

**Figure 2** (A) Individual plasma concentrations of PTX in seven patients following 1-h intravenous infusion of NK105 at a dose of 150 mg m<sup>-2</sup>. (B) Relationships between dose and C<sub>max</sub>, and (C) between dose and AUC<sub>0-inf</sub>, of PTX in patients following 1-h intravenous infusion of NK105. Regression analysis for dose vs C<sub>max</sub> was applied using all points except one patient at 80 mg m<sup>-2</sup> whose medication time became 11 min longer and one patient at 180 mg m<sup>-2</sup> who had medication discontinuation and steroid medication. (Plots were shown as open circle.) Regression analysis for dose vs AUC<sub>0-inf</sub> was applied using all points except one patient who had medication discontinuation and steroid medication. (Plot was shown as open circle.) Relationships between dose and C<sub>max</sub>, and AUC<sub>0-inf</sub> in patients following conventional PTX administration were plotted (closed square, see Tamura *et al*, 1995).

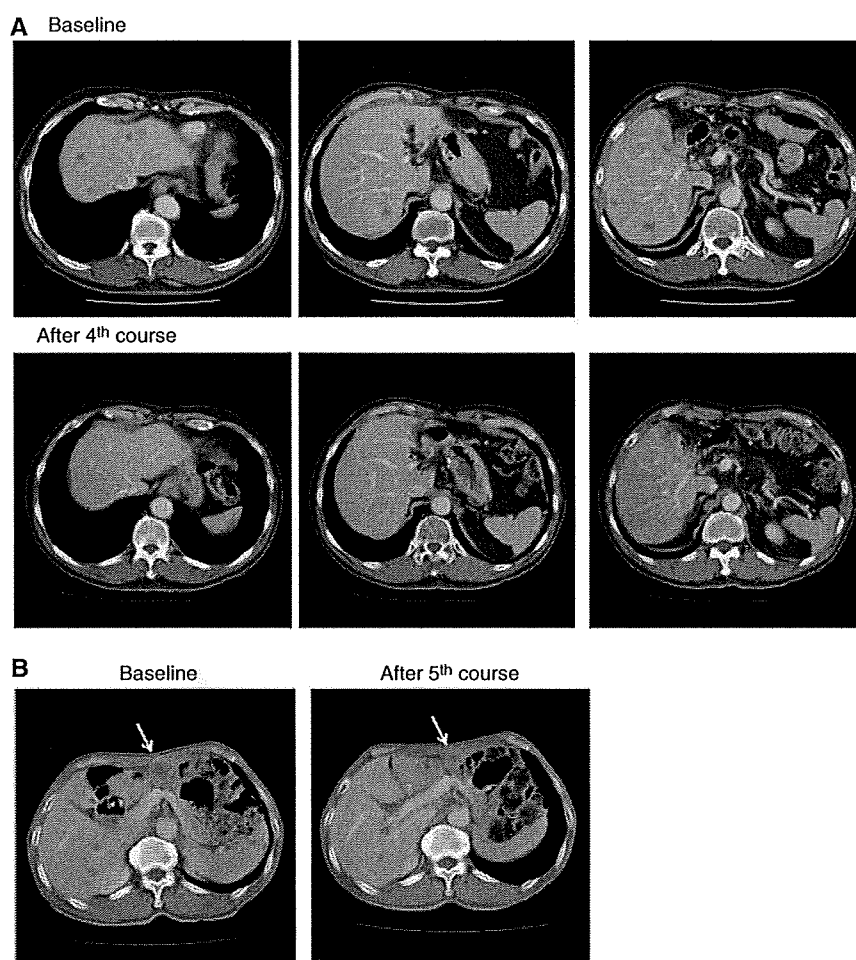
**Table 3** Pharmacokinetic parameters

	Dose (mg m <sup>-2</sup> )	n	C <sub>max</sub> (µg ml <sup>-1</sup> )	AUC <sub>0-inf</sub> (µg h ml <sup>-1</sup> )	t <sub>1/2</sub> (h)	CL <sub>tot</sub> (ml h <sup>-1</sup> m <sup>-2</sup> )	V <sub>ss</sub> (ml m <sup>-2</sup> )	UE <sup>a</sup> (%)	CL <sub>r</sub> (ml h m <sup>-2</sup> )	
NK105	10	1	0.9797	11.4	9	880.4	10400.3	7.5	66.4	
	20	1	2.8971	29.1	8.5	687.9	8027	8.6	59.4	
	40	1	8.8334	93.9	13.2	426.1	5389.8	5.2	22	
	80	1	18.4533	149.3	7	535.8	5875.8	4.7	25.3	
	110	3	23.3924	232	9.7	483.3	5881.2	7.6	35.6	
				±5.6325	±39.1	±1.6	±82.7	±1512.0	±1.7	±6.9
	150	7	40.1699	369.8	10.6	408.6	4527.1	5.3	21.6	
			±5.5334	±35.2	±1.3	±37.3	±639.5	±1.5	±6.5	
180	4 <sup>b</sup>	45.6278	454.5	11.3	416.5	4983.4	5.9	23.7		
			±8.6430	±119.1	±0.6	±104.7	±887.5	±1.4	±4.2	

<sup>a</sup>UE, urinary excretion. <sup>b</sup>One patient at 180 mg m<sup>-2</sup> level was omitted from the calculation of summary pharmacokinetic parameters, as there was administering interruption for developing allergic reactions.

Indeed, the results of this clinical trial show that NK105 can be administered safely as a short infusion (1 h) without the administration of antiallergic agents like dexamethasone and antihistamine, although one patient at 180 mg m<sup>-2</sup> developed transient grade 2 hypersensitivity at the first course. Therefore, NK105 may offer advantages in terms of safety and patient convenience and comfort.

The pharmacokinetic analysis of NK105 suggests that the distribution of PTX-incorporating micelles is mostly restricted to the plasma and, in part, to extracellular fluids in the body. This is consistent with data obtained in a preclinical study (Hamaguchi *et al*, 2005) showing that the distribution of NK105 in tissues is characterised by an EPR effect, similar to that of tumour and inflammatory lesions, or by the presence of a reticuloendothelial



**Figure 3** Serial CT scans. **(A)** A 60-year-old male with pancreatic cancer who was treated with NK105 at a dose level of  $150 \text{ mg m}^{-2}$ . Baseline scan (upper panels) showing multiple metastasis in the liver. Partial response, characterized by a more than 90% decrease in the size of the liver metastasis (lower panels) compared with the baseline scan. The antitumour response was maintained for nearly 1 year. **(B)** A 64-year-old male with stomach cancer who was treated with NK105 at a dose level of  $150 \text{ mg m}^{-2}$ . Baseline scan (left panel) showing a peritoneal metastasis and liver metastasis. About 40% reduction (right panel) was observed in peritoneal metastasis, but not in the liver metastasis after fifth course.

**Table 4** Pharmacokinetic parameters

	Dose ( $\text{mg m}^{-2}$ )	n	$C_{\text{max}}$ ( $\mu\text{g ml}^{-1}$ )	$\text{AUC}_{0-\text{inf}}$ ( $\mu\text{g h}^{-1} \text{ml}^{-1}$ )	$t_{1/2}$ (h)	$\text{CL}_{\text{tot}}$ ( $\text{ml h}^{-1} \text{m}^{-2}$ )	$V_{\text{ss}}$ ( $\text{ml m}^{-2}$ )	UE (%)	$\text{CL}_r$ ( $\text{ml h m}^{-2}$ )
NK105	150	7	40.1699 $\pm 5.5334$	369.8 $\pm 35.2$	10.6 $\pm 1.3$	408.6 $\pm 37.3$	4527.1 $\pm 639.5$	5.3 $\pm 1.5$	21.6 $\pm 6.5$
PTX	210	5	6.744 $\pm 2.733$	23.18 $\pm 10.66$	13.3 $\pm 1.5$	10740 $\pm 4860$	58 900 $\pm 24 700$	9.45 $\pm 3.76$	1020 $\pm 648$
XYOTAX <sup>a</sup>	233	4	NA	1583 $\pm 572$	120 $\pm 28$	276 $\pm 63$	6200 $\pm 2100$	NA	NA
Abraxane	300	5	13.52 $\pm 0.95$	17.61 $\pm 3.70$	14.6 $\pm 2.04$	17 700 $\pm 3894$	370 000 $\pm 85 100$	NA	NA
Genoxol-PM	300	3	3.107 $\pm 1.476$	11.58 $\pm 4.28$	11.4 $\pm 2.4$	29 300 $\pm 13 800$	NA	NA	NA

<sup>a</sup>Conjugated taxanes.

system. When compared with conventional PTX at a dose of  $210 \text{ mg m}^{-2}$  (conventional dose for a 3-week regimen in Japanese patients), NK105 at a dose of  $150 \text{ mg m}^{-2}$  (recommended phase II dose) exhibited more than 15-fold larger plasma AUC and a 26-fold lower  $\text{CL}_{\text{tot}}$ . The larger plasma AUC is consistent with the stability of the micelle formulation in plasma. The  $V_{\text{ss}}$  of NK105

was 13-fold lower than that of conventional PTX. This suggests that PTX may have a relatively lower distribution in normal tissue, including normal neural tissue, following NK105 administration. Regarding the drug distribution in tumours, nanoparticle drug carriers have been known to preferentially accumulate in tumour tissues utilising the EPR effect (Matsumura and Maeda, 1986;

Maeda *et al*, 2000; Duncan, 2003). We speculate that NK105 accumulates more in tumour tissues than free PTX, since NK105 is very stable in the circulation and exhibits a markedly higher plasma AUC than free PTX. Moreover, a polymeric micelle carrier system for a drug has the potential to enable the sustained release of the drug inside a tumour following the accumulation of micelles in the tumour tissue (Hamaguchi *et al*, 2005; Uchino *et al*, 2005; Koizumi *et al*, 2006). Regarding NK105 in particular, this sustained release may begin at a PTX-equivalent dose of  $<1 \mu\text{g ml}^{-1}$  (data not shown). Consequently, the released PTX is distributed throughout the tumour tissue where it kills the cancer cells directly.

In the present study, NK105 appeared to exhibit characteristic pharmacokinetics different from those of other PTX formulations including conventional PTX, Abraxane, Genexol-PM, and Xyotax. For example, previous clinical PK data at each phase II

recommended dose shown that plasma AUC and  $C_{\text{max}}$  were 11.58 and 3.1 in Genexol-PM (Table 4). The antitumour activities seen in two patients with intractable cancers are encouraging. In addition, we recently demonstrated in preclinical study that combined NK105 chemotherapy with radiation exerts a significantly more potent antitumour activity, compared with combined PTX therapy and radiation (Negishi *et al*, 2006). This data on NK105 justifies its continued clinical evaluation.

