Table 1. Global methylation level of leukocyte DNA in Japanese women according to factors used for adjustment

Factor	Level	Crude		Multivariate-adjusted*						
		n	Methylation level (%)	Methylation level (%)	95% CI		Effect	95% CI		*P*-value
Age, years	<40	25	69.9	70.4	68.7	72.1				
	40–49	109	70.0	70.6	69.5	71.7				
	50–59	129	70.3	70.9	69.8	72.1				
	60–69	96	70.4	71.2	70.0	72.4				
	≥70	25	69.7	70.5	68.8	72.1				
	Trend						0.15	−0.2	0.49	0.403
Body mass index (quartile category)	≤20.8	93	70.1	70.8	69.6	71.9				
	20.9–22.5	97	71.0	71.7	70.5	72.9				
	22.6–24.8	97	70.1	70.8	69.6	71.9				
	≥24.9	97	69.5	70.2	69.1	71.4				
	Trend						−0.26	−0.57	0.05	0.104
Smoking	Never	354	70.1	70.5	69.9	71.1				
	Past	8	70.8	70.7	68.3	73.1				
	Current	20	70.7	71.3	69.7	72.9				
	Trend						0.36	−0.40	1.13	0.351
Physical activity	No	231	69.9	70.4	69.3	71.4				
	≤2 days per week	33	70.9	71.4	70.0	72.8				
	≥3 days per week	120	70.5	70.8	69.7	71.9				
	Trend						0.25	−0.14	0.63	0.208

*Adjusted for age (continuous), body mass index (continuous), smoking (never smoker, past smoker, current smoker), alcohol drinking (non-drinker, occasional drinker, regular drinker of <150 g ethanol/week, regular drinker of ≥150 g ethanol/week), and physical activity in the past 5 years (no, ≤2 days/week, ≥3 days/week). Model for each factor listed in the table did not include the corresponding variable as adjustment. CI, confidence interval.

global methylation level significantly decreased by 0.70% (95% CI, 0.28–1.12) per quartile category for folate intake among non-drinkers, whereas no association was observed among drinkers (0.08% [95% CI, −0.40–0.55]). Additional analysis by the four categories of alcohol drinking used in Table 2 also found a statistically significant interaction ($P_{interaction}$ = 0.002). As stated above, we observed an inverse association among non-drinkers. In contrast, the global methylation level significantly increased by 1.32% (95% CI, 0.22–2.42) per quartile category for folate intake among regular drinkers of more than 150 g ethanol/week, but no association was seen among occasional drinkers and regular drinkers of less than this amount (data not shown). No statistically significant interactions were observed for vitamin B2, B6, or B12 intake, alcohol consumption, or five SNPs of *MTHFR*, *MTR*, and *MTRR*.

Discussion

In this cross-sectional study among Japanese women, we found that higher folate intake was significantly associated with a lower level of global methylation of peripheral blood leukocyte DNA. Subgroup analysis suggested that alcohol drinking modified the association between folate intake and global methylation level. Because of the cross-sectional nature of the study, we were not able to determine if higher dietary folate intake leads to global hypomethylation of leukocyte DNA. Considering the role of folate in one-carbon metabolism, however, our findings suggest that dietary folate intake might modulate the global methylation level of leukocyte DNA.

Our findings appear to contradict at least some previous studies of the association between folate level and global methylation level of peripheral blood DNA. Two intervention studies showed decreased methylation of leukocyte DNA in a folate-depleted diet group.[14,16] One of these studies provided an average of 118 μg folate per day to 33 postmenopausal women for 7 weeks,[16] and the second provided an average of 56–111 μg folate per day to eight postmenopausal women for 9 weeks.[14] Although these studies differed from our study in their method of methylation analysis (*in vitro* [3H]methyl incorporation assay by SssI CpG methylase) and subjects (postmenopausal or elderly women recruited in the USA), their data indicate that moderate folate depletion induces hypomethylation of leukocyte DNA. Regarding the effect of folate supplementation on the methylation level of leukocyte DNA, a randomized controlled trial of 400 μg folic acid supplementation per day (n = 15) or placebo (n = 16) for 10 weeks in patients with colorectal adenoma showed an increase in leukocyte DNA methylation level.[15] In contrast, supplementation with 2 mg folic acid and 20 μg vitamin B12 for 12 weeks did not change this variable.[13] These intervention studies suggest that the effect of folate on the methylation level of leukocyte DNA might depend on dose, but that a dose–response pattern might not be straightforward. For instance, it has been suggested that folates act as inhibitors of dihydrofolate reductase,[26] and that high folate levels could have the same functional effect as a low folate status under certain circumstances.[10,27] In fact, several animal studies showed that the effect of isolated folate deficiency on genomic DNA methylation in rodent liver and colon was either a decrease or increase.[28,29]

A recent cross-sectional study reported that a dietary pattern characterized by high intake of vegetables and fruits was associated with a lower prevalence of LINE-1 DNA hypomethylation.[30] In contrast, three other studies found no association between dietary folate intake and global methylation level of leukocyte DNA in the control groups of a head and neck cancer case–control study in the US, a bladder cancer case–control study in Spain, and a gastric cancer case–control study in Poland.[6–8] These findings should be interpreted cautiously, however, because the analyses of the bladder and gastric cancer case–control studies were primarily aimed at identifying potential confounders for assessing an association between

Table 2. Global methylation level according to five dietary factors and five single nucleotide polymorphisms of genes associated with folate metabolic enzymes

