imatinib in a group of patients who had received imatinib continuously at a daily dose of either 300 or 400 mg. The patients from whom blood samples were collected showed almost similar background characteristics to the entire study population. There was no significant difference in the mean C_{min} between patients receiving 300 or 400 mg imatinib, and there was no significant difference in the ratio of patients whose C_{min} was higher than 1000 ng/mL between the two groups. When pharmacokinetic analyses of patients receiving 400 mg imatinib in the present study are compared with the IRIS study, the C_{min} in the present study was distributed at higher concentrations than in the IRIS study (mean C_{min} 1165 vs. 979 ng/mL, respectively); however, the distribution of C_{min} in patients receiving 300 mg imatinib was similar between the studies.[23] Larson *et al.* reported a weak correlation between C_{min} and age, BW, or BSA in the IRIS study, but also suggested that the effects of body size and age on C_{min} were not likely to be of clinical significance because C_{min} showed large interpatient variability.[23] However, the C_{min} in their female patients was significantly higher than that in male patients, and they speculated that this may be due to the small body size of the female patients. The same tendency was seen in the present study, especially in terms of age and gender. Therefore, a small body size among Japanese old and/or female patients may partly account for the higher C_{min} of imatinib. Regarding

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Table 6. Patient characteristics and plasma trough levels of imatinib according to the daily dose of imatinib

	Imatinib daily dose†		*P*-value
	400 mg	300 mg	
No. patients	26	24	
No. men/women	19/7	12/12	0.092
Age (years)	49 (17–79)	58 (33–76)	0.012
Body weight (kg)	65.2 ± 10.6	59.5 ± 10.7	0.062
BSA (m^2)	1.68 ± 0.17	1.57 ± 0.17	0.034
Sokal risk group (*n*)			
Low	18	13	0.357
Intermediate	6	6	
High	2	5	
C_{min} (ng/mL)			
Mean ± SD	1165 ± 445	1113 ± 426	0.673
Median (range)	1035 (710–2420)	1130 (439–2140)	
% Patients on >1000 ng/mL imatinib	57.7 (15/26)	62.5 (15/24)	0.1
Best response (%)			
MCyR	26 (100)	23 (96)	
CCyR	26 (100)	22 (92)	
MMR	24 (92)	23 (96)	

Unless indicated otherwise, data are given as the mean ± SD, as the median with the range given in parentheses, or as the number of patients in each group with percentages given in parentheses, as appropriate. †Imatinib at a daily dose of 400 or 300 mg without any dose modification. BSA, body surface area; CCyR, complete cytogenetic response; C_{min}, plasma trough level; MCyR, major cytogenetic response; MMR, major molecular response.

the plasma concentration of imatinib in Japanese patients, there are other reports showing sufficient C_{min} in patients receiving imatinib at doses lower than 400 mg,[6,24] but it remains uncertain whether there are any individual or ethnic differences in the metabolism of imatinib.[24,25]

Another possible reason for the satisfactory outcomes seen for patients in the 300-mg group could be that, at this dose, imatinib could be administered continuously to some patients without serious adverse events. A recent study regarding imatinib dosage in Japanese patients reported that, based on multivariate analysis, older age and lower BW are significant risk factors for the discontinuation of imatinib therapy and that patients with these factors were less likely to achieve a CCyR.[18] Continuous and adequate dosage is essential for optimal outcome, and adherence to imatinib therapy is critical.[26,27]

In conclusion, the long-term follow-up of the JALSG CML202 study revealed almost similar excellent outcomes to those of the IRIS study and others. There were no significant differences in OS and EFS between the 300- and 400-mg imatinib groups. However, cumulative rates of cytogenetic or molecular responses in the 300-mg group were inferior to those in the 400-mg group. The results of the present study suggest that imatinib at a dose of 400 mg may be optimal for Japanese patients, but that 400 mg imatinib is not tolerable in a considerable number of patients, and that the measurement of C_{min} is useful in finding the optimal dose, especially in elderly and/or female patients. Nevertheless, excessive dose reductions to <300 mg imatinib should be avoided even in patients who are intolerant to 400 mg imatinib or have a small body size. We hope our findings are useful for the treatment of CML patients in other Asian countries.

Acknowledgments

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Disclosure Statement

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Correlation between Amp-CMLTM (FUJIREBIO Inc., Tokyo, Japan) and Fusion Quant M-BCRTM (Ipsogen, Marseille, France).

Data S1. Measurement of major *BCR-ABL1* transcript.

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Therapeutic Discovery

Molecular Cancer Therapeutics

Dll4-Fc, an Inhibitor of Dll4-Notch Signaling, Suppresses Liver Metastasis of Small Cell Lung Cancer Cells through the Downregulation of the NF-κB Activity

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Abstract

Notch signaling regulates cell-fate decisions during development and postnatal life. Little is known, however, about the role of Delta-like-4 (Dll4)-Notch signaling between cancer cells, or how this signaling affects cancer metastasis. We, therefore, assessed the role of Dll4-Notch signaling in cancer metastasis. We generated a soluble Dll4 fused to the IgG1 constant region (Dll4-Fc) that acts as a blocker of Dll4-Notch signaling and introduced it into human small cell lung cancer (SCLC) cell lines expressing either high levels (SBC-3 and H1048) or low levels (SBC-5) of Dll4. The effects of Dll4-Fc on metastasis of SCLC were evaluated using a mouse model. Although Dll4-Fc had no effect on the liver metastasis of SBC-5, the number of liver metastasis inoculated with SBC-3 and H1048 cells expressing Dll4-Fc was significantly lower than that injected with control cells. To study the molecular mechanisms of the effects of Dll4-Fc on liver metastasis, a PCR array analysis was conducted. Because the expression of NF-κB target genes was affected by Dll4-Fc, we conducted an electrophoretic mobility shift assay and observed that NF-κB activities, both with and without stimulation by TNF-α, were downregulated in Dll4-Fc–overexpressing SBC-3 and H1048 cells compared with control cells. Moreover, Dll4-Fc attenuates, at least in part, the classical and alternative NF-κB activation pathway by reducing Notch1 signaling. These results suggest that Dll4-Notch signaling in cancer cells plays a critical role in liver metastasis of SCLC by regulating NF-κB signaling. *Mol Cancer Ther; 11(12); 2578–87. ©2012 AACR.*

Introduction

Small cell lung cancer (SCLC) accounts for 15% to 20% of lung cancer cases and presents with aggressive clinical behavior characterized by rapid growth and metastasis to distant organs (1). The production of metastases in multiple organs such as the liver, bone, and brain during early stages frequently makes the prognosis of patients with these diseases poor. Therefore, novel effective therapies to control cancer metastases are necessary to improve the prognoses of patients with SCLC.

Notch signaling is composed of a family of 4 Notch receptors and 5 ligands. Notch receptors are proteolytically cleaved by γ-secretase upon ligand binding. The cytoplasmic portions of the receptors then directly transduce signals from the cell surface to the nucleus, thereby controlling the expression of target genes (2). Aberrations of Notch signaling are associated with lung cancer progression as well as T-cell acute lymphoblastic leukemia/lymphoma (T-ALL) and breast cancer progression (3–5). Current evidence provides that VEGF induces Delta-like-4 (Dll4)-regulated differentiation of tip and stalk cells in endothelial cells (6–9). Repression of Dll4-Notch signaling in tumor endothelial cells results in nonproductive angiogenesis and consequent suppression of tumor growth. On the basis of these reports, several Dll4-neutralizing antibodies have been developed that show anticancer effects in preclinical models (10–12). However, the effects of Dll4 expressed by cancer on tumor progression remain to be fully elucidated.

In this study, we investigated the role of Dll4 in metastasis using Dll4-Fc, an inhibitor of Dll4-Notch signaling, and cancer cell lines expressing either high or low levels of Dll4.