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## Novel SN-38–Incorporated Polymeric Micelle, NK012, Strongly Suppresses Renal Cancer Progression

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### Abstract

It has been recently reported that NK012, a 7-ethyl-10-hydroxy-camptothecin (SN-38)–releasing nanodevice, markedly enhances the antitumor activity of SN-38, especially in hypervascular tumors through the enhanced permeability and retention effect. Renal cell carcinoma (RCC) is a typical hypervascular tumor with an irregular vascular architecture. We therefore investigated the antitumor activity of NK012 in a hypervascular tumor model from RCC. Immunohistochemical examination revealed that Renca tumors contained much more CD34-positive neovessels than SKRC-49 tumors. Compared with CPT-11, NK012 had significant antitumor activity against both bulky Renca and SKRC-49 tumors. Notably, NK012 eradicated rapid-growing Renca tumors in 6 of 10 mice, whereas it failed to eradicate SKRC-49 tumors. In the pulmonary metastasis treatment model, an enhanced and prolonged distribution of free SN-38 was observed in metastatic lung tissues but not in nonmetastatic lung tissues after NK012 administration. NK012 treatment resulted in a significant decrease in metastatic nodule number and was of benefit to survival. Our study shows the outstanding advantage of polymeric micelle-based drug carriers and suggests that NK012 would be effective in treating disseminated RCCs with irregular vascular architectures. [Cancer Res 2008;68(6):1631–5]

### Introduction

Passive targeting of the drug delivery system is suited to combating the pathophysiologic characteristics present in many solid tumors: hypervascularity, irregular vascular architecture, potential for secretion of vascular permeability factors, and the absence of effective lymphatic drainage that prevents efficient clearance of macromolecules. These characteristics, unique to solid tumors, are believed to be the basis of the enhanced permeability and retention (EPR) effect (1). Polymeric micelle-based anticancer drugs have recently been developed (2, 3), and some were put under evaluation for clinical trials (4, 5).

7-Ethyl-10-hydroxy-camptothecin (SN-38), a biological active metabolite of irinotecan hydrochloride (CPT-11), has potent antitumor activity, but has not been used clinically because it is a water-insoluble drug. It has been recently shown that novel SN38-incorporated polymeric micelles, NK012, have the potential

to allow effective sustained release of SN-38 inside a tumor and possess potent antitumor activities especially in a vascular endothelial growth factor (VEGF)–secreting hypervascular tumor (6), because the supramolecular structures of NK012 which enable SN-38 to accumulate in the target tissue are based on the EPR effect (1).

Renal cell carcinoma (RCC) is a typical hypervascular tumor with an irregular vascular architecture. We therefore conducted an investigation to determine whether NK012 would be effective in treating RCC by using established RCC tumor models with pulmonary metastasis.

### Materials and Methods

**Drugs and cells.** CPT-11 was purchased from Yakult Honsha Co., Ltd. SN-38 and NK012 was prepared and supplied by Nippon Kayaku Co., Ltd. (6). Five human RCC lines (SKRC-49, Caki-1, 769P, 786O, and KU19-20) and murine Renca cells were maintained in DMEM or MEM supplemented with 2 mmol/L glutamine, 1% nonessential amino acids, 100 units/mL streptomycin and penicillin, and 10% FCS.

**In vitro growth inhibition assay.** The growth inhibitory effects of NK012, SN-38, and CPT-11 were examined with a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay, as described previously (6).

**In vivo growth inhibition assay.** The animal experimental protocols were approved by the Committee for Ethics of Animal Experimentation, and the experiments were conducted in accordance with the Guidelines for Animal Experiments in the National Cancer Center. Athymic nude mice (3–4 wk old) were maintained in a laminar air flow cabinet under aseptic conditions. 10<sup>7</sup> RCC cells were s.c. injected into the backs of the mice. NK012 at doses of 10 mg/kg/d or 20 mg/kg/d and CPT-11 at doses of 15 mg/kg/d or 30 mg/kg/d were given i.v. on days 0 (when tumors were allowed to grow until they became massive in size, around 1.5 cm), 4, and 8. Tumor volume was determined by direct measurement with calipers and calculated as  $\pi/6 \times (\text{large diameter})^2 \times (\text{small diameter})^2$ .

**Assessment of treatment effects of NK012 on murine pulmonary metastasis model.** A total of  $1 \times 10^5$  Renca cells were inoculated into male BALB/c mice via the tail vein. The mice were randomly divided into three groups of 10. NK012 at dose of 20 mg/kg/d and CPT-11 at dose of 30 mg/kg/d were given i.v. on days 0 (7 d after inoculation), 4, and 8. After that, the mice were sacrificed, their lungs were stained intratracheally with 15% India black ink solution, and the number of metastatic nodules in each mouse was counted. To determine the effect of NK012 on survival, an identical experiment to the one described above was done. After treatment, mice were maintained until each animal showed signs of morbidity (i.e., over 10% weight loss compared with untreated controls), at which point they were sacrificed. Kaplan-Meier analysis was done to determine the effect on time to morbidity, and statistical differences were ranked according to a Mantel-Cox log-rank test using the StatView 5.0 software package.

**Histologic and immunohistochemical analysis.** Histologic sections were taken from Renca tumor tissues. After extirpation, tissues were fixed with 3.9% formalin in PBS (pH 7.4), and the subsequent preparations and H&E staining were performed by Tokyo Histopathological Laboratory Co.,

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**Table 1.** *In vitro* growth inhibitory activity of SN-38, NK012, and CPT-11 in RCC lines (MTT assay)

Cell line	IC <sub>50</sub> (μmol/L)		
	SN-38	NK012*	CPT-11
SKRC-49	0.0064 ± 0.005	0.011 ± 0.008	4.14 ± 0.45
Caki-1	0.0062 ± 0.009	0.032 ± 0.006	8.45 ± 0.85
769P	0.015 ± 0.007	0.085 ± 0.014	34.54 ± 3.76
786O	0.031 ± 0.007	0.12 ± 0.012	28.14 ± 1.21
KU19-20	0.10 ± 0.006	0.34 ± 0.014	32.65 ± 1.25
Renca	0.045 ± 0.005	0.0096 ± 0.008	2.26 ± 0.05

\*The dose of NK012 is expressed as a dose equivalent to SN-38.

Ltd. Monoclonal anti-CD34 antibody (HyCult Biotechnology) was used to detect the tumor blood vessels. CD34-positive neovessels were counted in 10 high-power fields (×400) by two independent investigators who operated in a blinded fashion.

**Assay for free (polymer-unbound) SN-38 in lung tissues.** The Renca pulmonary metastasis model described above was used for the analysis of the biodistribution of NK012 and CPT-11. Ten days after Renca inoculation, NK012 (20 mg/kg) or CPT-11 (30 mg/kg) was given i.v. to the mice. The mice were sacrificed at 0, 24, 48, and 72 h after administration, and lung samples were taken and stored at -80°C until analysis. We prepared control mice without Renca inoculation as the nonmetastatic model; NK012 was administered as well, and lung samples were stored. Samples were then homogenized on ice using a Digital homogenizer (Iuchi) and suspended in the mixture of 100 mmol/L glycine-HCl buffer (pH 3)/methanol (1:1, v/v) at a concentration of 5% w/w. Proteins were precipitated with an ice-cold mixture of 1 mmol/L H<sub>3</sub>PO<sub>4</sub>/MeOH/H<sub>2</sub>O (1:1:4, v/v/v) containing camptothecin as an I.S. The sample was vortexed for 10 s and filtered through a MultiScreen Solvintert (Millipore Corporation), and the concentration of free SN-38 in the aliquots of the homogenates (100 μL) was determined using the high-performance liquid chromatography method (6).

**Statistical analysis.** Data were expressed as mean ± SD. Significance of differences was calculated using the unpaired *t* test with repeated measures of StatView 5.0. *P* < 0.05 was regarded as statistically significant.

## Results and Discussion

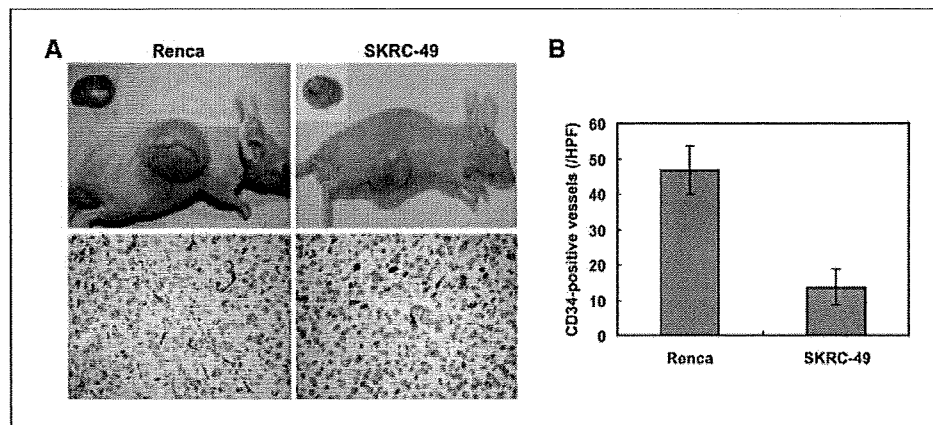
We first evaluated *in vitro* cellular sensitivity of RCC lines to SN-38, NK012, and CPT-11. The IC<sub>50</sub> values of each agent for RCC lines are shown in Table 1. NK012 exhibited higher cytotoxic effect

against each cell line compared with CPT-11 (96-fold to 406-fold sensitive).

It is essential to elucidate the correlation between the effectiveness of micellar drugs and tumor hypervascularity and hyperpermeability. Gross evaluation of those RCC tumors s.c. injected into the backs of mice revealed that Renca tumors were more reddish and grew faster than SKRC-49 tumors, and immunohistochemical examination showed that Renca tumors contained much more CD34-positive neovessels than SKRC-49 tumors (Fig. 1).

We allowed the tumors to grow until they became massive, around 1.5 cm, and then initiated treatment. A striking decrease in Renca tumor volume was observed on day 15 in mice treated with NK012 at 20 mg/kg/d compared with the untreated control (Fig. 2A). Renca bulky masses completely disappeared on day 21 in 6 of 10 mice treated with NK012 at 20 mg/kg/d. On the other hand, Renca tumors in mice treated with CPT-11 at 30 mg/kg/d were not eradicated and rapidly regrew after a partial response at day 15. An approximate 10% body weight loss occurred in mice treated with NK012 20 mg/kg, compared with the untreated controls, but there was no significant difference in comparison with tumor-free mice treated with NK012, suggesting that the decrease in body weight was likely to be due to tumor shrinkage rather than toxic effects. We next compared the antitumor activities of the NK012 and CPT-11 treatment in SKRC-49 and Renca tumors. The SKRC-49 tumor volume in mice treated with NK012 at 20 mg/kg/d on day 21 was over 70% smaller than in the untreated controls on day 21 and ~50% smaller than in mice on day 0 (Fig. 2B). However, the SKRC-49 tumors were not eradicated in mice treated with NK012. Considering that equivalent *in vitro* growth inhibitory effects by NK012 were observed for SKRC-49 and Renca cells (Table 1), our results suggest that the antitumor activity of NK012 *in vivo* might be affected by tumor environment factors, such as tumor vascularity.