Factor	Level	Crude		Multivariate-adjusted*						
		n	Methylation level (%)	Methylation level (%)	95% CI		Effect	95% CI		*P*-value
Folate (μg/day)	≤339.9	96	70.3	71.2	70.0	72.3				
	339.9–419.5	96	70.6	71.4	70.2	72.6				
	419.5–521.7	96	70.1	70.8	69.6	71.9				
	>521.7	96	69.7	70.2	69.0	71.4				
	Trend						−0.36	−0.69	−0.03	0.030
Vitamin B2 (mg/day)	≤1.4	96	69.8	70.6	69.5	71.7				
	1.4–1.6	96	70.0	70.8	69.6	72.0				
	1.6–1.8	96	70.9	71.4	70.2	72.6				
	>1.8	96	70.0	70.7	69.5	71.9				
	Trend						0.08	−0.24	0.39	0.636
Vitamin B6 (mg/day)	≤1.4	96	70.2	71.1	69.9	72.3				
	1.4–1.6	96	70.3	71.1	69.9	72.2				
	1.6–1.8	96	70.1	70.7	69.6	71.9				
	>1.8	96	70.1	70.6	69.4	71.7				
	Trend						−0.19	−0.53	0.15	0.268
Vitamin B12 (μg/day)	≤6.4	96	70.5	71.3	70.1	72.5				
	6.4–8.3	96	69.9	70.6	69.4	71.7				
	8.3–10.6	96	70.3	70.9	69.8	72.1				
	>10.6	96	70.0	70.7	69.5	71.8				
	Trend						−0.14	−0.46	0.17	0.370
Alcohol drinking	Non-drinker	232	69.9	70.4	69.4	71.5				
	Occasional drinker	39	70.9	71.4	70.0	72.8				
	Regular drinker of <150 g ethanol/week	87	70.8	71.3	70.1	72.4				
	Regular drinker of ≥150 g ethanol/week	26	69.9	70.3	68.8	71.9				
	Trend						0.23	−0.11	0.57	0.183
MTHFR rs1801131	AA	254	70.3	70.9	69.9	72.0				
	AC + CC	130	70.0	70.7	69.6	71.8				
	Dominant model						−0.25	−0.98	0.47	0.494
MTHFR rs1801133	CC	112	70.2	70.9	69.7	72.0				
	CT + TT	272	70.1	70.8	69.8	71.9				
	Dominant model						−0.04	−0.80	0.72	0.918
MTR rs1805087	AA	257	70.3	71.0	70.0	72.1				
	AG + GG	126	69.8	70.5	69.4	71.6				
	Dominant model						−0.53	−1.26	0.20	0.156
MTRR rs162049	GG	116	70.2	70.9	69.7	72.0				
	AG + GG	266	70.2	70.8	69.8	71.9				
	Dominant model						−0.05	−0.80	0.70	0.902
MTRR rs10380	CC	302	70.2	70.9	69.8	71.9				
	CT + TT	81	70.2	70.8	69.6	72.0				
	Dominant model						−0.09	−0.93	0.75	0.834

*Adjusted for age (continuous), body mass index (continuous), smoking (never smoker, past smoker, current smoker), alcohol drinking (non-drinker, occasional drinker, regular drinker of <150 g ethanol/week, regular drinker of ≥150 g ethanol/week), and physical activity in the past 5 years (no, ≤2 days/week, ≥3 days/week). CI, confidence interval; *MTHFR*, methylenetetrahydrofolate reductase; *MTR*, methionine synthase; *MTRR*, methionine synthase reductase.

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Table 3. Association between mean global methylation level in leucocyte DNA and folate intake by factors related to one-carbon metabolism

Factor	Level	Number	Multivariate-adjusted*				
			Effect	95% CI		*P*-value for trend	*P*-value for interaction
Alcohol	Non-drinker	232	−0.70	−1.12	−0.28	0.001	
	Drinker	152	0.08	−0.40	0.55	0.749	0.013
Vitamin B2, mg/day	≤1.6	192	−0.32	−0.82	0.18	0.208	
	>1.6	192	−0.67	−1.15	−0.19	0.006	0.304
Vitamin B6, mg/day	≤1.6	192	−0.07	−0.65	0.50	0.803	
	>1.6	192	−0.63	−1.17	−0.10	0.020	0.157
Vitamin B12, μg/day	≤8.3	192	−0.20	−0.64	0.25	0.389	
	>8.3	192	−0.53	−0.99	−0.08	0.022	0.287
MTHFR rs1801131	AA	254	−0.46	−0.86	−0.05	0.028	
	AC + CC	130	−0.18	−0.70	0.33	0.484	0.400
MTHFR rs1801133	CC	112	−0.25	−0.81	0.31	0.384	
	CT + TT	272	−0.41	−0.80	−0.02	0.037	0.627
MTR rs1805087	AA	257	−0.21	−0.61	0.19	0.298	
	AG + GG	126	−0.60	−1.13	−0.08	0.024	0.233
MTRR rs162049	GG	116	−0.49	−1.05	0.07	0.084	
	AG + GG	266	−0.30	−0.68	0.09	0.137	0.555
MTRR rs10380	CC	302	−0.37	−0.73	−0.01	0.042	
	CT + TT	81	−0.32	−1.03	0.39	0.374	0.892

*Adjusted for age (continuous), body mass index (continuous), smoking (never smoker, past smoker, current smoker), alcohol drinking (non-drinker, occasional drinker, regular drinker of <150 g ethanol/week, regular drinker of ≥150 g ethanol/week), and physical activity in the past 5 years (no, ≤2 days/week, ≥3 days/week). CI, confidence interval; *MTHFR*, methylenetetrahydrofolate reductase; *MTR*, methionine synthase; *MTRR*, methionine synthase reductase.

global methylation level and the risk of cancer based on univariate analyses.

The important messages from this and these previous studies may be that: (i) the mechanisms of individual variation in the global DNA methylation level of peripheral blood leukocytes are complex and multifactorial in nature; and (ii) in actual daily dietary life, in Japan, folate intake may not be the major single determinant of global methylation level and may not necessarily confound association analysis between leukocyte global methylation and the risk of cancers that are associated with folate intake. Only a few observational studies have examined associations of dietary and genetic factors related to one-carbon metabolism with global methylation level of leukocyte DNA among healthy individuals based on nutrient intake estimated from the usual diet alone.[6–8] None of the five candidate SNPs examined in this study showed a statistically significant association, although rs1801131 and rs1801133 in *MTHFR*, for instance, have been reported to be linked to altered enzymatic activity[31,32] and folate level.[33,34] Given the present result of rs1801131 in *MTHFR* (AA genotype group: number = 254, mean = 70.25, and SD = 3.3; AC + CC genotype group: number = 130, mean = 70.01, and SD = 3.3), for example, the expected power to detect an association was 10% with a two-sided and error level of 5%. Therefore, we cannot exclude the possibility that the null findings are explained by insufficient power, and additional larger studies are needed to clarify the association between these SNPs and global methylation level.