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Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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Materials and Methods

Cell lines

Human SCLC cell lines, SBC-3 and SBC-5, were kindly provided by Drs. M. Tanimoto and K. Kiura (Okayama University, Okayama, Japan). H1048 cells were purchased

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from the American Type Culture Collection. SCLC cell lines were authenticated by a Multiplex STR assay (BEX). Human umbilical vascular endothelial cells (HUVEC) were purchased from Lonza. The Platinum-E (PLAT-E) retroviral packaging cells were kindly provided by Dr. T. Kitamura (Tokyo University, Tokyo, Japan; ref. 13).

Reagents

Antimouse IL-2 receptor β-chain monoclonal antibody, TM-β1, was supplied by Drs. M. Miyasaka and T. Tanaka (Osaka University, Osaka, Japan). Antibodies against Notch1, p50, and RelB were purchased from Cell Signaling. Antibodies against p65 and β-actin were purchased from Santa Cruz Biotechnology. Antibody against CD-31 was purchased from BD Biosciences. Antibody against mouse Dll4 was purchased from R&D Systems.

Retroviral transfection

The murine Dll4 extracellular domain-Fc (Dll4-Fc; ref. 6) and its control vector was infected into SBC-3, H1048, and SBC-5 as previously described (14, 15), and infected cell populations were fluorescence-activated cell sorted (FACS) on a JSAN cell sorter (Bay bioscience). The proportion of GFP-positive cells was more than 85% of the cell population.

Animals

Male SCID mice, 5 to 6 weeks of age, were obtained from CLEA Japan and maintained under specific pathogen-free conditions throughout the study. All experiments were conducted in accordance with the guidelines established by the Tokushima University Committee on Animal Care and Use.

***In vivo* metastasis models**

To facilitate metastasis formation, SCID mice were pretreated with TMβ-1 to deplete NK cells. Two days later, the mice were inoculated with SBC-3, H1048, or SBC-5 cells into the tail vein (16–18). The mice were sacrificed humanely under anesthesia, and the major organs were then removed and weighed and the number of metastatic nodules on the surface of the organs was counted.

Analysis of micrometastasis

To detect micrometastasis in the liver by reverse transcription (RT)-PCR, whole livers were removed 7, 14, and 21 days after injection of the cells, and RNA was extracted using the RNeasy Mini Kit (Qiagen). Reverse transcription reaction was conducted as follows. RT-PCR was conducted using TaqMan Gene expression assays (Supplementary Table S1). The expression levels of human-*β2-microglobulin* (h*B2M*) in the murine livers were used as molecular markers for micrometastasis.

To evaluate the longest diameter or microvessel density in the liver micrometastases, whole livers were removed 21 days after inoculation of SBC-3-Dll4-Fc or control cells. Frozen tissue sections (8-μm thick) were fixed with 4% paraformaldehyde (PFA) and used to identify endothelial cells using rat antimouse CD31 monoclonal antibody (1:30 dilution; BD Biosciences). The highest numbers of staining within a section were selected for histologic quantification.

RT-PCR and TaqMan gene signature arrays

Total cellular RNAs were isolated using the RNeasy Mini Kit (Qiagen), and reversely transcribed using a TaqMan RNA-to-CT 2-Step Kit (Applied Biosystems). The primers for *Dll4-Fc* and *β-actin* were as follows: Dll4-Fc: 5′-ACAGGCACCCACTGTGAACT-3′ and 5′-CTGGGATAGAAGCCTTTGAC-3′; β-actin: 5′-AAGAGAGGCATCCTCACCCT-3′ and 5′-TACATGGCTGGGGTGTTGAA-3′. RT-PCR was conducted using Ampli Taq Gold (Applied Biosystems).

A quantitative PCR analysis of metastasis-associated genes was carried out using the TaqMan Array Gene Signature (Applied Biosystems).

Western blot analysis

Cells were cultured for 48 hours, and were lysed in M-PER reagent (Pierce) containing phosphatase and protease inhibitor cocktails (Roche). To detect protein in the conditioned medium, cultured medium was collected after incubation for 48 hours. The metastatic liver lesions were homogenized in T-PER (Pierce). The concentrations of protein were determined using a Bio-Rad Protein Assay Kit (Bio-Rad). Aliquots of 500 μg of total proteins were immunoprecipitated with the antibody against Notch1 (Cell Signaling Technology). Then, immunoblotting was conducted as previously described (19).

Luciferase assay

The NF-κB activities in SBC-3, H1048, and SBC-5 cells were measured as previously described (20). SCLC cells were cultured for 48 hours after transfection. Then, the luciferase activities in the cell extracts were measured.

Electrophoretic mobility shift assay

Cells were cultured for 4 hours with the presence or absence of TNF-α (100 ng/mL; R&D Systems). Then, nuclear extractions were prepared using the NE-PER Kit (Pierce). Electrophoretic mobility shift assay was conducted using the Lightshift EMSA Kit (Pierce) according to the manufacturer's protocols. A supershift assay was also conducted to confirm the DNA–NF-κB bound complexes.

Migration and invasion assay

Cell migration was determined as previously described (21). Invasion assay was conducted using Matrigel-coated transwell chambers (BD Biosciences) according to the manufacturer's protocols. In each analysis, the 4 areas containing the highest number of the cells within a chamber were counted under light microscopy at ×100 magnification.

Data analysis

The data are expressed as the mean ± SD. Welch *t* test and the Mann–Whitney *U* test were used for the statistical

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analyses. Differences were considered to be significant at $P < 0.05$.

Results

Retroviral transduction of Dll4-Fc in cancer cells suppresses Dll4-Notch signaling

On the basis of previous studies that used extracellular domain of Dll4 to inhibit Dll4-Notch signaling (6), we generated a retroviral vector encoding the extracellular domain of murine Dll4 fused to the human IgG1 Fc constant region (Dll4-Fc) with GFP. This construct was infected into human SCLC cell lines expressing either high levels of Dll4 (SBC-3 and H1048) or low levels of Dll4 (SBC-5; Supplementary Fig. S1A). The Dll4-Fc–transduced cancer cells were sorted with flow cytometry (FACS). *Dll4-Fc* mRNA was detected in the Dll4-Fc–transduced SBC-3 cells (SBC-3-Dll4-Fc) but not in the vector-transduced control cancer cells (Fig. 1A). Dll4-Fc proteins were detected in the cell lysate and conditioned medium of the SBC-3-Dll4-Fc cells but not in those of the control cells (Fig. 1B). Similar results were obtained in the H1048 and SBC5 cells (Supplemental Fig. S1B, S1C, S1E, and S1F).

To examine whether Dll4-Fc affected the behavior of cells, the growth rates of cells were examined using an MTT assay. The growth rates of the SBC-3-Dll4-Fc, H1048-Dll4-Fc, and SBC5-Dll4-Fc cells were similar to those of the counterpart of control cells (Fig. 1C and Supplementary Fig. S1D and S1G). We then examined whether transduced Dll4-Fc inhibits Dll4-Notch signaling. Confluent HUVECs were treated with the conditioned medium of the SBC-3-Dll4-Fc cells and the *Hes1* expression was measured as a surrogate marker of Notch activity. The conditioned medium of the SBC-3-Dll4-Fc cells suppressed the *Hes1* expression (Fig. 1D). This result indicates that the Dll4-Fc secreted from SBC-3-Dll4-Fc cells is functional.

Dll4-Fc suppresses liver metastasis of SCLC cells expressing high levels of Dll4

We examined whether Dll4 plays an important role in cancer metastasis using NK-cell–depleted SCID mice as a multiple-organ metastasis model. In this model, SBC-3 and H1048 cells expressing high levels of Dll4 metastasized to the liver, kidney, bone, and lymph node (17, 18). We observed that SBC-3-Dll4-Fc and H1048-Dll4-Fc cells produced significantly fewer numbers of metastatic nodules in the liver compared with the control cells (Table 1). The numbers of metastatic nodules in other organs, such as the kidney, bone, and lymph node were not affected by Dll4-Fc. The mice intravenously inoculated with SBC-5 cells expressing low levels of Dll4 had liver, lung, and bone metastases (16). The numbers of metastatic nodules produced by SBC-5-Dll4-Fc cells in those organs were comparable with those produced by control SBC-5 cells (Table 2). These results suggest that Dll4 reveals prometastatic function in the liver in proportion to its expression.