We next examined the distribution of free SN-38 in the metastatic or nonmetastatic (no inoculation of Renca cells) lung tissues after administration of NK012 or CPT-11. In the case of NK012 administration in mice with lung metastasis, free SN-38 was detectable at the concentration of >100 ng/g in metastatic lung tissues with a typical microvascular architecture (Fig. 3A) even at 72 hours after administration, whereas the concentrations of free SN-38 in nonmetastatic lung tissues after NK012 administration were much lower than those in metastatic lung tissues after treatment with NK012 (significant at 24, 48, and 72 hours; *P* < 0.05;



**Figure 1.** Comparison of tumor angiogenesis of Renca and SKRC-49 in athymic nude mice. *A*, representative photographs of massive tumors developed from Renca and SKRC-49 at 28 d after s.c. injection (inoculation). Immunohistochemical (CD34, ×400) examinations for each tumor are shown. *B*, tumor neovascularization in each tumor was quantified by counting CD34-positive neovessels. Bars, SD. Experiments were repeated twice with similar results.

**Figure 2.** Growth-inhibitory effect of NK012 and CPT-11 on bulky RCC tumors. I.v. administration of NK012 or CPT-11 was started when the mean tumor volumes of groups reached a massive 1,500 mm<sup>3</sup>. The mice were divided into test groups as indicated. *A*, representative of each group at day 15 in the Renca allograft model. Arrows, Renca allografts (top). Time profile of tumor volume in mice treated with NK012 or CPT-11 at indicated doses (bottom). Each group consisted of 10 mice. Bars, SD. *B*, the comparison of antitumor activities of CPT-11 and NK012 in SKRC-49 xenografts and Renca allografts. Representative of mice treated with NK012 at day 0 and day 21. Experiments were repeated twice with similar results. The mice at day 0 in the photograph belong to the group in the second experiment which started just at day 21 of the first experiment. Arrows, tumor grafts. The relative tumor volume values at day 21 to those at day 0 in each group set to 1 (bottom). Each group consisted of 10 mice.

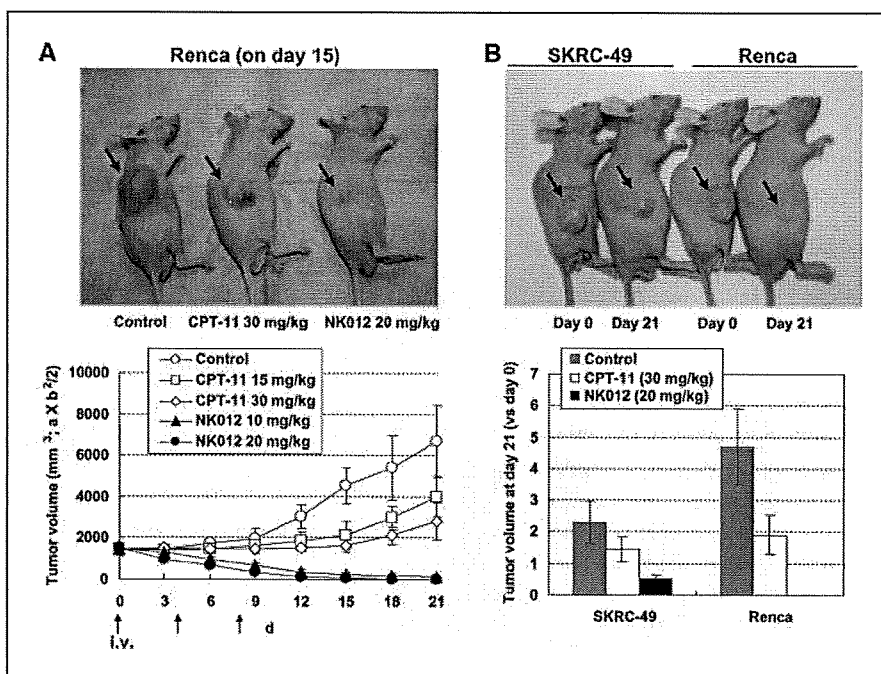
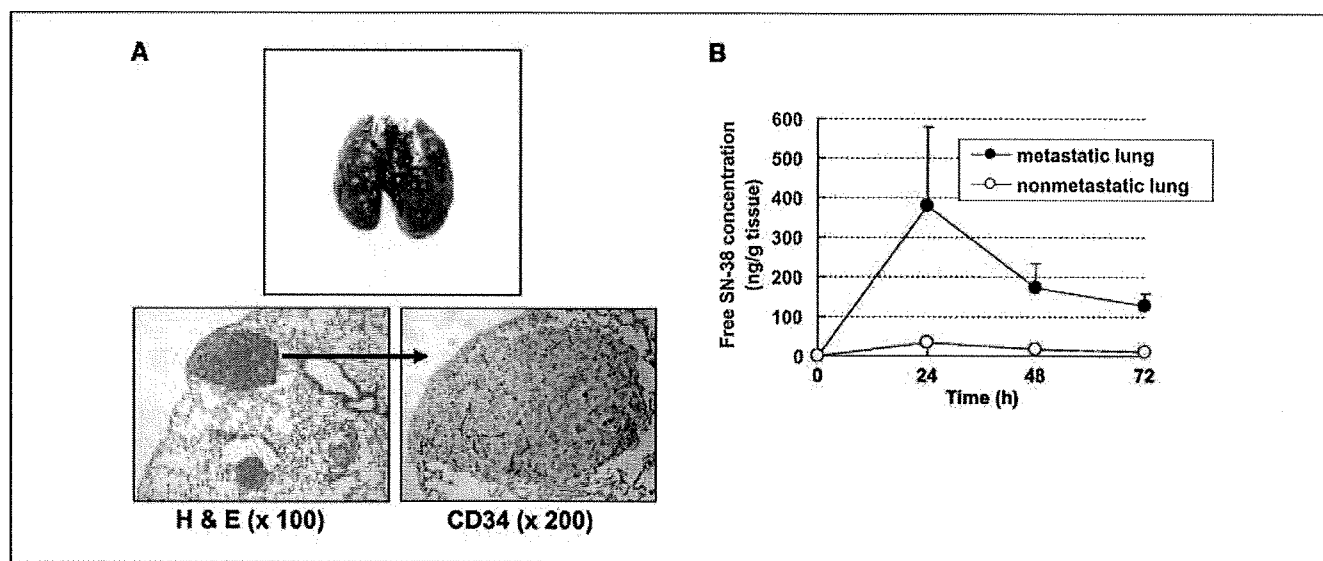


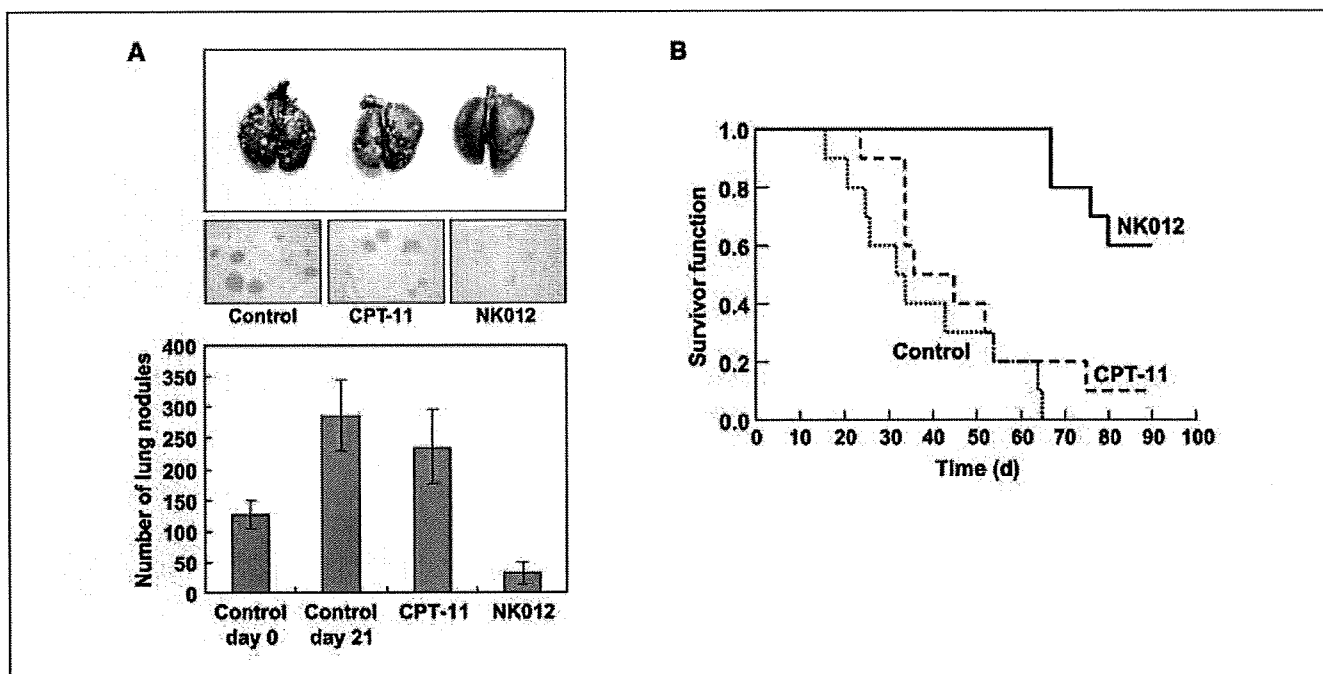
Fig. 3B). On the other hand, the concentrations of free SN-38 after administration of CPT-11 were almost negligible in metastatic lung tissues at all time points (data not shown). These results strongly suggest that SN-38 could be selectively released from NK012 and maintained in metastatic Renca tumor tissues.

Deviating from the ordinary experimental pulmonary metastasis prevention model, we initiated treatment 7 days after inoculation (day 0) when multiple lung nodules derived from Renca were observed in all mice in our preliminary study (Fig. 4A). On day 21, there was no significant difference between the mean number of

metastatic nodules in the control group (287 ± 56 nodules, n = 10) and in the group receiving CPT-11 treatment (236 ± 59 nodules, n = 10). Significant treatment effects were found, however, in the group receiving NK012 treatment (32 ± 18 nodules, n = 10) on day 21 compared with the control group on day 21 (P < 0.0001). Notably, a dramatic decrease in metastatic nodule number was observed in the NK012 treatment group on day 21 compared with the control group on day 0 (126 ± 23 nodules, n = 10, P < 0.001; Fig. 4A). Kaplan-Meier analysis showed that a significant survival benefit was obtained in the NK012 treatment group compared with



**Figure 3.** Pulmonary metastasis of Renca cells and lung tissue distribution of free SN-38 after administration of NK012 and CPT-11. *A*, gross appearances of pulmonary metastasis observed 7 d after Renca inoculation (top). Multiple metastatic nodules and neovascularization in metastatic lung tumor lesion (bottom). *B*, time profile of free SN-38 concentration in metastatic or nonmetastatic lung tissues in mice treated with NK012 (20 mg/kg/d). Bars, SD. Experiments were performed in tetraplicate.