Subgroup analyses in the present study showed that alcohol drinking modified the association between folate intake and global methylation level ($P_{interaction} = 0.01$). The association between folate intake and global methylation level varied by alcohol drinking status: higher folate intake was significantly associated with a lower global methylation level among non-drinkers; no association was observed among occasional and light drinkers; and higher folate intake was significantly associated with a higher global methylation level among relatively heavy drinkers. Alcohol consumption interferes with folate metabolism[35] and decreases levels of serum folate.[36] Although this interaction remained inexplicable, these findings might nevertheless provide hints about its biological mechanism. Furthermore, subgroup analysis by alcohol drinking was based on a relatively small number subjects, particularly with regard to heavy drinkers ($n = 26$), and thus replication of this interaction in a larger study is awaited.

Several limitations of the present study warrant mention. First, misclassifications due to inaccurate measurement would result in null associations. Although dietary intakes in the present study were assessed using a validated FFQ, misclassifications may have been unavoidable. However, as reproducibility of the assay for global methylation level was relatively high in the present study (intra-assay CV, 6.4), measurement errors during laboratory assay might have been minimal. Second, the present study made multiple comparisons, which might have led to false-positive results. In this regard, we observed a statistically significant association between higher folate intake and lower level of global methylation, which might nevertheless be explained by chance. Finally, because the sample size was limited, the study might not have had sufficient statistical power to detect small associations, as mentioned above, and this is one of the possible explanations for the observed absence of associations. In particular, the results of subgroup analysis and interaction tests should be interpreted carefully.

In this cross-sectional study in 384 healthy Japanese women with validated FFQ data, we found that a higher folate intake level was associated with a lower global methylation level of leukocyte DNA. Although the data of this study and others suggest that folate intake can modulate the global methylation level of leukocyte DNA, inconsistencies among the studies have been noted, and may reflect the complex and multifactorial nature of individual variation in the global DNA methylation level of peripheral blood leukocytes.

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Disclosure Statement

The authors have no conflict of interest.

Abbreviations

BMI	body mass index
CI	confidence interval
CV	coefficient of variation
FFQ	food frequency questionnaire
LUMA	LUminometric Methylation Assay
MTHFR	methylenetetrahydrofolate reductase
MTR	methionine synthase
MTRR	methionine synthase reductase
SD	standard deviation
SNP	single nucleotide polymorphism

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Review Article

Genetic factors related to gastric cancer susceptibility identified using a genome-wide association study

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Gastric cancer (GC) is one of the major malignant diseases worldwide, especially in Asia, where Japan and Korea have the highest incidence in the world. Gastric cancer is classified into intestinal and diffuse types. While the former is almost absolutely caused by *Helicobacter pylori* infection as the initial insult, the latter seems to include cases in which the role of infection is limited, if any, and a contribution of genetic factors is anticipated. Previously, we performed a genome-wide association study (GWAS) on diffuse-type GC by using single nucleotide polymorphisms (SNP) catalogued for Japanese population (JSNP), and identified a prostate stem cell antigen (*PSCA*) gene encoding a glycosylphosphatidylinositol-anchored cell surface antigen as a GC susceptibility gene. From the second candidate locus identified using the GWAS, 1q22, we found the Mucin 1 (*MUC1*) gene encoding a cell membrane-bound mucin protein as another gene related to diffuse-type GC. A two-allele analysis based on risk genotypes of the two genes revealed approximately 95% of Japanese population have at least one of the two risk genotypes, and approximately 56% of the population have both risk genotypes. The two-SNP genotype might offer ample room to further stratify a high GC risk subpopulation in Japan and Asia by adding another genetic and/or non-genetic factor. Recently, a GWAS on the Chinese population disclosed an additional three GC susceptibility loci: 3q13.31, 5p13.1 and 10q23. (*Cancer Sci* 2013; 104: 1–8)

Gastric cancer (GC) is one of the major malignant diseases and the second causal of cancer death worldwide.[1] It is usually classified into two types, intestinal and diffuse, a classification which was originally based on histological observation but is recently thought to reflect its pathogenesis.[2] The majority of intestinal-type GC (IGC) arises in the sequence of inflammatory change of the gastric epithelium resulting from bacterial infection; *Helicobacter pylori* (HP) infection – chronic inflammation – intestinal metaplasia – dysplasia – adenocarcinoma. In contrast, de novo diffuse-type GC (DGC) is thought to emerge in a histologically almost normal epithelium as a consequence of some genetic change that occurred in gastric stem cells and/or epithelial precursor cells, although some cases with DGC might represent dedifferentiated stages of IGC, and a contribution of HP is also suggested.[3] In other words, it is apparent that the pathogenesis of IGC is initiated by HP infection, a class I carcinogen acknowledged by WHO, and therefore IGC is essentially a preventable disease by eradicating HP infection. However, DGC might develop earlier in life than IGC,[4] and no definite DGC-specific environmental risk factor has been established. Therefore, so far we have neither solid strategy nor promising theory to envision a consistent diminution of the incidence of DGC.

The incidence of GC has strong geographical and ethnical characteristics. For example, it is one of the rare cancers in North America and Europe, while its incidence is significantly high in Japan and Korea. This can be explained roughly by the difference in regional prevalence of HP infection.[4] However, Japan has a high incidence of GC (age-standardized incidence rate 62.7/100 000) but lower HP seroprevalence (39.3%) than, for example, Bangladesh (92%) and India (79%), which have a much lower GC incidence, 1.6/100 000 and 5.7/100 000, respectively, suggesting the contribution of some other factor in the carcinogenesis of gastric epithelial cells.[5] Moreover, *Helicobacter* and Cancer Collaborative Group reported that HP infection was not associated with the overall risk of GC developing in the cardia of the stomach.[6]

Genome-wide association study (GWAS) of genetic factors for GC development

Genome-wide association study has been successful in exploring genetic susceptibility factors of a number of polygenic or so-called lifestyle-related diseases based on a common disease – common variant hypothesis.[7,8] The current choice of polymorphic markers in GWAS is single nucleotide polymorphisms (SNP), and the spectrum and frequency of SNP depend on each ethnic population. Japan preceded other countries in the preparation for conducting GWAS, because SNP in the Japanese population (JSNP) were already catalogued in the early 2000s by Dr Yusuke Nakamura at the Institute of Medical Science, The University of Tokyo. The JSNP database led to a number of fruitful harvests in the area of GWAS on genetic factors for common diseases in the late 2000s.[9] As a part of the so-called Millennium Project in Japan, GWAS on GC was performed with two steps of the association study.[10,11] The first step was performed on 85 576 SNP using 188 DGC cases and 752 references, and the second step was performed on 2753 selected SNP with 749 DGC cases and 750 controls. Finally, it listed the top 10 SNP related to DGC with statistical significance, which included four SNP located in chromosome 8q24.3 and two SNP in 1q22 (Table 1).[12] The subsequent linkage–disequilibrium (LD) analyses revealed two genes in the LD block at 8q24.3 and five genes at 1q22.[12,13]