Dll4-Fc suppresses liver micrometastasis of SCLC cells

The number of macroscopic metastatic nodules produced by the SBC-3-Dll4-Fc cells in the liver was less than that produced by the control cells; however, the size of the nodules produced by the SBC-3-Dll4-Fc cells was comparable with that of the nodules produced by the control cells (Fig. 2A). Furthermore, the proportion of metastatic nodules larger than 2 mm produced by the SBC-3-Dll4-Fc cells in the liver was comparable with that produced by the control cells (Fig. 2B). These results suggest that Dll4-Fc affects the early steps of metastasis such as survival in circulation, attachment to endothelial cells, migration, invasion, and extravasation, but not growth or neovascularization. To confirm this hypothesis, we evaluated

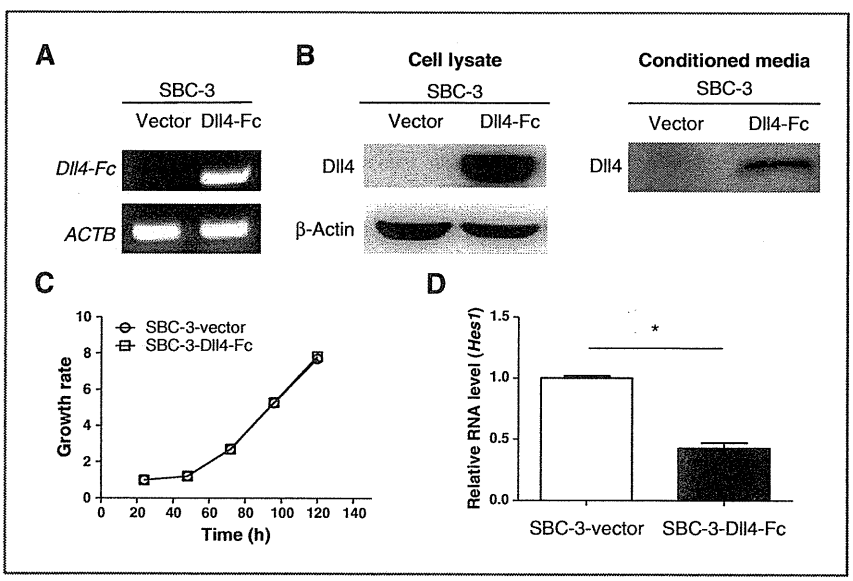

Figure 1. Extracellular domain of murine Dll4 fused to Fc (Dll4-Fc) expressed in cancer cells inhibits Notch signaling. A and B, the expression of *Dll4-Fc* mRNA (A) and Dll4-Fc protein in SBC-3 cells (B, left) and conditioned media (B, right) was determined. C, the effects of Dll4-Fc on the growth rates of the SBC-3 cells were measured using an MTT assay. D, the effects of the conditioned media of the SBC-3-vector and SBC-3-Dll4-Fc on *Hes1* mRNA expression in HUVECs; *, $P < 0.05$ (Welch *t* test).

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Table 1. Production of metastasis by SCLC cells expressing high level of Dll4 with or without Dll4-Fc in NK cell–depleted SCID mice

			No. of metastatic colonies[a]		
Cell line	**Incidence**	**Liver weight (g)**	**Liver**	**Kidney**	**Lymph node**
SBC-3-vector	7/7	2.6 (1.6–5.4)	15.6 (2–43)	4.0 (0–11)	2.1 (1–3)
SBC-3-Dll4-Fc	9/9	1.3 (1.1–1.5)[b]	4.0 (1–8)[c]	2.8 (0–5)	2.0 (1–3)
			No. of metastatic colonies[a]		
Cell line	**Incidence**	**Liver weight (g)**	**Liver**	**Kidney**	**Bone**
H1048-vector	6/6	1.1 (0.8–1.4)	4.8 (2–7)	59.8 (39–77)	9.5 (8–12)
H1048-Dll4-Fc	7/7	0.8 (0.6–1.1)	1.6 (1–3)[d]	49.9 (19–84)	7.7 (3–14)

NOTE: The statistical significance of difference was analyzed by one-way ANOVA and *post hoc* pairwise comparisons were done by Newman–Keuls multiple comparison test.

[a]Values are the mean (minimum–maximum).

[b]Statistically significant difference compared with SBC-3-vector ($P < 0.05$).

[c]Statistically significant difference compared with SBC-3-vector ($P < 0.001$).

[d]Statistically significant difference compared with H1048-vector ($P < 0.05$).

micrometastasis of SBC-3-Dll4-Fc and control cells in the liver to investigate the effects of Dll4-Fc on the early steps of SCLC metastasis. To detect micrometastasis, whole livers were removed on days 7, 14, and 21 after injection of the cells and the expression of h*B2M* as a molecular marker of liver metastasis was detected using RT-PCR. As shown in Fig. 2C, the levels of h*B2M* mRNA in the livers of mice inoculated with SBC-3-Dll4-Fc cells were significantly lower than those in the livers of mice inoculated with control cells, on day 21, suggesting that Dll4-Fc plays a role in the early steps of metastasis produced by SCLC cells (Fig. 2C). A histologic analysis was also done on day 21 using metastatic liver tissue after inoculation with either SBC-3-Dll4-Fc or control cells. All of the metastatic foci in the liver were smaller than 1.5 mm in diameter (Fig. 2D and E), and the diameters of the metastatic foci produced by the SBC-3-Dll4-Fc cells in the liver were comparable with those of the metastatic foci produced by the control cells (Fig. 2E). As a previous report showed that the Dll4-Fc suppresses tumor growth by promoting dysregulated angiogenesis (6), we evaluated the effects of Dll4-Fc on tumor angiogenesis in metastatic foci of the liver on day 21. The microvessel density in the micrometastatic foci produced by the SBC-3-Dll4-Fc cells was comparable with that in the micrometastatic foci produced by the control cells (Fig. 2F and G), thus indicating that Dll4-Fc does not affect angiogenesis in metastatic foci. Taken together, these *in vivo* results suggest that blockage of Dll4-Notch signaling in cancer cells suppresses the early steps of liver metastasis produced by SBC-3 cells.

Dll4-Fc reduces migration ability and invasiveness

Cancer metastasis is a multistep process requiring tumor cell migration, intravasation, survival in circulation, extravasation, and colonization to a secondary site. Especially, the activities of migration and invasion play important roles in the early steps of metastasis. To examine the effect of Dll4-Fc on the cell migration, migration assay was conducted using SBC-3 and SBC-5 cells expressing Dll4-Fc. Although the cell migration of the SBC-5 expressing Dll4-Fc was comparable with that of the control cells (data not shown), the migration ability of the SBC-3 cells was suppressed by Dll4-Fc (Fig. 3A and B). Moreover, to evaluate the effects of Dll4-Fc on the ability

Table 2. Production of metastasis by SCLC cells expressing low level of Dll4 with or without Dll4-Fc in NK cell–depleted SCID mice

			No. of metastatic colonies[a]		
Cell line	**Incidence**	**Liver weight (g)**	**Liver**	**Lung**	**Bone**
SBC-5-vector	8/8	1.4 (0.7–2.0)	43.8 (24–91)	54.3 (8–90)	4.0 (1–5)
SBC-5-Dll4-Fc	9/9	1.3 (1.1–1.5)	30.8 (15–68)	69.7 (47–102)	2.8 (1–5)

NOTE: The statistical significance of difference was analyzed by one-way ANOVA and *post hoc* pairwise comparisons were done by Newman–Keuls multiple comparison test.