**Figure 4.** Treatment effect of NK012 on established pulmonary metastasis and survival. NK012 (20 mg/kg/d) and CPT-11 (30 mg/kg/d) were given i.v. to mice with established pulmonary metastasis on days 0 (7 d after Renca inoculation), 4, and 8. *A*, gross and histologic appearances of pulmonary metastases at day 21 (*top*). The metastatic nodules in each mouse were counted. Each group consisted of five mice. *B*, mice were maintained for 90 d after each treatment and survival was assessed by a Kaplan-Meier analysis. Each group consisted of five mice. Experiments were repeated twice with similar results.

the control group ( $P < 0.001$ ), but no significant survival benefit was obtained in CPT-11 treatment group ( $P = 0.239$ ; Fig. 4*B*). Although no severe toxic effects were observed in any mouse treated with NK012, 3 of 10 mice treated with NK012 were sacrificed during the observation period according to the 'Guidelines for Animal Experiments because their body weights had become 10% lower than those of the other mice. However, the sacrificed mice were a little bit smaller than others when they started treatment, and they showed no disseminated lung metastasis (data not shown).

Our results presented here strongly support recent findings reported by us that the macromolecular drug distribution throughout the tumor site was enhanced by the hypervascularity and hyperpermeability, and subsequently higher antitumor activity was achieved (6). We assume that conventional low molecular size anticancer agents almost disappear from the bloodstream without being subjected to the EPR effect before they can reach the target organs (solid tumor). The clinical importance of angiogenesis in human tumors has been shown in several reports indicating a positive relationship between the blood vessel density in the tumor mass and poor prognosis with chemoresistance in patients with various cancers (7–9). Furthermore, recent reports showing that anticancer agents were less active against VEGF-overexpressing tumors (10, 11) may support the idea that low-molecular drugs are not so effective in the treatment of solid tumors which are rich in blood vessels.

Our study thus far has several limitations about clarifying whether extensive angiogenesis in the tumor is an essential determinant for the susceptibility to NK012. In our ongoing study, we found that NK012 also has a striking antitumor activity against some hypovascular tumor models of human pancreatic cancer

xenografts.<sup>5</sup> It also remains unclear whether NK012 possesses strong antitumor activity in other metastatic sites besides the lung. It is known that the EPR effect is affected by various permeability factors, such as bradykinin (12), nitric oxide (13), and various cytokines independent of VEGF and hypervascularity (14). Among solid tumors with rapid progression potential, irregularity occurs not only in blood flow and vascular density, but also in the vascular network and anatomic architecture (15, 16), suggesting that EPR effect may be predominantly promoted in rapid-progressive tumor phenotypes and influenced by organ-specific tumor microenvironment. Hoffman and coworkers (17, 18) have developed a technique of surgical orthotopic implantation (SOI) with more clinical features of systemic and aggressive metastases than our conventional animal models. Further preclinical studies using such models as SOI might clarify cancer phenotypes and metastatic organs to which we can apply NK012 more precisely.

The results of chemotherapy in RCCs have been disappointing, as indicated by the low response proportions. However, clinical trials using gemcitabine-containing regimens have been encouraging, with major responses occurring in 5% to 17% of patients (19, 20), suggesting the possibility that chemotherapy is promising as a modality for RCC therapy if anticancer agents can be selectively delivered, released, and maintained around tumor tissues. Our current report highlights the advantages of polymeric micelle-based drug carriers like NK012 as promising modalities for treatment, rather than prevention, of disseminated RCCs with abnormal vascular architecture. The results of our ongoing phase-I

<sup>5</sup> Y. Saito, M. Yasumaga, J. Kuroda, Y. Koga, and Y. Matsumura. Unpublished data.



clinical trial and future phase-II trials of NK012 in patients with advanced solid tumors including RCC might meet or even exceed our expectations.

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# Autosomal loci associated with a sex-related difference in the development of autoimmune phenotypes in a lupus model

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Sex-related differences (SrD) are a general characteristic of human autoimmune diseases. There is an increasing body of evidence that suggests a link between sex-related hormones and autoimmune onsets. Here, through a genetic approach using a lupus mouse model, we attempted to show the involvement of genetic factors in the development of SrD in autoimmune diseases. Using MRL/lpr × (MRL/lpr × C57BL/6.Fas<sup>lpr</sup>)F1 (MBN2) mice, the whole genome was searched to identify linkage loci to autoimmune phenotypes inherited from a lupus MRL/Mp.Fas<sup>lpr</sup> (MRL/lpr) strain of mice, which exhibits glomerulonephritis, splenomegaly and antinuclear autoantibody. The genome-wide association study confirmed four linkage loci on chromosomes 4, 7, 13, and 17. Furthermore, differential analyses performed using male and female groups of MBN2 mice revealed that two loci located on chromosomes 4 (41–72 cM, MRL/lpr allele) and 7 (4–21 cM, B6/lpr allele) were male specific and suppressed autoimmune phenotypes. Notably, the sum effect of the two loci adequately explained a range of SrD developed in the MBN2 mice. Our present findings suggest the presence of a male-predominant mechanism underlying the development of SrD in autoimmunity, depending on the effects of autosomal loci under an undefined male-specific condition.

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**Key words:**  
 Animal models  
 · Autoimmunity  
 · Genetics  
 · Rheumatology

## Introduction

Human autoimmune diseases occur due to interactions between genetic and environmental factors. Although these factors vary depending on every entity of autoimmune disease, female predominant onset is a general characteristic [1]. Indeed, the female to male incidence ratios for systemic lupus erythematosus (SLE), Sjögren's syndrome, Hashimoto disease, and rheumatoid arthritis (RA) are estimated to be 9:1, 9:1, 10:1, and 4:1, respectively. It is widely accepted that a crucial mechanism underlies the female susceptibility or male resistance to the development of an autoimmune disease.

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**Abbreviations:** GN: glomerulonephritis · LOD: logarithm of odds · **Marc4**: male-specific autoimmune-resistant locus on chromosome 4 · **Marc7**: male-specific autoimmune-resistant locus on chromosome 7 · **MBF1**: (MRL/lpr × B6/lpr)F1 · **MBN2**: (MRL/lpr × MBF1)N2 · **QTL**: quantitative trait locus · **SpM**: splenomegaly · **SrD**: sex-related difference

Past studies conducted using animal autoimmune models have shown that an increased but non-toxic load of sex hormone [2–4], treatment with agonistic or antagonistic agent related to a sex hormone receptor [2, 4–6], or removal of sex glands [7, 8] affects the incidence or severity of autoimmune phenotypes. In humans, polymorphisms of genes encoding sex hormone receptors were shown to be associated with the onset of SLE [9] and RA [10, 11]. Transcriptional regulation of immunologically important genes by a sex hormone has been documented [12, 13]. These previous findings indicate the important role of sex hormones in the development of a sex-related difference (SrD) in autoimmune diseases.

The other lines of study have suggested a direct link between abnormality in sex chromosome and incidence of autoimmune disease. Congenital X-chromosome monosomy, *i.e.*, 45 XO Turner's syndrome, has been shown to render marked susceptibility to autoimmune disorders [14, 15]. A recent report demonstrated a high incidence of somatic X-chromosome monosomy in peripheral immune cells of patients with systemic sclerosis and autoimmune thyroiditis [16]. In this report, we have suggested the possible role of haploinsufficiency of X-linked genes in the onset of autoimmune diseases. These findings underscore the direct effect of sex chromosome-linked genes on the development of SrD in autoimmune diseases.

Autoimmune animal models have provided opportunities for understanding the genetic predisposition of autoimmune diseases. We have been using an autoimmune-prone strain of mice – MRL/Mp.*Fas*<sup>lpr</sup> (MRL/lpr) – to investigate the etiology of autoimmune disorders through genetic approaches. This strain was selected because it has the advantage of spontaneous development of a spectrum of autoimmune phenotypes that resemble human SLE. This strain carries a defective mutation in the *Fas* gene, denoted as *lpr* (for lymphoproliferation). The *Fas* protein is a cell-surface receptor that mediates apoptotic signals in activated lymphocytes. Therefore, the *lpr* mutation explains persistent autoimmune responses and lymphoproliferation due to the accumulation of abnormal CD4<sup>+</sup>, CD8<sup>+</sup>, CD45R (B220)<sup>+</sup>, and Thy1<sup>+</sup> T cells in the MRL/lpr strain [17, 18]. On the other hand, it has been shown that other *lpr* congenic strains of mice – C3H/HeJ.*Fas*<sup>lpr</sup> and C57BL/6J.*Fas*<sup>lpr</sup> (B6/lpr) – barely develop autoimmune diseases [19–21], suggesting that the onset of the ultimate *lpr*-induced phenotypes depends on the genetic background of *lpr*. Genetic studies that relied on these *lpr*-related strains have provided explanations on the constitution of autoimmune susceptibility other than *lpr*.

Previous studies have suggested the lack of SrD in MRL/lpr mice [22–25]. In the early stage of this study,

we carefully confirmed that MRL/lpr mice hardly display any SrD related to the severity of autoimmune phenotypes, including glomerulonephritis (GN), splenomegaly (SpM), and autoantibody to nuclear antigens (ANA). In contrast, the descendent strains – (MRL/lpr × B6/lpr)F1 (MBF1) and (MRL/lpr × MBF1)N2 (MBN2) – were found to exhibit significant SrD, suggesting the presence of strain-specific genetic factors responsible for the development of SrD in the MBF1 and MBN2 strains. Here, we attempted to identify sex-specific loci to elucidate the development of SrD in the MBN2 model. Differential genetic analyses performed using the male and female groups of MBN2 mice revealed two male-specific autosomal loci associated with the suppression of autoimmune phenotypes. These findings suggest the presence of a male-specific mechanism underlying the SrD in autoimmune disorders.

## Results

### Histopathological characterization of glomerulonephritis in MBN2 mice

Fully developed GN in the MRL/lpr and MBN2 mice was similarly characterized by increases in the cellular and matrix components of the glomerulus (data not shown). As described in the *Materials and methods*, two different indexes – cellular and matrix grades – were used to quantify a pathological change in the glomerulus in the MBN2 mice independently. We observed a significant correlation between the two grades of GN (Supporting Information Fig. 1). This finding suggests the presence of a mutual dependence of the cellular and matrix proliferations in the development of GN in the MBN2 mice. Hereafter, we adopted a log<sub>10</sub>-transformed index based on the two grades as an individual GN index.