In the 8q24.3 locus, prostate stem cell antigen (*PSCA*) gene was identified as a DGC susceptibility gene, with a significant association between DGC and two SNP, rs2976392 and rs2294008, in the gene (rs2976392: 926 cases, 1397 controls, allele-specific odds ratio = 1.71, 95% confidence interval = 1.50 –1.94, $P = 1.5 \times 10^{-16}$; rs2294008: 925 cases, 1396 controls, allele-specific odds ratio = 1.67, 95% confidence interval = 1.47 –1.90, $P = 2.2 \times 10^{-15}$).[12] The association was replicated in

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Table 1. **Gastric cancer susceptibility loci identified using a genome-wide association study**

Locus	Representative SNP (major/minor allele)	Odds ratio (95% CI)	*P*-value	Ethnicity	Cancer type	Nearest gene	Primary report
1q22	rs2070803 (G/A)	1.63 (1.33–1.98)†	1.2×10^{-6}†	Japanese	Diffuse	*MUC1*	Sakamoto *et al.*[12]
3q13.31	rs9841504 (C/G)	0.76 (0.69–0.83)‡	1.7×10^{-9}‡	Chinese	Non-cardia	*ZBTB20*	Shi *et al.*[23]
5p13.1	rs13361707 (T/C)	1.41 (1.32–1.49)‡	7.6×10^{-29}‡	Chinese	Non-cardia	*PRKAA1*	Shi *et al.*[23]
8q24.3	rs2976392 (A/G)	1.62 (1.38–1.89)†	1.1×10^{-9}†	Japanese	Diffuse	*PSCA*	Sakamoto *et al.*[12]
10q23	rs2274223 (A/G)	1.31 (1.19–1.43)†	8.40×10^{-9}†	Chinese	Cardia, non-cardia	*PLCE1*	Abnet *et al.*[24]

CI, confidence interval; SNP, single nucleotide polymorphism. †Allelic model. ‡Additive model.

the Korean population (rs2976392: 449 cases, 390 controls, allele-specific odds ratio = 1.90, 95% confidence interval = 1.56–2.33, $P = 8.0 \times 10^{-11}$; rs2294008: 454 cases, 390 controls, allele-specific odds ratio = 1.91, 95% confidence interval = 1.57–2.33, $P = 6.3 \times 10^{-11}$) and it also showed a relatively weak correlation to IGC in both populations from Japan (rs2976392: 599 cases, 1397 controls, allele-specific odds ratio = 1.29, 95% confidence interval = 1.12–1.49, $P = 5.0 \times 10^{-4}$) and Korea (rs2976392: 416 cases, 390 controls, allele-specific odds ratio = 1.37, 95% confidence interval = 1.12–1.68, $P = 0.0017$). Later, the association of rs2976392 or rs2294008 with GC was validated in other Japanese and Korean panels and in Chinese and Caucasian studies (Table 2).[14–21] Intriguingly, *PSCA* was also identified as a gene related to bladder-cancer susceptibility for Caucasians by GWAS.[22]

Our subsequent analyses revealed that the 1q22 region contains 13 SNP with strong LD over five genes, but we have concluded that the Mucin 1 (*MUC1*) gene is responsible for the observed association as the second DGC susceptibility gene; rs2070803 with $P = 2.20 \times 10^{-6}$, adjusted per allele odds ratio = 1.63 (606 cases and 1264 controls), which was replicated in additional Japanese ($P = 3.93 \times 10^{-5}$, odds ratio = 1.81, 304 cases and 1465 controls) and Korean ($P = 2.19 \times 10^{-4}$, odds ratio = 1.82, 452 cases and 372 controls) case-control panels.[13] While rs2070803 was one of the original LD mapping markers, which we found to have an association with DGC, we later identified rs4072037 in the *MUC1* gene as a functional SNP. [13]

In addition, the combined genotype association data of rs2294008 in *PSCA* and rs4072037 in *MUC1*, both of which were shown to have biological functions (discussed in sections *PSCA* gene and *MUC1* gene), revealed that 66.5% of the Japanese control subjects had the risk genotype of rs4072037 (risk allele = A, in a recessive model of the allele effect), 84.6% had the risk genotype of rs2294008 (risk allele = T, in a dominant model) and 55.8% had both, showing an odds ratio = 8.4 (Fig. 1).[13] This suggests approximately 95% of Japanese population possess at least one of the two risk genotypes. The risk allele of rs2294008 is a major allele in the Japanese population, but the allele is minor in some other ethnic populations including Caucasians (Supporting Information Table S1).[12,13] Moreover, Korea has the highest GC incidence almost equivalent to that of Japan,[5] where both the risk alleles are similarly major, and it was estimated that more than 90% of the Korean population has at least one risk genotype of the two SNP.[13] In Japanese population and other ethnic groups, the association was demonstrated between one or two of the risk alleles, and it seems possible that the ethnic prevalence of GC development is influenced by the risk allele frequency of the SNP with proven biological functions.[12,13]

The majority of IGC arises in the chronic inflammatory lesion of the gastric epithelium resulting from HP infection, but some genetic contribution has also been suggested, and, indeed, the association of *PSCA* with IGC was demonstrated.[12] Independent of the DGC GWAS described above, an IGC GWAS was also initiated in Japan. The first screening (1600 cases, 3400 controls, 501 909 SNP) has already been performed by the National Cancer Center in collaboration with RIKEN, in which, intriguingly, rs2294008 and three other SNP in *PSCA* were included in the six SNP showing the most statistically significant association ($P < 1 \times 10^{-6}$, Hiromi Sakamoto, Teruhiko Yoshida and Yusuke Nakamura, unpublished data). In the first screening, even *PSCA*, which showed the strongest association, showed a relatively low odds ratio, for example, rs2294008 with odds ratio = 1.27 for IGC.