[a]Values are the means (minimum–maximum).

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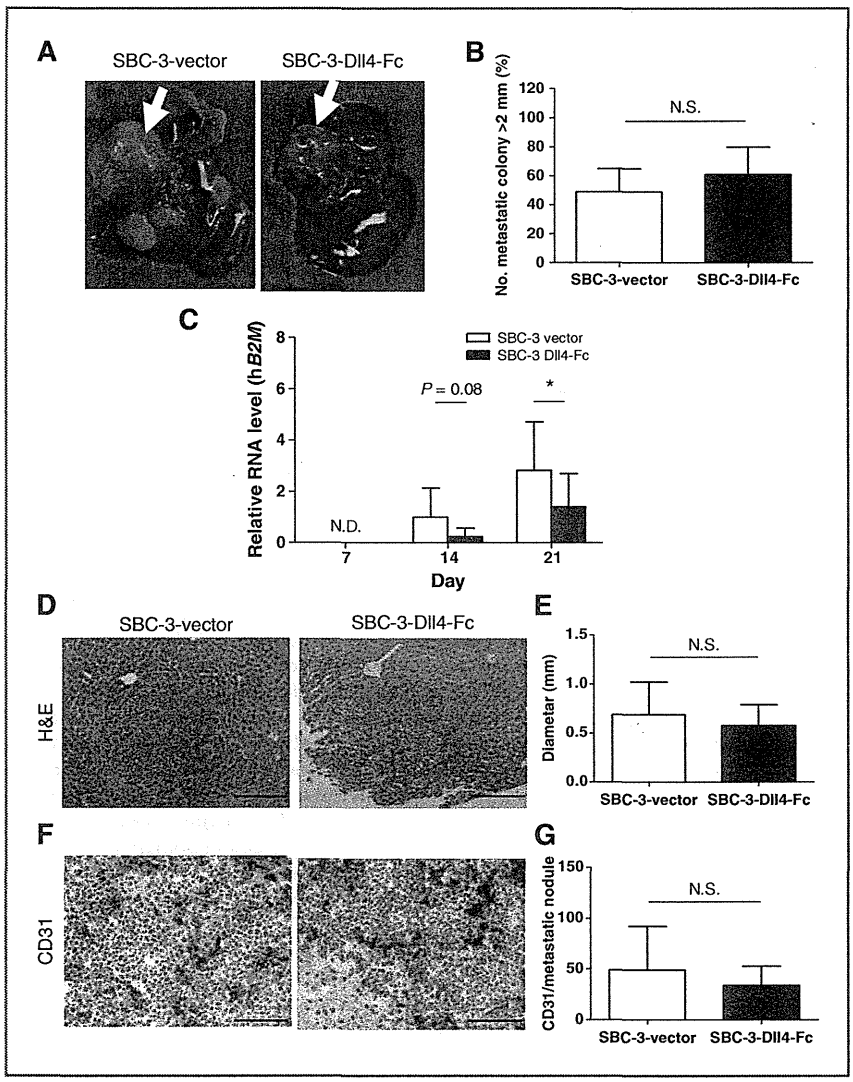

Figure 2. Dll4-Fc suppresses micrometastasis of SBC-3 cells in the liver. A, representative macroscopic metastatic nodules on the surface of the livers 42 days after inoculation of control (left) and SBC-3-Dll4-Fc cells (right) in SCID mice are shown. The size of the tumor produced by SBC-3-Dll4-Fc cells was comparable with that of the tumor produced by control cells (arrow). B, the proportion of metastatic nodules larger than 2 mm on the surface of the liver was measured 42 days after inoculation of control and Dll4-Fc–expressing SBC-3 cells. C, micrometastasis in the liver was assessed by RT-PCR. The relative expression was calculated according to the expression of h*B2M* 14 days after inoculation of SBC-3-vector as 1.0. D and E, the longest diameter of each metastatic foci in the liver was measured after hematoxylin and eosin staining. D, photographs of the representative metastatic foci of SBC-3-vector and SBC-3-Dll4-Fc cells are shown (H&E; magnification, ×40). E, the longest diameter of each metastatic foci was measured and plotted. Bar, 1.0 mm. F and G, staining of the microvessels in the metastatic foci in the liver with antimouse CD31 monoclonal antibody 21 days after injection of SBC-3-Dll4-Fc and control cells. F, representative CD31 staining patterns. G, the analysis of the number of microvessels in the metastatic foci. Bar, 200 μm; *, $P < 0.01$ (Mann–Whitney *U* test); N.D., not detected; N.S., not significant.

of the invasion, we conducted an invasion assay using Matrigel-coated Transwell chambers (Fig. 3C and D). The invasion activity of the SBC-3 cells was also attenuated by Dll4-Fc. These results suggest that Dll4-Fc inhibits the early steps of liver metastasis through the attenuation of cell migration and invasion.

Dll4-Fc suppresses the expression of metastasis-associated genes through the inhibition of NF-κB activity

To elucidate the molecular mechanisms underlying the effects of Dll4-Fc on liver metastasis, the expression levels of genes associated with metastasis were determined using a PCR array system. Six genes (*SERPINE1, MMP10, CXCR4, MMP7, S100A4,* and *MMP1*) were downregulated in Dll4-Fc–overexpressing SBC-3 and H1048 cells, but not in SBC-5 cells, compared with control cells (Fig. 4A and Supplementary Table S2). Several signaling pathways were involved in these genes. However, because the most selected genes, *SERPINE1, CXCR4, S100A4,* and *MMP1,* were regulated by NF-κB, we explored whether NF-κB was involved in Dll4-Notch signaling in these cells (22–25). By conducting a luciferase assay using a luciferase reporter plasmid carrying 6 tandem NF-κB binding sites, we observed that the level of NF-κB activity was decreased in Dll4-Fc–overexpressing SBC-3 and H1048 cells, but not in SBC-5 cells, compared with that observed in control cells (Fig. 4B). In addition, to confirm the suppression of NF-κB activity by Dll4-Fc, the effects of recombinant Dll4-Fc on NF-κB activity of SBC-3-vector cells were measured by luciferase assay (Supplementary Fig. S2). Similar results were observed with SBC-3-Dll4-

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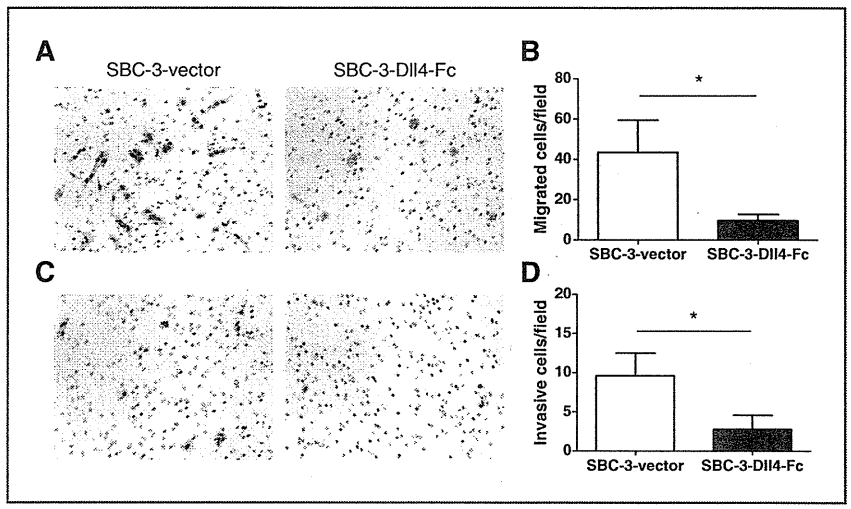

Figure 3. Dll4-Fc suppresses the migration and invasion abilities in SBC-3 cells. The effects of Dll4-Fc on the migration and invasion abilities of SBC-3 cells were measured using a Transwell assay. A and C, representative stainings of migrated (A) and invasive (C) cancer cells are shown. B and D, the cells that migrated (B) and invaded (D) to the lower surface of the insert were counted; *, $P < 0.01$ (Welch t test).