### Strain-specific onset of SrD in autoimmune phenotypes

Four autoimmune phenotypes including GN, SpM, ANA of IgM class, and ANA of IgG class were quantified in the MRL/lpr, B6/lpr, MBF1, and MBN2 mice. The results showed that the MRL/lpr mice developed all the phenotypes without any evidence of SrD (Table 1). On the other hand, the MBN2 mice developed all the phenotypes with significant SrD. The MBF1 and B6/lpr mice also exhibited significant SrD for some phenotypes. The severity of any of these phenotypes in the male and female groups was found to be normally distributed (Fig. 1). These findings suggest the contribution of complex genetic factors, including B6/lpr-derived factor(s), to the onset of SrD in MBF1 and MBN2 mice.

**Table 1.** Severity of autoimmune phenotypes in male and female groups of MRL/lpr, B6/lpr, MBF1 and MBN2 mice<sup>a)</sup>

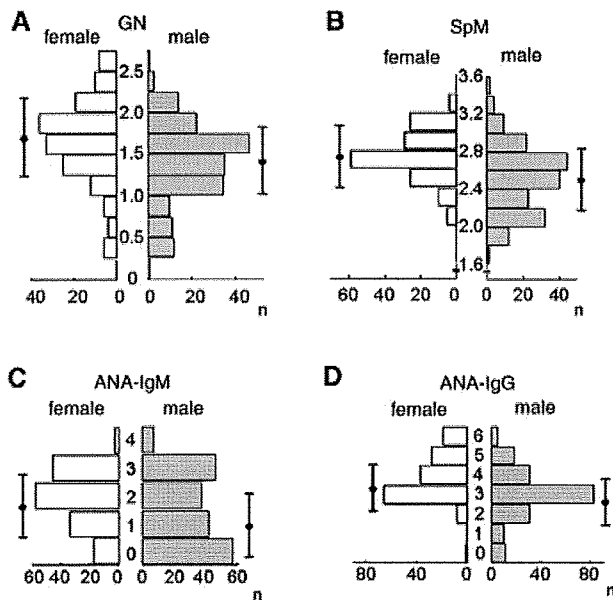
strain	group	trait <sup>b)</sup>			
		GN log <sub>10</sub> index	ANA-IgM titer score	ANA-IgG titer score	SpM mg
MRL/lpr	total	2.29 (0.198, 23)	2.80 (0.847, 30)	4.90 (0.803, 30)	557 (284, 28)
	male	2.25 (0.182, 12)	2.75 (0.860, 12)	5.00 (0.852, 12)	514 (180, 13)
	female	2.33 (0.213, 11)	2.83 (0.857, 18)	4.83 (0.786, 18)	594 (353, 15)
B6/lpr	total	0.803 (0.414, 25)	0.696 (0.635, 23)	2.48 (0.70, 23)	213 (98.0, 17)
	male	0.648 (0.234, 9)	0.688 (0.704, 16)	2.50 (0.730, 16)	132 (34.5, 9) ***
	female	0.891 (0.471, 16)	0.714 (0.488, 7)	2.43 (0.535, 7)	305 (51.7, 8)
MBF1 <sup>c)</sup>	total	1.52 (0.554, 56)	1.94 (1.15, 53)	3.87 (0.680, 53)	725 (338, 56)
	male	1.13 (0.445, 22) ***	1.77 (1.11, 22)	3.46 (0.583, 22) ***	482 (221, 22) ***
	female	1.76 (0.475, 34)	2.06 (1.18, 31)	4.16 (0.596, 31)	881 (308, 34)
MBN2 <sup>d)</sup>	total	1.51 (0.518, 348)	1.67 (1.17, 348)	3.39 (1.32, 348)	549 (440, 348)
	male	1.38 (0.480, 189) ***	1.49 (1.25, 189) *	2.99 (1.30, 189) ***	460 (458, 189) ***
	female	1.67 (0.520, 159)	1.88 (1.01, 159)	3.87 (1.17, 159)	656 (394, 159)

<sup>a)</sup> Value denotes a mean (S.D., number of mice) of trait index. Difference of averages between male and female groups were estimated by Student's *t*-test. \**p* < 0.05; \*\*\**p* < 0.0001.

<sup>b)</sup> GN, glomerulonephritis; ANA, antinuclear autoantibody; SpM, splenomegaly.

<sup>c)</sup> (MRL/lpr × B6/lpr) F1.

<sup>d)</sup> MRL/lpr × (MRL/lpr × B6/lpr) N2.



**Figure 1.** SrD in severity-stratified distribution of the autoimmune traits in MBN2 mice. (A) GN index represents the disease severity, estimated by log<sub>10</sub> [(mean of cellular grades) + (mean of matrix grades)]. The distribution of log<sub>10</sub>-transformed GN indexes was estimated as normal. (B) SpM index denotes log<sub>10</sub>-transformed spleen weight. (C, D) Titers of ANA were determined by immunofluorescence analysis. A diagonal dot with lines by each bar graph indicates a mean with SD. For all the indexes, difference between the male and female groups is statistically significant.

### Identification of four loci associated with autoimmune phenotypes

By genome-wide screening, we evaluated the difference in the average severity of each autoimmune phenotype between two subgroups of 95 MBN2 mice that were sorted depending on the genotype at every microsatellite position: MRL/MRL homozygote and MRL/B6 heterozygote. The *t* test (*p* < 0.05) preliminarily qualified nine regions on chromosomes 1, 2, 4, 5, 7, 10, 11, 13, and 17 as candidate loci for any of the autoimmune phenotypes (Supporting Information Fig. 2). We next examined other approximately 100 MBN2 mice that were randomly selected, and re-evaluated the preliminary nine loci using the accumulated data in the same manner, except that the threshold *p* value for acceptance was < 0.02. At this stage, we differentially examined the male and female groups. Four regions on chromosomes 4, 7, 13, and 17 were estimated as significant. Finally, association studies and quantitative trait locus (QTL) analyses performed using 348 MBN2 mice confirmed their statistical significance (Table 2). Analysis with relative spleen weight (mg/g of body weight) reproduced extremely similar results.

**Table 2.** Association of microsatellite genotypes<sup>a)</sup> and autoimmune phenotypes in MBN2 mice

trait <sup>b)</sup>	chr.	marker	position cM	Male		<i>p</i> <sup>c)</sup>	Female		<i>p</i> <sup>c)</sup>
				mean (S.D., number of mice) MM	mean (S.D., number of mice) MB		mean (S.D., number of mice) MM	mean (S.D., number of mice) MB	
GN (log <sub>10</sub> index)	4	D4Mit124	57.4	1.24 (0.50, 89)	1.52 (0.42, 100)	<b>0.00004**</b>	1.59 (0.56, 85)	1.76 (0.46, 74)	0.0050
		D4Mit71	61.9	1.43 (0.56, 85)	1.60 (0.46, 104)	<b>0.00023*</b>	1.61 (0.56, 90)	1.74 (0.46, 69)	0.11
	7	D7Mit57	4	1.50 (0.46, 98)	1.26 (0.47, 91)	<b>0.00082*</b>	1.67 (0.55, 82)	1.69 (0.55, 77)	0.83
		D7Mit267	11	1.48 (0.48, 99)	1.28 (0.46, 90)	0.0043	1.69 (0.56, 86)	1.68 (0.51, 73)	0.95
		D7Mit155	15	1.47 (0.48, 99)	1.29 (0.46, 90)	0.0087	1.69 (0.56, 88)	1.65 (0.48, 71)	0.62
	13	D13Mit24	43	1.36 (0.45, 85)	1.41 (0.51, 104)	0.48	1.79 (0.54, 76)	1.56 (0.48, 83)	0.0046
		D13Mit147	49	1.37 (0.45, 87)	1.40 (0.50, 102)	0.67	1.79 (0.49, 74)	1.56 (0.52, 85)	0.0048
	17	D17Mit197	9.65	1.42 (0.44, 95)	1.35 (0.51, 94)	0.35	1.70 (0.51, 84)	1.64 (0.53, 75)	0.47
D17Mit214		18.7	1.41 (0.46, 97)	1.36 (0.50, 92)	0.45	1.70 (0.51, 83)	1.63 (0.54, 76)	0.41	
ANA-IgM (titer score)	4	D4Mit124	57.4	1.21 (1.10, 89)	1.74 (1.33, 100)	0.0037	1.80 (1.02, 85)	1.99 (1.00, 74)	0.25
		D4Mit71	61.9	1.17 (1.13, 85)	1.76 (1.29, 104)	<b>0.0010*</b>	1.76 (1.04, 90)	2.06 (0.95, 69)	0.062
	7	D7Mit57	4	1.77 (1.27, 98)	1.20 (1.18, 91)	<b>0.0017*</b>	1.94 (1.02, 82)	1.83 (1.01, 77)	0.60
		D7Mit267	11	1.79 (1.28, 99)	1.17 (1.14, 90)	<b>0.00057*</b>	1.92 (1.00, 86)	1.85 (1.04, 73)	0.85
		D7Mit155	15	1.77 (1.28, 99)	1.19 (1.16, 90)	<b>0.0014*</b>	1.93 (1.00, 88)	1.83 (1.04, 71)	0.70
	13	D13Mit24	43	1.73 (1.31, 85)	1.30 (1.17, 104)	0.018	1.93 (1.02, 76)	1.84 (1.00, 83)	0.57
		D13Mit147	49	1.67 (1.27, 87)	1.34 (1.22, 102)	0.077	1.97 (0.96, 74)	1.81 (1.01, 85)	0.32
	17	D17Mit197	9.65	1.63 (1.20, 95)	1.35 (1.29, 94)	0.12	1.92 (0.92, 84)	1.85 (1.11, 75)	0.70
D17Mit214		18.7	1.63 (1.21, 97)	1.35 (1.29, 92)	0.12	1.92 (0.91, 83)	1.86 (1.12, 76)	0.71	
ANA-IgG (titer score)	4	D4Mit124	57.4	2.73 (1.21, 89)	3.22 (1.34, 100)	0.0089	3.77 (1.09, 85)	4.00 (1.25, 74)	0.21
		D4Mit71	61.9	2.68 (1.24, 85)	3.24 (1.30, 104)	<b>0.0030*</b>	3.70 (1.16, 90)	4.10 (1.15, 69)	0.031
	7	D7Mit57	4	3.20 (1.17, 98)	2.76 (1.40, 91)	0.018	4.00 (1.16, 82)	3.74 (1.16, 77)	0.50
		D7Mit267	11	3.20 (1.16, 99)	2.76 (1.41, 90)	0.018	3.95 (1.16, 86)	3.78 (1.18, 73)	0.67
		D7Mit155	15	3.18 (1.16, 99)	2.78 (1.41, 90)	0.032	3.96 (1.17, 88)	3.77 (1.16, 71)	0.53
	13	D13Mit24	43	3.20 (1.29, 85)	2.82 (1.29, 104)	0.044	3.83 (1.19, 76)	3.92 (1.15, 83)	0.74
		D13Mit147	49	3.15 (1.36, 87)	2.85 (1.24, 102)	0.12	3.92 (1.13, 74)	3.83 (1.20, 85)	0.65
	17	D17Mit197	9.65	3.08 (1.26, 95)	2.89 (1.34, 94)	0.31	3.68 (1.05, 84)	4.08 (1.25, 75)	0.025
D17Mit214		18.7	3.10 (1.26, 97)	2.87 (1.34, 92)	0.22	3.69 (1.08, 83)	4.08 (1.23, 76)	0.034	
SpM (log <sub>10</sub> mg)	4	D4Mit124	57.4	2.42 (0.34, 89)	2.60 (0.34, 100)	<b>0.00039*</b>	2.70 (0.26, 85)	2.79 (0.25, 74)	0.033
		D4Mit71	61.9	2.42 (0.35, 85)	2.59 (0.34, 104)	<b>0.0011*</b>	2.70 (0.27, 90)	2.80 (0.24, 69)	0.012
	7	D7Mit57	4	2.59 (0.34, 98)	2.43 (0.35, 91)	<b>0.0025*</b>	2.76 (0.27, 82)	2.73 (0.26, 77)	0.52
		D7Mit267	11	2.58 (0.34, 99)	2.44 (0.35, 90)	0.0060	2.76 (0.27, 86)	2.72 (0.25, 73)	0.41
		D7Mit155	15	2.58 (0.35, 99)	2.45 (0.35, 90)	0.012	2.76 (0.27, 88)	2.72 (0.25, 71)	0.27
	13	D13Mit24	43	2.58 (0.39, 85)	2.46 (0.31, 104)	0.013	2.81 (0.28, 76)	2.68 (0.22, 83)	<b>0.0020*</b>
		D13Mit147	49	2.59 (0.39, 87)	2.45 (0.30, 102)	0.0055	2.80 (0.28, 74)	2.69 (0.23, 85)	0.0073
	17	D17Mit197	9.65	2.60 (0.39, 95)	2.43 (0.30, 94)	<b>0.0011*</b>	2.80 (0.28, 84)	2.68 (0.22, 75)	0.0060
D17Mit214		18.7	2.60 (0.39, 97)	2.42 (0.29, 92)	<b>0.00051*</b>	2.80 (0.29, 83)	2.68 (0.22, 76)	<b>0.0012*</b>	