Besides the Japanese study, two GC GWAS were recently conducted on the Chinese population; one unveiled 3q13.31 and 5p13.1 as a GC-related chromosomal region, in addition to 1q22 and 8q24, and the other 10q23 (Table 1).[23,24] The 5p13.1 includes eight genes in the vicinity of rs13361707, and the susceptibility gene is yet to be identified in this region. Recombination hotspot analyses suggested *PLCE1*, a member of the phospholipase C family, in the 10q23, and *ZBTB20*, encoding zinc finger and BTB domain-containing protein 20, in the 3q13.31, are likely to be the causal for the association in the GWAS.[23,24]

PSCA gene

As mentioned above, *PSCA* was identified as a DGC susceptibility gene by the Japanese GWAS, although it was originally reported as the gene upregulated in prostate cancer.[25] It is also upregulated in many types of other cancers including urinary bladder cancer, renal cell carcinoma, hydatidiform mole, ovarian mucinous tumor, pancreatic cancer, non-small-cell lung cancer and glioma (Table 3).[26–32] In those cancers, PSCA can act to promote tumor progression and it was actually demonstrated that suppression of the gene with siRNA resulted in growth inhibition of prostate cancer cells.[33] In contrast, downregulation of the gene was reported only in esophageal and gastric cancers.[12,34] Recently, we have reported that the gene is also downregulated in gallbladder cancer.[35] Intriguingly, both gallbladder cancer and IGC develop in a similar sequence of chronic inflammation, intestinal metaplasia, dysplasia and cancer. Moreover, PSCA is downregulated in intestinal metaplasia in both gallbladder and gastric epithelia.[12,35] There could be other cancers in which PSCA is silenced during carcinogenesis.

It was demonstrated that PSCA has growth inhibition activity on GC cells, which is concordant with the finding of frequent downregulation in the cancer.[12] In the stomach, PSCA is expressed in the isthmus/neck region, a middle portion of the gastric epithelium, in which rapidly amplifying pre-pit cells are present to support the rapid turnover of mucus-secreting pit cells (Fig. 2). It is speculated that PSCA has a role in regulating cell growth of the pre-pit cells and that reduction of its function predisposes the pre-pit cells to abnormal cell division and carcinogenesis (Fig. 2). It is thought that the initial lesion of DGC arises in the isthmus/neck region, based on a detailed

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Table 2. Association studies of prostate stem cell antigen (*PSCA*) and gastric cancer

Report	SNP (major> minor)†	Ethnicity	Cases	Controls	Model‡	Reference§	Odds ratio	95% confidence interval	*P* value	Analyzed histology or subclasses
Sakamoto *et al.*[12]	rs2294008 T>C	Japanese	749	750	Per-allele	C	1.58	1.35–1.85	6.3×10^{-9}	Diffuse
			925 (diffuse) 599 (intestinal)	1396	Per-allele	C	1.67/1.29/1.30	1.47–1.90/1.11–1.49/ 1.10-1.52	2.2×10^{-15}/ 5.1×10^{-4}/0.0015	Diffuse/intestinal/ intestinal versus diffuse
					Dominant	CC	4.18/1.59/2.70	2.88–6.21/1.15–2.21/ 1.64–4.50	1.5×10^{-17}/ 0.0041/4.7×10^{-5}	
					Recessive	CC + CT	1.62/1.24/1.35	1.35–1.93/1.01–1.52/ 1.06–1.71	9.4×10^{-8}/ 0.040/0.013	
		Korean	454 (diffuse) 417 (intestinal)	390	Per-allele	C	1.91/1.37/1.39	1.57–2.33/1.12–1.68/ 1.14–1.69	6.3×10^{-11}/ 0.0017/7.9×10^{-14}	
					Dominant	CC	3.61/1.85/1.81	2.41–5.51/1.27–2.71/ 1.17–2.83	3.2×10^{-11}/ 0.0011/0.0066	
					Recessive	CC + CT	1.61/1.22/1.39	1.15–2.26/0.84–1.77/ 1.00–1.94	0.0051/0.31/0.050	
			749	750	Per-allele	G	1.62	1.38–1.89	1.1×10^{-9}	Diffuse
	rs2976392 A>G	Japanese	926 (diffuse) 599 (intestinal)	1397	Per-allele	G	1.71/1.29/1.32	1.50–1.94/1.12–1.49/ 1.13–1.56	1.5×10^{-16}/5.0×10^{-4}/6.0×10^{-4}	Diffuse/intestinal/ intestinal versus diffuse
					Dominant	GG	4.24/1.55/2.73	2.92–6.29/1.13–2.16/ 1.67–4.56	6.4×10^{-18}/ 0.0059/3.3×10^{-5}	
					Recessive	GG + GA	1.66/1.24/1.35	1.39–1.99/1.02–1.52/ 1.07–1.71	1.5×10^{-8}/ 0.035/0.012	
		Korean	449 (diffuse) 416 (intestinal)	390	Per-allele	G	1.90/1.37/1.39	1.56–2.33/1.12–1.68/ 1.14–1.69	8.0×10^{-11}/ 0.0017/9.0×10^{-4}	
					Dominant	GG	3.47/1.86/1.75	2.32–5.27/1.27–2.72/ 1.13–2.74	1.1×10^{-10}/ 0.0010/0.010	
					Recessive	GG + GA	1.64/1.24/1.41	1.17–2.30/0.86–1.80/ 1.01–1.97	0.0036/0.26/0.041	
Wu *et al.*[14]	rs2294008 C>T	Chinese	1020 (non-cardia) 716 (cardia)	1020	CT	CC	1.37/1.28	1.14–1.64/1.05–1.58	0.001/0.017	Non-cardia/cardia
					TT	CC	1.26/0.94	0.90–1.76/0.63–1.39	0.176/0.738	
					Dominant	CC	1.35/1.23	1.13–1.61/1.01–1.49	0.001/0.042	
	rs2976392G>A				GA	GG	1.21/1.09	1.01–1.45/0.89–1.34	0.041/0.402	
					AA	GG	1.14/0.99	0.82–1.59/0.69–1.44	0.430/0.968	
					Dominant	GG	1.20/1.08	1.01–1.43/0.88–1.31	0.041/0.470	
Matsuo *et al.*[15]	rs2294008 T>C	Japanese	708	708	Per-allele	C	1.4	1.19–1.65	3.7×10^{-5}	Gastric cancer
					Dominant	CC	2.07	1.45–2.95	6.4×10^{-5}	
					Recessive	CC + CT	1.31	1.11–1.65	0.003	
	rs2976392 A>G				Per-allele	G	1.4	1.19–1.65	4.1×10^{-5}	
					Dominant	GG	2.09	1.46–2.99	5.7×10^{-5}	
					Recessive	GG + GA	1.36	1.10–1.67	0.004	