Fc. To explore the mechanisms underlying the attenuation of NF-κB signaling by Dll4-Fc, we conducted an Electrophoretic Mobility Shift Assay (EMSA). Although we could not detect a specific DNA–protein complex in the nuclear extracts of the SBC-5 cells (data not shown), 2 or 3 complexes were detected in the nuclear extracts of the SBC-3 and H1048 cells, which were, at least in part, reduced by Dll4-Fc (Supplementary Fig. S3A and S3B). Because the H1048 cells showed higher expression levels of *TNFA* mRNA than the SBC-3 cells (data not shown), we suggested that Dll4 affected NF-κB activity either with or without TNF-α. To confirm this hypothesis, we conducted EMSA with stimulation of TNF-α (Fig. 4C). In accordance with Fig. 4B, we represented that Dll4-Fc, at least in part, suppressed basal level of DNA-bound complexes (Fig. 4C and Supplementary Fig. S3C; lane 1, 6, 8, and 13). Moreover, we found that the upper DNA-bound complex of the SBC-3 cells was elevated with TNF-α treatment (Fig. 4C and Supplementary Fig. S3C; lanes 1 and 2) and partially suppressed by Dll4-Fc (Fig. 4C and Supplementary Fig. S3C; lanes 2 and 7). Similar results were obtained with stimulation of the H1048 cells by TNF-α (Fig. 4C and Supplementary Fig. S3C; lanes 9 and 14). To reveal the molecular mechanisms underlying suppression of NF-κB signaling by Dll4-Fc with TNF-α treatment, we examined supershifts using antibodies against p65 and Notch1. The upper complexes elevated by TNF-α were attenuated when antibody against p65 was added to the SBC-3 and H1048 cells (Fig. 4C and Supplementary Fig. S3C; lanes 2, 4, 9, and 11). In addition, all complexes were partially suppressed by antibody against Notch1 (Fig. 4C and Supplementary Fig. S3C; lanes 2, 5, 9, and 12). These results imply that Dll4-Notch signaling is involved in NF-κB activity both with and without TNF-α stimulation. Although antibody against Notch1 partly suppressed the upper, middle, and bottom of DNA-bound complexes, antibody against p65 only attenuated upper bound complex with TNF-α stimulation. To determine the lower 2 bound complexes, we conducted supershifts using antibodies against p50 and RelB (Fig. 4D). The upper and bottom DNA bound complexes were reduced by antibody against p50, and middle band was attenuated by antibody against RelB. These results suggested that p65, p50, and RelB were interacted with Notch1. To confirm these results, we examined the interaction of Notch1 with p65, p50, and RelB in 3 nodules of the liver metastasis produced by SBC-3 (Fig. 4E). We found that Notch1 bound to p65, p50, and RelB in metastatic liver lesions. Moreover, Dll4-Fc attenuated these interactions. To investigate the effects of Dll4-Fc on Notch1 signaling, we measured the cleavage of the Notch1 intracellular domain (N1-ICD) as a marker of Notch1 activity in Dll4-Fc expressing SBC-3, H1048, and SBC-5 cells. An immunoblot analysis showed that the levels of N1-ICD was reduced in Dll4-Fc–transduced SBC-3 and H1048 cells, but not in Dll4-Fc–transduced SBC-5 cells, compared with that observed in control cells (Fig. 4F). These results indicate that a reduction of N1-ICD induced by Dll4-Fc attenuates the binding of p65, p50, and RelB to DNA and consequently reduces the level of NF-κB activity. Moreover, we determined the levels of migration activity of SBC-3 expressing Dll4-Fc and control cells stimulated by TNF-α (Fig. 4G). In accordance with Fig. 3B, Dll4-Fc suppressed the migration ability of SBC-3 cells. In addition, we found that the migration ability of SBC-3 cells was elevated with TNF-α treatment and significantly reduced by Dll4-Fc. These results suggest that downregulation of NF-κB activity induced by the reduction of N1-ICD is involved in the inhibition of liver metastasis through the repression of cell migration.

Discussion

In this study, we showed that Dll4-Fc inhibits the liver metastasis caused by the suppression of early steps of metastasis in SCLC cells expressing high levels of Dll4 (Supplementary Fig. S1A, Table 1 and Fig. 2C). Moreover,

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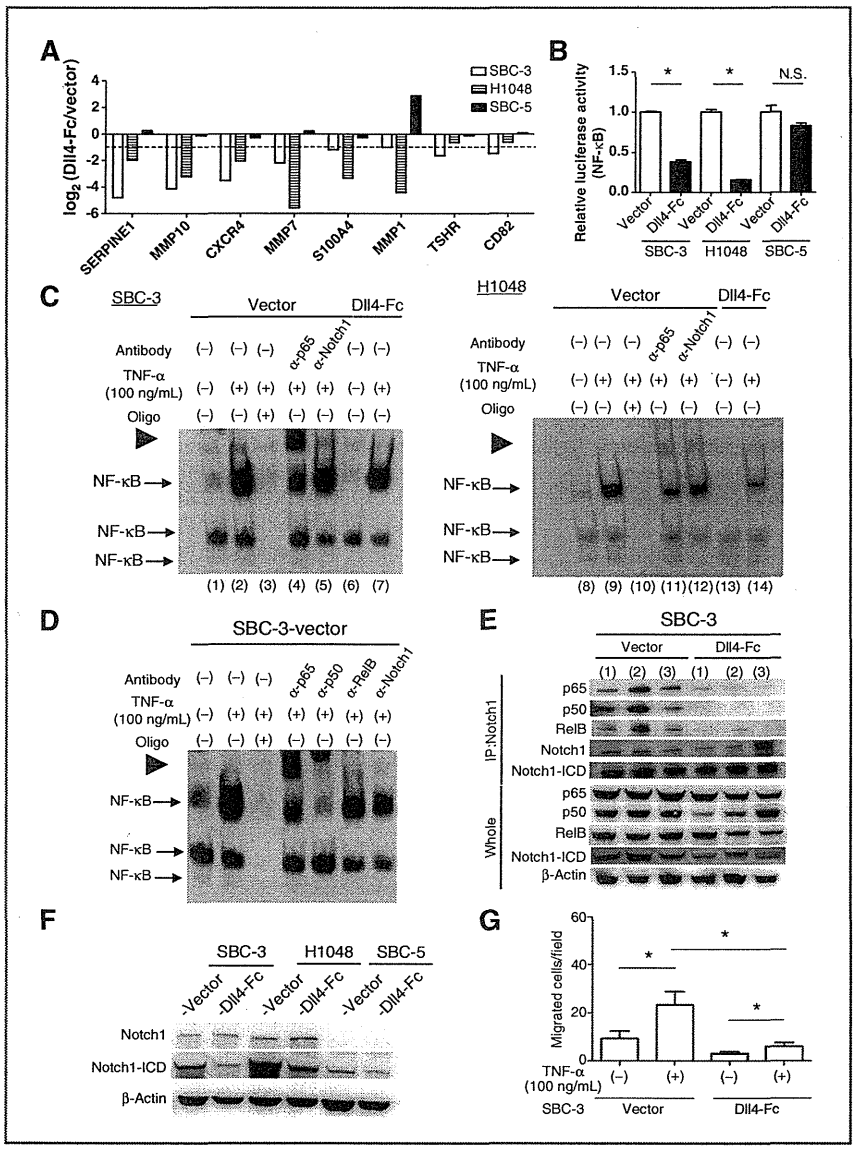