<sup>a)</sup> MM and MB denote MRL/MRL homozygote and MRL/B6 heterozygote, respectively.

<sup>b)</sup> GN, glomerulonephritis; ANA, antinuclear antibody; dsDNA, anti-double stranded DNA antibody; SpM, splenomegaly.

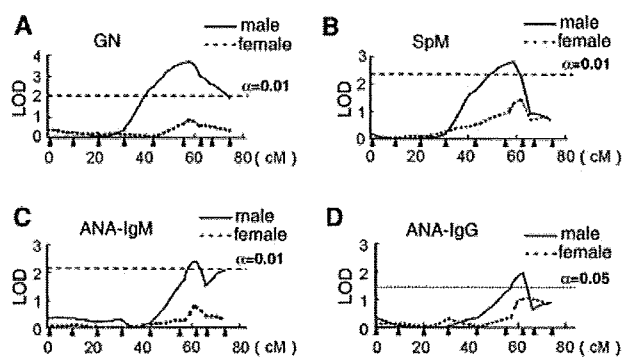
<sup>c)</sup> Student's t-test. \* *p* < 0.0034 (suggestive); \*\* *p* < 0.0001 (significant).

### Male-predominant autoimmune-resistant locus on chromosome 4

Data from the 348 MBN2 mice revealed male-specific associations of the MRL/lpr allele with all the autoimmune phenotypes examined at *D4Mit124* (57.4 cM) and/or *D4Mit71* (61.9 cM). These associations were observed in the male group; the phenotypes of the MRL/lpr allele demonstrated a recessive resistance inheritance (Table 2). The QTL analyses of the male group demonstrated a single logarithm of odds (LOD) peak beyond the threshold of significance for GN, SpM, ANA-IgM, and ANA-IgG at positions 57.3, 59.4, 61.7, and 61.9 cM, respectively (Fig. 2). In contrast, such common associations were not observed in the female group (Fig. 2). Here, we designated the linkage interval that included all significant QTL points (41–72 cM) as *Marc4* (for male-specific autoimmune-resistant locus on chromosome 4).

### Male-predominant autoimmune-resistant locus on chromosome 7

Suggestive associations of the genotypes with GN, ANA-IgM, and SpM were observed at *D7Mit57* (4 cM) or *D7Mit267* (11 cM) in the male group; the phenotypes of the B6/lpr allele demonstrated a dominant resistance inheritance (Table 2). Although these markers also showed tendencies for association with ANA-IgG, the tendencies were not statistically supported. The QTL analyses of the male group demonstrated a single LOD peak beyond the threshold of significance for GN, ANA-IgM, and SpM at positions 5.8, 9.6, and 4.7 cM,

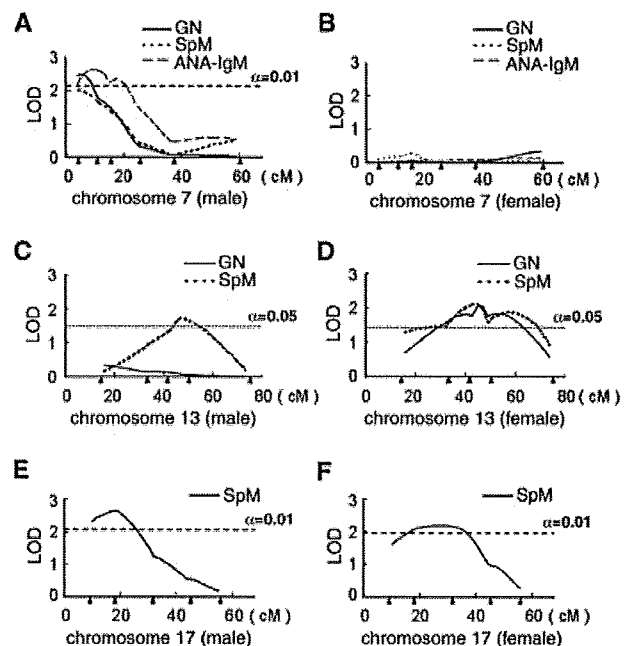


**Figure 2.** Plots of LOD scores of QTL on chromosome 4 for an autoimmune trait in MBN2 mice. The genetic positions (cM) of microsatellite markers used in QTL analysis are indicated on horizontal axis with arrowheads. A peak of LOD for GN (A), SpM (B), ANA-IgM (C), ANA-IgG (D) was identified at the position 57.3, 59.4, 61.7, and 61.9 cM, respectively. We adopted interval mapping in the Windows QTL Cartographer (V2.5) software. Horizontal broken and dotted line lines indicate the threshold level of statistic significance at  $\alpha=0.01$  and  $\alpha=0.05$ , respectively.

respectively (Fig. 3). Here, we designated the linkage interval that included all significant QTL points (4–21 cM) as *Marc7* (for male-specific autoimmune-resistant locus on chromosome 7).

### Autoimmune susceptibility loci on chromosomes 13 and 17

We detected two suggestive associations of the genotypes with SpM at *D13Mit24* (43 cM) and *D17Mit214* (18.7 cM) (Table 2). The association at *D13Mit24* was observed only in the female group; however, the QTL analyses for SpM demonstrated a single LOD peak between *D13Mit24* and *D13Mit147* (49 cM) in both male and female groups (Fig. 3), suggesting that this locus is not exclusively female predominant. The association at *D17Mit214* was observed without any sex-related predominance (Table 2). This was supported by the significant QTL that included this marker position (Fig. 3). A significant QTL for GN was observed between *D13Mit24* and *D13Mit147* in the female group (Fig. 3).



**Figure 3.** Plots of LOD scores of QTL for an autoimmune trait on chromosomes 7, 13, and 17. The genetic positions (cM) of microsatellite markers used in QTL analysis are indicated on horizontal axis with arrowheads. The male and female results are shown on the left and right side, respectively. (A) Peaks of LOD for GN, ANA-IgM, and SpM were identified in the male group of MBN2 mice at the position 5.8, 9.6, and 4.7 cM, respectively, on chromosome 7. (B) No significant peak was observed in the female group on this chromosome. The genetic linkages to SpM and GN were observed on chromosome 13 (C, D) and 17 (E, F). The threshold levels are indicated as in Fig. 2.

### Effects of *Marc4* and *Marc7* on SrD in autoimmune phenotypes

The male and female groups of MBN2 mice were each divided into four subcohorts based on the genotype combinations of *D4Mit124* and *D7Mit57*, which are included in *Marc4* and *Marc7*, respectively. The averages and SD of the trait index of each subcohort are summarized in Table 3. Significant variance in the average indexes among the four subcohorts was evident in the male group ( $p < 0.001$ ). Except the effect of *Marc4* on SpM, each locus exhibited a significant effect on the SrD for every autoimmune phenotype of interest in the male group. The two loci exhibited an additive suppressive effect on these phenotypes, which was accountable for the entire range of SrD developed in the MBN2 mice. To evaluate the interaction between two QTL of interest, we adopted the R/qlt program [26]. The strength of the interaction between two QTL was estimated by the LOD score in the interaction mode. An interaction was not observed for any two QTL identified on chromosomes 1, 4, 7, 13, and 17, which included *Marc4* and *Marc7*. The results on *Marc4* and *Marc7* are presented in Table 4.

### Discussion

Female preponderance in the incidence of human autoimmune diseases implies that sex-specific conditions yield susceptibility to autoimmune diseases. It has been principally considered that a sex hormone directly or indirectly affects immune cell functions and that this effect results in autoimmune reaction enhancement in females or its suppression in males. The present study revealed two male-specific autosomal loci – *Marc4* and *Marc7* – that dominated the suppression of autoimmune phenotypes in the present autoimmune model. These findings suggest a male-specific mechanism underlying SrD in the onset of autoimmune diseases. Concurrently, a new question arose on the interaction between sex hormones and these loci. Although this should be investigated by further studies, the effect of *Marc4* cannot be explained by this interaction.