Table 2 (continued)

Report	SNP (major> minor)†	Ethnicity	Cases	Controls	Model‡	Reference§	Odds ratio	95% confidence interval	*P* value	Analyzed histology or subclasses
Lu *et al.*[16]	rs2294008 C>T	Chinese	1053	1100	CT	CC	1.16/1.24/1.17	0.97–1.39/0.89–1.73/ 0.95–1.42		Gastric cancer/ diffuse/intestinal
					TT	CC	1.05/1.21/0.94	0.74–1.47/0.66–2.24/ 0.64–1.39		
					Dominant	CC	1.14/1.24/1.13	0.96–1.36/0.90–1.70/ 0.93–1.37		
	rs2976392G>A				GA	GG	1.40/1.34/1.37	1.17–1.67/0.96–1.87/ 1.12–1.66		
					AA	GG	1.23/1.87/1.03	0.88–1.72/1.10–3.20/ 0.70–1.52		
					Dominant	GG	1.37/1.43/1.31	1.15–1.62/1.04–1.96/ 1.09–1.58		
Ou *et al.*[17]	rs2294008C>T	Tibetan	196	246	Per-allele	C	1.34	1.00–1.79	0.049	Gastric cancer
					CT	CC	1.5	1.01–2.23	0.042	
					TT	CC	1.55	0.77–3.15	0.221	
	rs2976392G>A				Per-allele	G	1.07	0.80–1.45	0.645	
					GA	GG	1.09	0.74–1.61	0.650	
					AA	GG	1.13	0.50–2.54	0.776	
Lochhead *et al.*[18]	rs2294008C>T	Caucasian	312	383	CT	CC	1.9/2.9/1.6	1.2–2.9/1.0–10.1/ 1.0–2.6	0.003/0.028/0.040	Gastric cancer/ diffuse/intestinal
					TT	CC	1.9/3.7/1.6	1.2–3.0/1.3–12.9/ 1.0–2.7	0.004/0.008/0.058	
					Dominant	CC	1.9/3.2/1.6	1.3–2.8/1.2–10.7/ 1.0–2.6	0.001/0.011/0.029	
					Recessive	CC + CT	1.2/1.7/1.2	0.9–1.7/0.9–3.2/ 0.8–1.7	0.184/0.089/0.431	
			309	211	CT	CC	0.8/0.5/0.8/0.7	0.5–1.5/0.3–0.9/ 0.4–1.7/0.3–1.7	0.521/0.008/0.493/ 0.419	Non-cardia/cardia/ non-cardia diffuse/ non-cardia intestinal
					TT	CC	1.7/0.6/1.7/1.2	0.9–3.0/0.3–1.2/ 0.8–3.6/0.5–2.9	0.069/0.105/0.155/ 0.651	
					Dominant	CC	1.1/0.5/1.1/0.9	0.7–1.8/0.3–0.9/ 0.6–2.1/0.4–1.9	0.689/0.010/0.849/ 0.717	
					Recessive	CC + CT	1.9/0.9/1.9/1.5	1.2–3.0/0.5–1.6/ 1.1–3.5/0.7–2.9	0.005/0.766/0.018/ 0.246	
Zeng *et al.*[19]	rs2294008C>T	Chinese	460	549	CT	CC	1.38	1.06–1.78	0.018	Gastric cancer
					TT	CC	1.66	1.03–2.69	0.038	
					Dominant	CC	1.42	1.10–1.82	0.006	
Song *et al.*[20]	rs2294008 C>T	Korean	3245	1700	Per-allele	C	1.29	1.18–1.41	<0.01	Gastric cancer
					CT	CC	1.50	1.28–1.76	<0.01	
					TT	CC	1.71	1.43–2.04	<0.01	
Sala *et al.*[21]	rs2294008 C>T	Caucasian	411	1530	Log-additive		1.42/1.47/1.54/1.52	1.23–1.66/1.19–1.81/ 1.20–1.96/1.20–1.93	6.5×10^{-6}/0.0003/ 0.0005/0.0005	Gastric cancer/ non-cardia/diffuse/ intestinal
					CT	CC	1.46/1.43/1.32/1.68	1.23–1.66/0.98–2.10/ 0.84–2.07/1.08–2.62	3.7×10^{-5}/0.0015/ 0.0018/0.0022	
					TT	CC	2.02/2.15/2.31/2.34	1.49–2.76/1.41–3.26/ 1.43–3.73/1.43–3.83		

†Major, major allele; minor, minor allele. ‡Genetic model for the biological effect of risk alleles (rs2294008:T, rs2976392:A). §Genetic model as reference (odds ratio = 1).

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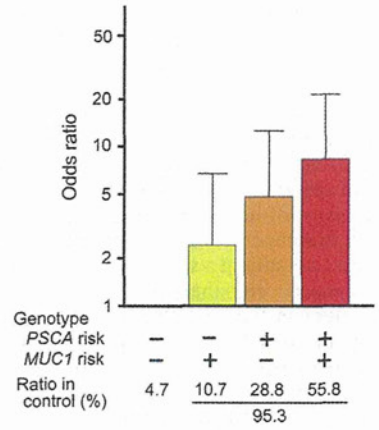

Fig. 1. Prostate stem cell antigen (*PSCA*) and Mucin 1 (*MUC1*) genotypes are associated with risk for diffuse-type gastric cancer (DGC). Association studies were performed with a distinct model for each risk allele's effect, dominant for rs2294008 (risk genotype: TT and TC; protective genotype: CC) and recessive for rs4072037 (risk genotype: AA; protective genotype: GG and GA), using genotype data of rs2294008 in *PSCA* and rs4072037 in *MUC1* (Japanese 605 DGC cases and 1264 controls). Bar, upper bound of 95% confidence interval.