Figure 4. Dll4-Fc suppresses NF-κB activity via prevention of Notch signaling. A, the expression of metastasis-associated genes of SBC-3, H1048, and SBC-5 were measured using RT-PCR array systems. The data represent differences (log_2 fold changes) in the expression levels of the genes between Dll4-Fc–expressing and control cells. The dashed line indicates log_2 (fold changes) of −1.0. B, the effects of Dll4-Fc on NF-κB activity in SBC-3, H1048, and SBC-5 cells were determined using a luciferase assay. C, EMSAs were conducted on nuclear extracts of SBC-3 (left) and H1048 (right) cells. Competition assays were done with excesses of unlabeled NF-κB oligonucleotides (oligo). D, a supershift assay using antibodies against p65, p50, RelB, and Notch1 were conducted to confirm the DNA-bound complexes in the SBC-3-vector cells. C and D, DNA-bound complexes were identified (arrow). The arrowhead indicates the supershift. E, the protein extractions of liver metastasis produced by SBC-3 cells were immunoprecipitated (IP) with anti-Notch1 antibody, and immunoprecipitates or total extractions were immunoblotted. F, the levels of intracellular domain (ICD) of Notch1 were measured by immunoblotting. G, the effects of Dll4-Fc on the migration ability of SBC-3 cells treated with TNF-α (100 ng/mL) were measured using Transwell; *, $P < 0.01$ (Welch t test).

Dll4-Fc attenuated, at least in part, the classical and alternative NF-κB activation pathway by reducing Notch1 signaling (Fig. 4). These findings highlight the importance of Dll4-Notch1 signaling in liver metastasis from lung cancer.

Several studies showed that VEGF-induced Dll4 in tip cells regulated the formation of stalk cells (6–9). Noguera-Troise and colleagues revealed that blockage of Dll4-Notch signaling by Dll4-Fc in endothelial cells suppressed tumor growth by promoting nonproductive angiogenesis. In accordance with these reports, we showed that suppression of Dll4-Notch signaling by Dll4-Fc in cancer cells resulted in increase of endothelial cell density in liver metastasis produced by SBC-3 cells (Supplementary Fig. S4A and S4B). However, the number of endothelial cells in liver metastasis produced by H1048-Dll4-Fc cells tended to be higher than that of control cells, whereas the difference was statistically not significant (Supplementary Fig. S4C and S4D). Because the transduction of Dll4-Fc did not affect VEGFA expression and its receptor activity of HUVECs (Supplementary Fig. S4E and S4F), these results suggest that the suppression of Dll4-Notch signaling by Dll4-Fc in cancer cells may have an effect to promote nonproductive angiogenesis, but the effect may vary depending on the tumor microenvironment. However, this study indicates that Dll4-Fc suppresses the early steps of liver metastasis of the SBC-3 cells without affecting angiogenesis, implying that Notch signaling of cancer cells stimulated by adjacent cancer cells

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derived from Dll4 is implicated in metastasis. A previous report showed that Dll4 expressed in tumor cells regulated cancer growth and differentiation (26), and improved tumor vascular function and promoted tumor growth (27, 28). Moreover, Zhang and colleagues showed that Dll1-Notch signaling in cancer cells regulates invasion and metastasis *in vitro* and *in vivo* (29). These results suggest that Dll4-Notch signaling in cancer cells may be involved in the progression of cancer. Recent evidence showed that Notch signaling between cancer and stromal cells also play an important role in metastasis. Sonoshita and colleagues showed that stromal Dll4 and Jagged1 facilitated local tumor invasion and intravasation through the inhibition of amino-terminal enhancer of split (AES) as an endogenous metastasis suppressor (30). Although the current experiments did not reveal the effects of stromal-derived Dll4 on metastasis, these findings indicate that the activation of Notch signaling of cancer cells stimulated by adjacent cancer and stromal cells expressing Dll4 is implicated in metastasis.

NF-κB plays an important role in lung cancer progression, including metastasis (31). In this study, we showed that Dll4-Notch1 axis regulated NF-κB signaling irrespective of TNF-α stimulation. Several reports described the interactions between Notch and NF-κB signaling in normal and cancer cells; however, the underlying molecular mechanisms remain unclear (32). Previous studies showed that downregulation of Notch1 inhibited invasion of pancreatic cancer cells by inactivation of classical NF-κB (33). These studies showed that knockdown of Notch1 reduced p65/DNA binding activity. In accordance with these results, we showed that Dll4-Fc suppressed the NF-κB activity, and attenuated the migration and invasion abilities (Figs. 3 and 4). Moreover, Shin and colleagues revealed that Notch1 enhanced NF-κB activity by facilitating nuclear retention (34). They showed that a direct interaction between N1-ICD and NF-κB subunits can override the cytoplasmic sequestration of NF-κB by IκB proteins by promoting nuclear retention of NF-κB. Although we indicated interaction between N1-ICD and p65, the retention of NF-κB subunits in the nuclei was not detected irrespective of TNF-α stimulation (Supplementary Fig. S5). These discrepancies might be because of differences in the types of cells and ligands of stimulation. Although further studies are required to clarify the mechanism, these findings indicate that interactions of Notch1 with p65 and p50 with stimulation of TNF-α may play an important role in the formation of metastasis of SCLC. In addition, we found that Dll4-Notch1 signaling was involved in the alternative NF-κB activation pathway through the interaction of RelB. The role of alternative NF-κB activation pathway in cancer cells remained unclear. Several reports showed that Notch1 regulated the expression of p52 and RelB (35, 36). Because, as far as we know, it is the first report to show the interaction of Notch1 and RelB, these findings may be a novel mechanism for regulation of the alternative NF-κB activation pathway by Notch1.

In this study, we showed that Dll4-Fc suppressed cancer cell migration, but not cell growth or proliferation, through the downregulation of NF-κB. However, several reports showed that inhibition of NF-κB reduced tumor cell growth and proliferation (37). These discrepancies might be due to differences in the type of cells. We indicated that SBC-3 and H1048 cells showed weak activation of NF-κB activity without any stimulation, suggesting that the growth of these cells might not be dependent on the activation of NF-κB *in vitro* (Fig. 4C and Supplementary Fig. S3C).

Dll4-Fc suppressed metastasis in the liver but not in the kidney, lymph node, or bone. It remained unclear why Dll4-Fc preferentially suppressed liver metastasis (Table 1). In this study we showed that Dll4-Fc suppressed the CXCR4 expression in SBC-3 and H1048 cells (Fig. 4A). CXCR4 is a crucial gene belonging to the chemokine-receptor super family associated with the preference of metastatic sites (38). The CXCL12–CXCR4 axis is involved in regulating the liver, adrenal gland, bone marrow, and brain metastasis of non–small cell lung cancer, and the expression level of CXCL12 in the liver is higher than that in the kidney (39). The attenuation of the expression of CXCR4 by Dll4-Fc may cause an obvious suppression of liver metastasis. Further study is needed to elucidate the detailed mechanisms underlying the preferential suppression of liver metastasis by Dll4-Fc.