There are inconsistent facts that MBN2 mice show remarkable SrD in autoimmune phenotypes, while their ancestral MRL/lpr mice do not show significant SrD. Past studies have readily confirmed little SrD in the autoimmune phenotypes of an MRL/lpr strain [22–25]. Here, a question that arose was why the male-specific

**Table 3.** Genetic effects of *Marc4* and *Marc7* on sex-related difference in autoimmune phenotypes<sup>a)</sup>

trait <sup>b)</sup>	sex	subcohorts by <i>Marc4/Marc7</i> genotypes <sup>d)</sup>				
		all	MM/MM	MM/MB	MB/MM	MB/MB
GN (log <sub>10</sub> index)	male <sup>§ c)</sup>	1.38 (0.48, 189)***	1.34 (0.46, 37)*	1.16 (0.52, 52)***	1.59 (0.44, 61)	1.40 (0.36, 39)***
	female	1.67 (0.52, 159)	1.64 (0.59, 44)	1.55 (0.53, 41)	1.70 (0.47, 38)	1.82 (0.46, 36)
ANA-IgM (titer score)	male <sup>§</sup>	1.49 (1.25, 189)***	1.41 (1.17, 37)*	1.08 (1.04, 52)**	1.98 (1.28, 61)	1.36 (1.33, 39)*
	female	1.88 (1.01, 159)	1.91 (1.03, 44)	1.68 (1.01, 41)	1.97 (1.03, 38)	2.00 (0.99, 36)
SpM (mg)	male <sup>§</sup>	460 (458, 189)***	443 (578, 37)	326 (325, 52)**	592 (484, 61)	447 (398, 39)**
	female	656 (394, 159)	646 (450, 44)	565 (366, 41)	726 (401, 38)	703 (331, 36)

<sup>a)</sup> Value denotes a mean (S.D., number of mice) of the indicated trait index. Difference of averages between male and female groups was estimated by Student's t-test. \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ .

<sup>b)</sup> GN, glomerulonephritis; ANA, antinuclear autoantibody; SpM, splenomegaly.

<sup>c)</sup> Variance among the genotype subcohorts was statistically evaluated by one-way ANOVA. §  $p < 0.01$ .

<sup>d)</sup> MM and MB denote MRL/MRL homozygote and MRL/B6 heterozygote, respectively.

**Table 4.** Interaction *Marc4* and *Marc7* in the male group<sup>a)</sup>

trait <sup>b)</sup>	locus1 <sup>c)</sup>	locus2 <sup>d)</sup>	lod.full <sup>e)</sup>	lod.add <sup>e)</sup>	lod.int <sup>e)</sup>
GN	c4 (50 cM)	c7 (9 cM)	5.36 (4.79)*	5.29 (3.58)*	0.08 (3.07)
SpM	c4 (50 cM)	c7 (4 cM)	4.11 (4.59)	4.09 (3.46)*	0.02 (2.62)
ANA-IgM	c4 (55 cM)	c7 (19 cM)	4.45 (3.91)*	4.24 (3.11)*	0.21 (2.83)

<sup>a)</sup> Value represents a calculated LOD with a threshold in parenthesis ( $\alpha = 0.05$ ). \*  $p < 0.05$ .

<sup>b)</sup> GN, glomerulonephritis; ANA, antinuclear antibody; SpM, splenomegaly.

<sup>c)</sup> Calculated position of maximum LOD on chromosome 4.

<sup>d)</sup> Calculated position of maximum LOD on chromosome 7.

<sup>e)</sup> lod.full, LOD score in the full mode; lod.add, LOD score in the additive mode; lod.int, LOD score in the interactive mode.

suppression by *Marc4* had attenuated in the male mice of this strain. A possible answer is that *Marc4* requires interaction with a B6/lpr-derived factor to exert its effect on SrD. *Marc7* could be a candidate B6/lpr-derived factor. However, as shown in Table 4, *Marc7* had no epistatic effect on *Marc4*, suggesting that another B6-derived factor activates *Marc4*. We further searched chromosomes 1, 13, and 17 with genotyping data obtained from all MBN2 mice for another epistatic locus to *Marc4*, but no such locus was detected (data not shown). We cannot rule out a possibility that an epistatic autosomal locus exists and that this locus is so weakly associated with the examined autoimmune phenotypes that it was overlooked in our genome-wide screening. Alternatively, another possibility is that a B6/lpr-derived Y chromosome is epistatic to *Marc4*. In this regard, there is a noteworthy report that documented SrD in the incidence of GN observed in two closely related backcross mice – MRL/lpr × (B6/lpr × MRL/lpr)F1 (BMN2) and MBN2 [21]. This report showed that MBN2 mice developed a clear tendency toward SrD, whereas the BMN2 mice developed little SrD. These findings have supported the contribution of the B6/lpr-derived Y chromosome or MRL/lpr-derived X chromosome to the development of SrD.

A mutation in the mitochondrial genome and abnormalities in sex chromosome, *i.e.*, Turner's syndrome and Klinefelter's syndrome, have been shown to predispose humans to autoimmune disorders. For example, mitochondrial DNA mutations cause a type of non-insulin-dependent diabetes and encephalomyelitis with an autoimmune etiology [27, 28]; Turner's syndrome and Klinefelter's syndrome predispose the patients to autoimmune thyroid disease and SLE-like disease, respectively [14, 15, 29, 30]. Some mitochondria-genomic disorders critically involve the female population [28]. Although the mechanism for this female preponderance remains unclear, this fact suggests a link between a mitochondrial phenotype and female gender. The association of sex chromosome abnormalities with the onset of autoimmune disease is indirect evidence that a sex chromosome directly contributes to autoimmune disease onset. Previous studies on this line have suggested a pathogenic impact of the number of an unidentified X allele on an autoimmune disease. This could be further implicated in the mechanism underlying SrD in autoimmune disease onset. The mitochondria and X chromosome in the present male MBN2 mice are derived from MRL/lpr females. Therefore, these genetic components are not implicated in the mechanism of male-specific autoimmune suppression that was observed in the MBN2 but not in the MRL/lpr mice. Instead, a direct role of the Y chromosome is considered. Although little is known about this role, a recent study conducted using a model

for experimental allergic encephalomyelitis has demonstrated this role of Y chromosome [31]. The Y-chromosome consomic strain, a murine model, will provide an answer to this question.

A number of studies performed using murine autoimmune models have documented autoimmune-linkage loci. *Marc4* and *Marc7* overlap well with some of these loci associated with SpM [32, 33], GN [34–38], vasculitis [39], anti-dsDNA [32, 36, 37], or mortality [33]. Sex-related preponderance was not examined for these loci. It is particularly noteworthy that a study conducted using (MRL/lpr × B6/lpr)F2 mice demonstrated two resistant loci MRL/lpr and B6/lpr alleles associated with SpM and anti-dsDNA; these alleles correspond to *Marc4* and *Marc7*, respectively [32].

There are genetic or functional implications of the genes included in *Marc4* and *Marc7* in the pathogenic context of autoimmune diseases. For example, the *lck* gene (60 cM of chromosome 4), which encodes for an src-family tyrosine kinase and plays a role in TCR signal transduction, was recently found to be down-regulated in the T cells of patients with SLE [40]. The *c1q* gene (66.1 cM of chromosome 4) encoding C1q, a complement component, is believed to be involved in the pathogenesis of SLE because it has been reported that approximately 90% of patients with congenital defects in C1q develop SLE early [41]. C1q-deficient mice were shown to develop autoantibody and GN [42]. A genetic polymorphism in the promoter region of the *c1qa* gene was identified in a lupus NZB strain [43]. The platelet-activating factor (PAF) receptor (*ptafr*, 62.4 cM on chromosome 4) has been suggested to contribute to the pathogenesis of GN through platelet activation. Administration of the PAF receptor antagonist flaxseed ameliorated proteinuria and SpM in MRL/lpr mice [44]. Patients with SLE demonstrated decreased activity of PAF acetylhydrolase, which is a degradation enzyme of PAF [45].

In the proximal region of chromosome 7, receptor *cd22* (9 cM) that regulates B cell survival and activation is underscored as a candidate that affects *Marc7* as previous studies indicated the association between a CD22 allotype (*cd22<sup>a</sup>*) and autoimmune disease onset in a *Yaa*-related autoimmune condition [46]. MRL/lpr represents lupus-prone *cd22<sup>a</sup>* that is different from B6/lpr (*cd22<sup>b</sup>*) (Nose *et al.*, unpublished data). In humans, the therapeutic use of anti-CD22 is under trial for the treatment of SLE [47].

This study revealed two loci on chromosomes 13 and 17 that yield susceptibilities to GN and SpM, and only SpM, respectively, in the MBN2 mice. These loci also overlap with previously defined loci in other autoimmune models [33, 34, 36, 37, 48]. Past studies have highly implicated the chromosome 17 interval because it includes the *H-2* region. This study, however, could not



show significant linkage between *H-2* and major autoimmune traits examined. These results indicate a minor influence of dimorphism – *H-2<sup>k</sup>* (MRL/lpr haplotype) or *H-2<sup>b</sup>* (B6/lpr haplotype) – on the development of autoimmune diseases. These, in turn, suggest a major contribution of the non-*H-2* loci; however, a sufficient set of such susceptibility loci remains undermined. MBN2 mice develop autoimmune disorders under varied genetic conditions, wherein a number of minor susceptibility loci influence the onset of autoimmune diseases, probably, in an interactive fashion of multiple loci. It should be considered that a conventional method for genome-wide screening, as performed in this study, limits the sensitivity for minor and/or interactive linkage loci.

A majority of human autoimmune diseases occur in a female-predominant fashion [1]. This study suggests the presence of a male-specific mechanism underlying the SrD in autoimmune diseases. It is further suggested that this mechanism is achieved by some autosomal gene functions controlled under undetermined male-specific conditions associated with sex-related hormones and/or the Y chromosome. Further understanding of the molecular details of this mechanism will provide a clue with regard to the general therapeutic approach for human autoimmune diseases.

## Materials and methods

### Mice

MRL/lpr and B6/lpr mice were purchased from the Charles River Japan, Yokohama, Japan. These mice were bred under specific pathogen-free condition in the Integrated Center of Science in Ehime University, Toon, Japan, or the Institute for Animal Experimentation Tohoku University, Sendai, Japan. MBF1 and the backcross MBN2 were prepared and housed in these centers. In all animal experiments in this study, we observed the Ehime University and Tohoku University guidelines for animal experimentation.

### Histological evaluation of GN

At 19–20 weeks of age, mice were killed under ether anesthesia. After sampling blood for serum isolation, the removed kidneys were fixed with 10% formalin in 0.01 M phosphate buffer, pH 7.2, and embedded in paraffin. Tissue sections were stained with periodic acid-Schiff. GN was microscopically graded under the criteria for an increase in a cellular or matrix component in the glomerulus (Supporting Information Fig. 1). An increase in a cellular component was graded on a scale of 0–3: 0, normal cellularity; 1, slight cellular proliferation; 2, enlargement of glomerulus with remarkable cellular proliferation; and 3, crescent formation. An increase in a matrix component was graded on a scale of 0–3: 0, normal; 1, slight mesangial increase; 2, lobulation or segmental sclerosis;

and 3, global sclerosis. No less than 30 glomeruli for an individual were examined, then an individual GN index was defined by  $\log_{10} [(a \text{ mean of cellular grades}) + (a \text{ mean of matrix grades})]$ .