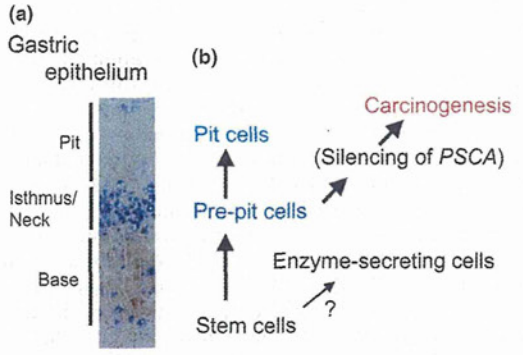

Fig. 2. Prostate stem cell antigen (*PSCA*) regulates proliferation of pre-pit cells in gastric epithelium? (a) PSCA is mainly expressed in the epithelium in the middle portion, the isthmus and neck regions, of the gastric gland (immunohistochemical double stain; blue for PSCA and brown for proliferating cell nuclear antigen). Together the two regions are called the isthmus/neck region as the boundary between the two regions is often ambiguous. Weak PSCA expression is also observed in the epithelium of the pit region. (b) The isthmus/neck region harbors pre-pit cells, a precursor of pit cells, which are rapidly proliferating to compensate for rapid turnover of pit cells. It is hypothesized that PSCA regulates proliferation of pre-pit cells, which also contributes to prevention of carcinogenesis in the epithelium.

Table 3. Cancer-type dependent expression status of the prostate stem cell antigen (*PSCA*) gene

Upregulation	Downregulation
Prostate cancer[25]	Esophageal cancer[34]
Urinary bladder cancer[26]	Gastric cancer[12,34]
Renal cell carcinoma[27]	Gallbladder cancer[35]
Hydatidiform mole[28]	
Ovarian mucinous tumor[29]	
(Aberrant expression)†	
Pancreatic cancer[30]	
Non-small-cell lung cancer[31]	
Glioma[32]	

†Upregulation in cancer but no expression in normal tissue.

histopathological investigation in Japan, which revealed that the smallest lesions of dysplasia and carcinoma seem to be confined to the region.[36]

It was demonstrated that the rs2294008 in the gene is the functional SNP affecting transcriptional activity of the *PSCA* promoter. However, the biological effect of the T allele still seems to be considerably controversial. The rs2294008 determines the position of the translation initiation codon; the T allele makes itself part of the codon encoding as the first methionin (ATG); in contrast, the C allele replaces the encoded amino acid from methionine to threonin (ACG), resulting in a change of the first methionine position. The T allele associated with the risk for GC has a negative effect on the promoter activity in gastric, urinary bladder and gallbladder cancer cell lines.[12,22,35] Therefore, it is anticipated that people possessing the T allele have a lower amount of PSCA protein in the organs than those possessing the C allele. However, recent microarray transcriptome analyses showed that normal and malignant urinary bladder tissues from people with the T allele contained more *PSCA* transcripts than those from people with the C allele.[37] The discrepancy between the *in vitro* reporter assay and the *in vivo* expression data further suggests a complexity of the PSCA regulation, which might be influenced by tissue-specific transcriptional factors and DNA methylation, as shown in gastric and gallbladder cancer cell lines.[35] In contrast, a recent study reported that the C allele of rs2294008 in *PSCA* was associated with an increased risk of duodenal ulcer (DU; odds ratio = 1.84; $P = 3.92 \times 10^{-33}$ in a recessive model).[38] Moreover, the results of functional analyses showed that the C allele changes the subcellular localization of PSCA protein from the cell surface to the cytoplasm and also reduced the protein's stability.[38] As PSCA might have several functions, some of which could be contradictory, in the context of tissues and pathological states, it is likely that the functional effect of rs2294008 might also differ. In particular, the reciprocal association between the rs2294008 alleles and two major HP-related gastrointestinal diseases is notable; the T allele predisposes to GC, while the C allele confers an increased risk for DU. It is known that patients with DU have a decreased risk for GC, but it depends on the location of gastritis; DU patients with chronic corpus gastritis have an increased risk of GC.[39] This could be explained by the relation between the location of gastritis and the amount of acid secretion; corpus-predominant gastritis is accompanied by hypochlorhydria and results in the highest risk for GC, whereas antrum-predominant gastritis is associated with hyperchlorhydria and predisposes to DU disease.[40,41] As PSCA is not expressed in the duodenum, its function in the stomach might affect DU development, possibly through such effects as those related to acid secretion, location and extent of HP infection or gastritis. It was also reported that the T allele was especially associated with non-cardia GC in Chinese and Caucasian patients but with cardia GC in Korean patients.[14,18–21]

The cell growth inhibition activity of PSCA was also demonstrated on gallbladder cancer (GBC) cells.[35] The GBC cells introduced with *PSCA* cDNA showed lower *in vitro* and *in vivo* growth than controls, and their invasion ability assayed with a Matrigel chamber was also attenuated.[35] PSCA is expressed homogenously in normal gallbladder epithelium, which is characterized by a mono-layer of columner cells and functions by absorbing water and electrolytes. It is possible that PSCA has a role in cell-division control and/or other activities such as active transport of molecules in gallbladder epithelium.

The product of the *PSCA* gene is glycosylphosphatidylinositol (GPI)-anchored membrane protein with unknown biological

function.[42] It is believed that, as with other GPI-anchored proteins, PSCA might be located in a special microdomain called lipid raft, enriched in glycosphingolipids, cholesterol and other lapidated proteins, on the outer surface of the cell membrane. The lipid raft is known as the domain where molecular interaction for subcellular signaling is processed.[43] However, there have been no reports on the elucidation of the PSCA ligand or the molecule on which PSCA makes some modification. Our attempt to co-immunoprecipitate PSCA-associating molecules has not been successful, even with cross-linking of proteins using DSP (dithiobis[succinimidyl-proprionate]) or DTSSP (3,3′-dithiobis[sulfosuccinimidylpro-prionate]). The function of PSCA might be restricted to a preparation of the microenvironment on the cell membrane by changing the local composition of lipid and other molecules, which supports some molecular interaction required for subcellular signal transduction. If that is the case, its apparently organ-dependent opposing function, either tumor promotion or suppression, could be highly comprehensible.