In summary, we have shown that Dll4-Fc suppressed liver micrometastasis of cancer cells expressing high levels of Dll4. In addition, we found that the microvessel densities of metastatic foci in the liver 21 days after inoculation of SBC-3-Dll4-Fc cells were comparable with those of the metastatic foci in control cells. The suppression of liver metastasis by Dll4-Fc may be caused by the attenuation of the classical and alternative activation pathway of NF-κB signaling through the inhibition of the Notch1 activity. Whether other Notch receptors implicate in the metastasis of SCLC, and which NF-κB pathways regulated by Notch1 are implicated in metastasis of SCLC requires further investigations. However, because several genes expression was specifically regulated in each of metastatic lesion (40), suggesting that the elucidation of mechanisms of each organ metastasis is necessary to develop effective therapies to control cancer metastasis, our findings could be useful for therapy of SCLC patients with multiorgan metastasis. Many Notch inhibitors and antibodies against Dll4 have been emerging as novel anticancer drugs. They may therefore be efficacious against liver metastasis in SCLC patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T. Kuramoto, H. Goto, S. Tabata, Y. Nishioka

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T. Kuramoto, H. Goto, S. Tabata
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Role of chemotherapy in treatments for biliary tract cancer

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Abstract The purpose of chemotherapy in patients with advanced solid cancers, including biliary tract cancer, is generally to improve the survival and quality of life of the patients. Also, adjuvant chemotherapy is expected to increase the curability of surgery in patients scheduled to undergo surgery. Most patients with unresectable biliary tract cancer develop obstructive jaundice, and biliary drainage is needed before any of the aforementioned treatments. Once jaundice is resolved by stenting of the bile duct or bilio-intestinal bypass, cholangitis often develops, leading to rapid deterioration of the patient's general condition. Therefore, the beneficial effect of chemotherapy in such patients remains controversial. A few randomized controlled trials have demonstrated the survival benefit of chemotherapy as compared with supportive care. In one of these trials, improvement of the quality of life was also confirmed. Recently, since the survival benefit of combined gemcitabine plus cisplatin therapy over gemcitabine alone has been demonstrated in randomized controlled clinical trials, this combined regimen has been recognized as a standard therapy for unresectable biliary tract cancer. A second-line regimen is now expected to be established for patients with gemcitabine-refractory biliary tract cancer, although the significance of second-line therapy remains unclear. One of the next issues in relation to chemotherapy for biliary tract cancer is the development of molecular-targeted agents; however, few large clinical trials of such agents have been conducted for biliary tract cancer. Various issues in chemotherapy for biliary tract cancer remain to be investigated, and global cooperation is necessary to conduct large clinical trials.

Keywords Biliary tract cancer · Chemotherapy · Survival benefit · Quality of life · Gemcitabine · Cisplatin

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Introduction

Biliary tract cancer is a common cause of cancer-related death in Asia, including Japan, and Latin America. In Japan, the mortality is estimated to be 17,000 deaths annually. While surgery remains the only potentially curative treatment, the curative resection rate remains low, at approximately 40% [1]. Most patients, furthermore, develop recurrence even after curative surgery. The poor prognosis is due to the difficulty in the diagnosis of biliary tract cancer in the earlier stages and the lack of satisfactory treatments for advanced disease.

Biliary tract cancer consists of cholangiocarcinoma, gallbladder cancer, and ampulla of Vater cancer; intrahepatic cholangiocarcinoma is also often included in clinical trials of treatments for biliary tract cancer. Each of these types of cancer has characteristic features and the treatment strategies and prognoses also differ. This heterogeneity has made it difficult to evaluate the efficacy of chemotherapy for biliary tract cancer, and randomized controlled trials (RCTs) with an appropriate stratification strategy, including by the tumor type, are required. Recently, a large RCT comparing combined gemcitabine plus cisplatin therapy with gemcitabine treatment alone demonstrated a survival benefit of the combined regimen over gemcitabine alone [2]. As a result, combined gemcitabine plus cisplatin therapy has come to be recognized as standard therapy for unresectable biliary tract cancer.

The purpose of chemotherapy in patients with advanced solid cancers, including biliary tract cancer, is generally to improve their survival and quality of life (QOL), and not to achieve a cure. Also, adjuvant chemotherapy is expected to increase the curability of surgery in patients scheduled to undergo surgery. There are some difficulties in the chemotherapy of patients with biliary tract cancer. Most patients present with obstructive jaundice at diagnosis, and biliary drainage is generally needed before any of the aforementioned treatments. Once the jaundice has been resolved by stenting of the bile duct or bilio-intestinal bypass, cholangitis often develops, resulting in rapid deterioration of the patient's general condition. Thus, the beneficial effect of chemotherapy for patients with unresectable biliary tract cancer remains controversial.

In this review, based on the recent advances in chemotherapy for biliary tract cancer, the significance and roles of systemic chemotherapy for patients with unresectable biliary tract cancer are discussed.

Improvement of survival in patients with unresectable biliary tract cancer

To assess the efficacy of chemotherapy in patients with advanced biliary tract cancer, some small RCTs comparing it with supportive treatment alone have been conducted (Table 1) [3–5]. Glimelius et al. [3] reported a comparative study between chemotherapy and supportive care in 90 patients with unresectable pancreatic cancer and biliary tract cancer. In this study, 5-fluorouracil (5-FU) + leucovorinor 5-FU + leucovorin + etoposide was compared with supportive care. For all the patients, the overall survival was significantly longer in the chemotherapy group than in the supportive care group (median 6.0 vs. 2.5 months, $P < 0.01$). In only the patients with biliary tract cancer, no significant difference in survival between the two groups was noted, due to the small number of patients (37 patients), and the survival in the two groups was similar (6.5 months in the chemotherapy group and 2.5 months in the supportive care group; $P = 0.1$). Takada et al. [4] conducted a comparative study in Japan comparing 5-FU + doxorubicin + mitomycin C (FAM) with palliative treatment, such as bypass, in patients with unresectable pancreatic cancer, gallbladder cancer, and bile duct cancer. No improvement in the prognosis was demonstrated in either treatment group overall, but longer survival was achieved in the chemotherapy group than in the control group for the 18 patients with gallbladder cancer (median 5.16 months in the chemotherapy arm and 2.4 months in the control arm).

Recently, a comparative study of modified gemcitabine/oxaliplatin (Gemox), 5-FU/folinic acid (FA),and best supportive care was reported in patients with unresectable gallbladder cancer [5]. The modified Gemox regimen yielded a statistically significantly higher response rate, progression-free survival, and overall survival as compared with 5-FU/FA chemotherapy and best supportive care. Although these studies included only a small number of patients, these results suggest a survival benefit of chemotherapy in patients with unresectable biliary tract cancer and adequate organ and bone marrow function, as long as obstructive jaundice and cholangitis can be controlled.

Promising agents for biliary tract cancer were examined in retrospective studies before a large RCT was conducted. In a pooled analysis of 104 phase II studies, significant correlations of the response rate and tumor control rate with the survival times were observed, and the response rate and tumor control rate were highest in the patients treated with a gemcitabine–platinum combination [6]. Four hundred thirteen consecutive patients administered non-surgical treatments were reviewed in a Japanese retrospective study [7]. To clarify the impact of systemic chemotherapy on the survival and identify promising agents for biliary tract cancer, the hazard ratios and 95% confidence intervals (CI) were estimated by Cox regression by subgroup of chemotherapeutic regimen as compared with best supportive care. The median overall survival in the best supportive care group was 3.12 months and that in the chemotherapy group was 7.38 months, and a statistically significant difference in survival was noted between the two groups ($P = 0.0001$). The adjusted hazard ratio in the Cox regression model using confounders for gemcitabine was 0.53 (95% CI 0.34–0.82) and for cisplatin -based regimens it was 0.49 (95% CI 0.36–0.99). Thus, gemcitabine and platinum were identified as promising agents for the treatment of biliary tract cancer.

A randomized phase II study (ABC-01) comparing gemcitabine alone with gemcitabine plus cisplatin was conducted in the United Kingdom [8]. It demonstrated superior 6-month progression-free survival (57.1 vs. 45.5%) with acceptable toxicity in the gemcitabine 1,000 mg/m^2 plus cisplatin 25 mg/m^2 group as compared with that in the gemcitabine 1,000 mg/m^2-alone group, and was therefore expanded to a phase III study (ABC-02). The results revealed a statistically significant improvement in the overall survival in the gemcitabine-plus cisplatin group as compared with that in the gemcitabine-alone group (Table 2) [2]. The BT-22 study was planned in Japan following the promising results of the ABC-01 study, and results similar to those of the ABC-02 study were demonstrated in Japanese patients with biliary tract cancer (Table 2) [9].