### Measurement of ANA

ANA titer was measured with the indirect immunofluorescence (IF) method applying to nuclear samples prepared from a normal mouse liver as described previously [49]. Nuclear samples were incubated with serum dilution and antibody of FITC-conjugated anti-mouse IgG,  $\gamma$  chain specific (Zymed, South San Francisco, CA) or anti-mouse IgM,  $\mu$  chain specific (Zymed). ANA titer was determined by a fluorescence detection of nuclear signal with following standards: 0, no staining with 1:100 serum dilution; 1, faintly stained with 1:100 serum dilution; 2, clearly stained with 1:100 serum dilution; 3, stained with 1:1000 serum dilution; 4, stained with 1:10000 serum dilution; 5, stained with 1:30 000 serum dilution; 6, stained with 1:90 000 serum dilution.

### Mapping of autoimmune-associated loci

The genotypes of MBN2 mice were determined using mouse microsatellite markers whose genetic positions are determined by the Mouse Genome Informatics (MGI), The Jackson laboratory. PCR was performed under the following conditions: 94°C for 2 min, 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 5 min. PCR products were visualized on 4% agarose gels by staining with ethidium bromide after electrophoresis.

In genome-wide screenings, we determined 117 microsatellite genotypes of randomly selected 95 MBN2 mice (Supporting Information Fig. 2). These markers provide a full coverage of mouse autosomes with an average of 12.9 cM apart (maximum distance is 23 cM). Both X chromosomes of every female MBN2 mouse were derived from MRL/lpr; therefore, we did not test X chromosome in this study. In QTL analysis, we determined the genotypes of another 253 MBN2 mice (a total of 348 mice) on candidate chromosomes.

### Statistical analysis

We adopted  $\log_{10}$ -transformed values of spleen weights (mg) and GN indexes to obtain normal distributions. In association studies,  $p < 0.0034$  and  $p < 0.0001$  were accepted as suggestive and significant linkage, respectively [50]. A QTL analysis was performed using Windows QTL Cartographer (V2.5) software with each autoimmune traits in MBN2 mice [51]. Interval mapping program was adopted and walk-speed was 2 cM. A significant and suggestive threshold level of LOD score was determined by the permutation test (1000 permutation with  $\alpha = 0.01$  or 0.05, respectively). To evaluate interaction of two QTL, we used the R/qtl program available at <http://www.biostat.jhsph.edu/~kbroman/qtl> [26]. Significant threshold level was determined by the permutation test installed in this program (100 permutations,  $\alpha = 0.05$ ). Spearman's rank correlation, Student's *t*-test, and one-way analysis of variance (ANOVA) were also used in the present study.  $p < 0.05$  was estimated as significant.

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# Association of Gankyrin Protein Expression with Early Clinical Stages and Insulin-Like Growth Factor-Binding Protein 5 Expression in Human Hepatocellular Carcinoma

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Gankyrin (also known as PSMD10) is a liver oncoprotein that interacts with multiple proteins including MDM2 and accelerates degradation of the tumor suppressors p53 and Rb. We produced a monoclonal anti-gankyrin antibody and immunohistochemically assessed the clinicopathological significance of gankyrin overexpression in 43 specimens of human hepatocellular carcinoma (HCC). Specific cytoplasmic staining for gankyrin was observed in 62.8% (27/43) of HCCs, which was significantly associated with low TNM stage ( $P = 0.004$ ), no capsular invasion ( $P = 0.018$ ), no portal venous invasion ( $P = 0.008$ ), and no intrahepatic metastasis ( $P = 0.012$ ). The cumulative survival rate of patients with gankyrin-positive HCC was significantly higher than that with gankyrin-negative HCC ( $P = 0.037$ ). p53 and MDM2 were positively stained by antibodies in 30.2% and 23.3%, respectively, of HCCs, but neither was inversely associated with gankyrin expression. In the Huh-7 human HCC cell line, overexpression of gankyrin up-regulated expression of insulin-like growth factor binding protein 5 (IGFBP-5), whereas suppression of gankyrin expression by siRNA down-regulated it. Suppression of IGFBP-5 expression inhibited proliferation of Huh-7 cells as well as U-2 OS osteosarcoma cells. In HCC specimens, positive staining for IGFBP-5 was observed by immunohistochemistry in 41.9% (18/43), and the level of expression was significantly correlated with that of gankyrin ( $r = 0.629$ ,  $P < 0.001$ ). **Conclusion:** These results suggest that gankyrin plays an oncogenic role(s) mainly at the early stages of human hepatocarcinogenesis, and that IGFBP-5 inducible by gankyrin overexpression may be involved in it. (HEPATOLOGY 2008;47:493-502.)

*Abbreviations:* 3A6C2, mouse monoclonal anti-gankyrin antibody; cDNA, complementary DNA; HCC, hepatocellular carcinoma; IGF, insulinlike growth factor; IGFBP-5, insulin-like growth factor-binding protein 5; MDM2, mouse double minute 2; mRNA, messenger RNA; RT-PCR, reverse transcription polymerase chain reaction; siRNA, short interfering RNA; TNM, tumor-node-metastasis.

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Liver cancer is the sixth most common cancer worldwide (626,000 or 5.7% of new cancer cases) and the third most common cause of death from cancer (598,000) in 2002.<sup>1</sup> Eighty-two percent of cases are in developing countries, and the areas of high incidence are sub-Saharan Africa, eastern and southeastern Asia, and Melanesia. Histologically, more than 90% of the primary liver cancers are hepatocellular carcinomas (HCCs). Although there are several modalities of treatment for HCC, most patients present with unresectable tumors, and non-surgical treatments are minimally effective at best.<sup>2,3</sup> Even for those patients who undergo surgical resection, the recurrence rate is very high and the prognosis is poor.<sup>2,4-6</sup> It is therefore important to clarify the mechanisms of human hepatocarcinogenesis and identify molecular targets to develop novel diagnostic, therapeutic, and preventive strategies.

By constructing subtracted complementary DNA (cDNA) libraries, we have previously identified 19 genes overexpressed in HCCs including 2 novel genes.<sup>7,8</sup> One of them was named gankyrin (gann-ankyrin repeat pro-

tein; "gann" in Japanese means cancer).<sup>9</sup> Gankyrin (also called PSMD10) consists of 7 ankyrin repeats, and its messenger RNA (mRNA) was overexpressed in 34 of 34 HCCs analyzed.<sup>9,10</sup> Independently, gankyrin was isolated as the p28 component or the interactor of the S6b subunit of the 19S regulator of the 26S proteasome.<sup>11,12</sup> The ankyrin repeat is the functional domain involved in protein-protein interactions, and gankyrin has been shown to interact with multiple proteins in addition to S6b. Gankyrin binds to retinoblastoma protein (Rb) and cyclin-dependent kinase (Cdk4), and accelerates phosphorylation and degradation of Rb, which results in release of the E2F transcription factor to activate DNA synthesis genes.<sup>9,13</sup> Gankyrin seems to play a role in cell cycle progression in noncancerous cells as well. Overexpression of gankyrin shortens population doubling time of NIH/3T3 mouse fibroblasts,<sup>9</sup> and its up-regulation correlates with cell cycle progression in normal rat primary hepatocytes, oval cells, and human hepatocytes.<sup>14,15</sup>

Overexpression of gankyrin confers tumorigenicity to NIH/3T3 cells and inhibits apoptosis in cultured human tumor cells exposed to chemotherapeutic agents.<sup>10</sup> The anti-apoptotic activity is attributable, at least partly, to increased degradation of p53, resulting in the reduced transcription of the p53-dependent proapoptotic genes.<sup>16</sup> Gankyrin binds to the E3 ubiquitin ligase MDM2 *in vitro* and *in vivo*, which increases p53-MDM2 association, thereby facilitating the ubiquitination and subsequent proteasomal degradation of p53 by MDM2. Gankyrin also controls MDM2 auto-ubiquitination and degradation, especially in the absence of p53.<sup>16</sup>

We produced a mouse monoclonal antibody against human gankyrin and assessed the expression of gankyrin protein in surgically resected HCC specimens by immunohistochemistry. Correlation of gankyrin positivity with clinicopathological findings and expression of p53 and MDM2 in HCC was analyzed. Furthermore, we demonstrated that expression of insulin-like growth factor-binding protein 5 (IGFBP-5) is inducible by overexpression of gankyrin in HCCs.

## Patients and Methods

**Patients and Specimens.** HCC tissues and their corresponding noncancerous liver tissues were obtained from 43 and 32 patients, respectively, who had undergone curative hepatectomy at the University Hospital of Kyoto Prefectural University of Medicine between 1992 and 2000. The specimens used were routinely processed, formalin-fixed, and paraffin-embedded. After hematoxylin-eosin staining, all samples were diagnosed as HCC and the tumor-node-metastasis (TNM) classification was

**Table 1. Patient and Tumor Characteristics**

Characteristic	Number (Percentage)
Number of patients	43
Sex distribution	
Male	27 (62.8%)
Female	16 (37.2%)
Age (years)	25-78, median 65
Virus marker	
HBV(+)/HCV(-)	6 (14.0%)
HBV(-)/HCV(+)	28 (65.0%)
HBV(+)/HCV(+)	3 (7.0%)
HBV(-)/HCV(-)	6 (14.0%)
AFP(ng/mL)	3.5-39999, median 90
Tumor size (cm)	1.6-17.0, median 4.0
Liver cirrhosis	
Yes	29 (67.5%)
No	14 (32.5%)
Chronic hepatitis	13 (30.2%)
Normal	1 (2.3%)
TNM stage	
I	4 (9.3%)
II	22 (51.1%)
III	8 (18.6%)
IV	9 (21.0%)
Histological differentiation	
Well	12 (27.9%)
Moderate	25 (58.1%)
Poor	6 (14.0%)
Capsular formation	
Yes	36 (83.7%)
No	7 (16.3%)
Capsular invasion	
Yes	14 (32.6%)
No	29 (67.4%)
Portal venous invasion	
Yes	9 (20.9%)
No	34 (79.1%)
Intrahepatic metastasis	
Yes	16 (37.2%)
No	27 (62.8%)

Abbreviations: HCV(+), anti-hepatitis C virus antibody positive; HBV(+), hepatitis B surface antigen positive; (-), negative; AFP, serum alpha-fetoprotein.

made according to the fourth edition of the general rules for the clinical and pathological study of primary liver cancer proposed by the Liver Cancer Study Group of Japan.<sup>17</sup> The demographic profiles of the patients are summarized in Table 1. For western blot analysis, HCCs and noncancerous liver tissues were obtained from 3 patients undergoing liver transplantation at the University Hospital of Kyoto Prefectural University of Medicine between 2004 and 2006. No donor organs were obtained from executed prisoners or other institutionalized persons. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the institutional review boards. Written informed consents were obtained from all patients for subsequent use of their resected tissues.