Improvement of the quality of life

It is difficult to assess efficacy based on the QOL, especially in patients with biliary tract cancer, because there are

Table 1 Trials comparing chemotherapy and supportive care in patients with unresectable biliary tract cancer

	n	Median OS (months)	*P* value	References
5-FU/leucovorin or 5-FU/leucovorin/etoposide	47	6	<0.01	Glimelius et al. [3]
Supportive care	43	2.5		
5-FU/doxorubicin/mitomycin C	42	4.96	0.283	Takada et al. [4]
Control	41	4.7		
Gemcitabine/oxaliplatin	27	9.5	0.039	Sharma et al. [5]
5-FU/folinic acid	28	4.6		
Best supportive care	27	4.5		

5-FU fluorouracil, *OS* overall survival

Table 2 Efficacy of first-line chemotherapy for unresectable biliary tract cancer

Regimen	*n*	Response rate (%)	Median PFS (months)	Median OS (months)	References
Gemcitabine	206	15.5	5.0	8.3	Valle et al. [2]
Gemcitabine/cisplatin	204	26.1	8.0	11.7	
Gemcitabine	42	11.9	3.7	7.7	Okusaka et al. [9]
Gemcitabine/cisplatin	41	19.5	5.8	11.2	
Gemcitabine/capecitabine	45	32	6.0	14.0	Cho et al. [13]
Gemcitabine/capecitabine	75	29	6.2	12.7	Riechelmann et al. [14]
Gemcitabine/capecitabine	44	25	7.2	13.2	Koeberle et al. [15]
Gemcitabine/S-1	35	34.3	5.9	11.6	Sasaki et al. [16]

PFS progression-free survival, *OS* overall survival

many specific symptoms due to tumor progression and/or obstruction of the bile duct in patients with advanced biliary tract cancer. In the ABC-02 study, it was demonstrated that patients who received gemcitabine had a significantly increased incidence of grade 3 or 4 liver function test abnormalities, possibly as a result of inferior disease control and biliary drainage as compared with that in the group administered combined gemcitabine plus cisplatin therapy [2]. This finding suggests that a higher efficacy of chemotherapy against tumor progression in patients with biliary tract cancer might contribute to maintaining the patency of the bile duct and prevent cholangitis due to re-obstruction of the bile duct.

Improvement in the QOL was also examined in a trial comparing chemotherapy and supportive care, with QOL assessed by using the European Organization for Research and Treatment of Cancer quality of life questionnaire (EORTC QLQ-C30) version 1.0 questionnaire [3]. The results revealed a significant QOL improvement in the chemotherapy group ($P < 0.01$); a 36% improvement was observed in the chemotherapy group (pancreatic cancer 38%, biliary tract cancer 33%) and a 10% (pancreatic cancer 13%, biliary tract cancer 5%) improvement was shown in the supportive care group. In the analysis by symptom, significant improvements of pain, fatigue, appetite, and dyspnea after 2 months, and that of pain and dyspnea after 4 months were observed in the chemotherapy group.

Second-line therapy for unresectable biliary tract cancer

Now that first-line chemotherapy has been established, the development of a second-line therapy would be the next logical step to improve survival in patients with unresectable biliary tract cancer. New oral fluoropyrimidines, such as capecitabine and tegafur/gimeracil/oteracil potassium (S-1), which are prodrugs of FU, have been investigated as monotherapy or in combination with gemcitabine. There are few prospective clinical trials that have focused on second-line therapy only in patients with biliary tract cancer. S-1 was investigated in patients with gemcitabine-refractory biliary tract cancer in a phase II study [10]. There were 3 confirmed partial responses (7.5%) among the 40 patients assessed, and 22 patients (55%) with stable disease. The median progression-free and overall survivals were 2.5 and 7.5 months, respectively. Despite the acceptable toxicity of S-1, its efficacy was modest, and these results could serve as reference data for the development of second-line therapy.

The impact of second-line therapy after gemcitabine-based chemotherapy was assessed in the ABC-02 and BT-22 studies [11]. In the ABC-02 trial conducted in the United Kingdom, the treatment of patients with disease progression was left to individual clinicians' discretion, and was best supportive care for the majority, with only

17% of patients receiving further chemotherapy, mostly 5-FU-based chemotherapy. On the other hand, in the BT-22 study conducted in Japan, 73% of the patients in the gemcitabine-plus-cisplatin group and 78% of the patients in the gemcitabine-alone group received post-study chemotherapy. S-1 was approved for the treatment of biliary tract cancer based on the results of a first-line phase II study in Japan [12], and, consequently, more than 60% of the patients in the BT-22 study were treated with S-1 as second-line therapy [9]. This difference in the rate of application of second-line therapy could have potentially improved the overall survival in the BT-22 study as compared with that in the ABC-02 study, although the overall survival in the two studies was quite similar. So far, therefore, the impact of second-line therapy in patients with gemcitabine-refractory biliary tract cancer remains unclear.

Future perspectives on chemotherapy for unresectable biliary tract cancer

Gemcitabine has been recognized as a key drug for the treatment of biliary tract cancer, and many phase II studies of gemcitabine have been conducted. The combination of gemcitabine plus the oral fluoropyrimidines capecitabine or S-1 also showed promising activity in phase II studies. These phase II studies yielded response rates of 25–34% and a median overall survival of 11.6–14.0 months (Table 2) [13–16]. A randomized phase II study comparing combined gemcitabine plus S-1 chemotherapy and S-1 monotherapy was conducted by the Japanese Clinical Oncology Group (JCOG) to evaluate the efficacy and safety of the two regimens and to determine which was the more promising regimen as a test arm regimen for comparison with the current standard regimen; namely, gemcitabine plus cisplatin [17]. If the combination of gemcitabine plus S-1 is found to be promising, a large RCT comparing this combination with gemcitabine plus cisplatin would be warranted.

One of the next issues that need to be addressed is whether molecular-targeted agents might also exert activity against biliary tract cancer. To date, no large clinical trials of molecular-targeted agents have been conducted for biliary tract cancer; however, some of these agents appear to offer promise. A combination of Gemox plus bevacizumab, a recombinant humanized monoclonal antibody directed against vascular endothelial growth factor (VEGF), yielded promising results in a phase II study, with a response rate of 40% and median overall survival of 12.7 months [18]. A phase II study of Gemox plus cetuximab, an anti-epidermal growth factor receptor (EGFR) antibody, also demonstrated promising efficacy, with a response rate of 63% and median overall survival of 15.2 months [19]. A randomized phase II study comparing Gemox plus cetuximab and Gemox alone has been conducted in France [20], and has been expanded to a phase III study.

Usage of monotherapy with targeted agents as second-line chemotherapy is also expected. Some preclinical experiences show that VEGF receptor or EGFR inhibitors administered alone may exert efficacy against biliary tract cancer. In many patients with progressive disease receiving first-line chemotherapy with the relatively toxic regimen of gemcitabine plus cisplatin or Gemox, the general condition may be poor, and serious cholangitis can easily develop. Less toxic therapy, such as monotherapy with a targeted agent, may be useful in such patients.

Conclusions

Effective chemotherapy is necessary to improve the survival and QOL of patients with biliary tract cancer. Because biliary tract cancer is a relatively rare disease as compared with other gastrointestinal cancers, such as colorectal cancer or gastric cancer, large clinical trials of treatments for this cancer are difficult to conduct. Efforts to establish new standard chemotherapies are ongoing, and international collaboration is necessary for the success of these efforts.

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

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資　料

がん診療の経済的な負担に関するアンケート調査

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「がんの医療経済的な解析を踏まえた患者負担の在り方に関する研究」

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＜調査の趣旨＞

新しいがん対策推進基本計画では、療養する患者さんが安心して働き暮らせる社会の構築が目ざされています。このアンケートは、経済的な負担ができるだけ少ない、がん医療の実践に向けた基礎資料を得ることを目的としています。

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○ アンケートに参加されなくても、今後の診療に不利益は生じません。

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