MUC1 gene

The Mucin family (MUC1 to MUC21) consists of secretary and membrane-bound types, and MUC1 belongs to the latter.[44] After being translated, a single MUC1 peptide was cleaved to N-terminal and C-terminal subunits, designated as MUC1-N and MUC1-C, respectively, by autoproteolysis, but both the subunits remain associated by non-covalent binding and are localized to the cell membrane in the apical side of the epithelial cells. MUC1-N present on the cell surface has multiple glycosylation sites and is thought to be the second line of protection for cells against many types of insults, after the front layer of defense by the secretary mucins in mucus.[45] However, MUC1-C has a transmembrane domain and a cytoplasmic tail (CT), which is involved in subcellular signal transduction. The CT contains several phosphorylation sites and a β-catenin binding site. Phosphorylation of Thr in the TDRSPYEKV sequence within the CT stimulates interactions between the CT and β-catenin, which leads to nuclear localization of the complex for regulating genes including *p53*.[46,47] Previously, and maybe at present, MUC1 has been considered as an oncoprotein, because there are several studies demonstrating the correlation of MUC1 expression and the poor prognosis of cancer patients. It was also reported that MUC1 acts as a growth factor receptor on undifferentiated human embryonic stem cells.[48–50]

In addition to the GWAS conducted in Japan, the recently conducted GWAS on the Chinese population also listed 1q22 as a candidate for GC-related locus (rs4072037; odds ratio = 0.75, $P = 4.22 \times 10^{-7}$).[12,24] The association between noncardia GC and the GC-related locus was also demonstrated in imputation analyses on large-scale Chinese case-control samples (rs4072037; odds ratio = 0.73, $P = 1.0 \times 10^{-4}$).[23] Moreover, an association between *MUC1* gene polymorphisms and GC has also been reported by other groups previously.[51,52] The *MUC1* gene contains a polymorphic number of tandem repeats, a variable number of tandem repeats (VNTR), which are shown by an electrophoresis pattern after restriction enzyme digestion. When the polymorphic allele is divided into large (L) and small (S) alleles, the latter was shown to associate with GC in Caucasians.[51,52] The association between the A allele of rs4072037 and DGC identified in Japanese patients was also found in Chinese and Caucasian patients.[53,54] The A allele of rs4072037 identified using the GWAS is in a linkage disequilibrium with the S allele in Japanese and Caucasian patients.[13,55] The results of these association studies on different ethnic populations strongly support the result of the Japanese GWAS; *MUC1* is a GC susceptibility gene.

It was demonstrated that the rs4072037 in *MUC1* has a biological function. In the gastric epithelium, variants 2 and 3 are the major *MUC1* transcript.[13] The rs4072037 is located in the 5′ side of the second exon of *MUC1* and determines the splicing acceptor site in the second exon, which in turn defines the type of variants; the G and A alleles result in the expression of variants 2 and 3, respectively (Fig. 3).[13,55] Consequently, the nine amino acid deletion in the second exon changes the supposed cleavage site of the N-terminal signal peptide, which might lead to a difference in the function of the encoded protein between the two splicing variants. It is understood that, in GC and other cancer cells that have lost cell polarity, the MUC1 protein interacts freely with other molecules including membrane receptors involved in cell growth and, consequently, it acts as an oncoprotein; in contrast, in normal epithelial cells, MUC1 is restricted to the apical surface of the cells where the interaction with other molecules is limited and it acts as a barrier against exogenous insults to the cells.[56] It is speculated that the rs4072037 affects the barrier function in the stomach of individuals through the determination of a major variant expressed in the stomach, which results in the difference in GC susceptibility. From a different viewpoint, *MUC1* was identified as associating with the serum magnesium level using GWAS.[57] Because a correlation between a low serum magnesium level and GC was suggested, it is possible that MUC1 affects GC susceptibility by playing a role in magnesium homeostasis.[58] However, it was demonstrated using the GWAS that hypomagnesaemia was correlated with the G allele of rs4072037, the protective allele for GC development.[57] As no correlation was observed between *MUC1* and IGC in the present study ([13] and Hiromi Sakamoto, Teruhiko Yoshida

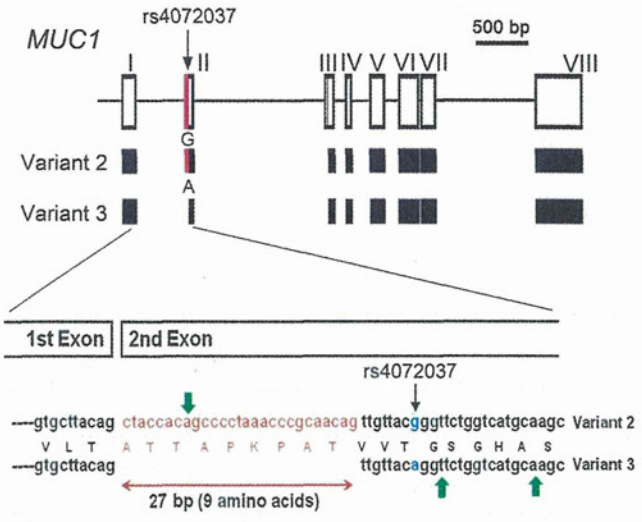

Fig. 3. Single nucleotide polymorphism (SNP) rs4072037 determines the major splicing variants expressed in the gastric mucosa. In the gastric mucosa, major splicing forms were variant 2 (NM_001018016) and variant 3 (NM_001018017). The allele of SNP rs4072037 is related to the splicing acceptor site selection in the second exon (upper panel). Nucleotide sequences of the first/second exon boundary of Mucin 1 (*MUC1*) variants 2 and 3 revealed using RNA ligase-mediated rapid amplification of the 5′ cDNA end on RNA samples from normal stomach and gastric cancer cell lines (lower panel).[13] In the present study, all variant 2 transcripts containing the first 27 bp of the second exon (double-headed red arrow) had a G allele at rs4072037, while all variant 3 lacking the 27 bp had A allele. This result is concordant with a previous report.[55] It is anticipated that deletion of the 27 bp, corresponding to nine amino acids, changes cleavage sites (green arrows) of the signal peptide among the variants. One-letter amino acid abbreviation is shown just below or above the second nucleotide of each codon.

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and Yusuke Nakamura, unpublished data), *MUC1* probably has a role specifically in DGC, contrary to *PSCA* whose association was revealed in both DGC and IGC (Table 2). This difference might derive from the difference in their pathogenesis.

Perspective

It is expected that identification of GC susceptibility genes will contribute to the development of a new approach in GC prevention in the future. The risk genotypes of the two genes identified by the previous Japanese GWAS classified the majority of Japanese people into a high-risk group (Fig. 1). This finding is supported by HapMap Project data on 11 ethnic populations, which show, for example, that the Japanese population has the lowest frequency of the protective genotype (C/C) of rs2294008 in the 11 populations (Supporting Information Table S1). This offers a good starting point for the development of a personalized DGC prevention, because it means that we could add other layers of risk factors to capture even higher DGC risk subgroups without too much size diminution and restriction of the target population. Adding non-genetic risk factors for a further stratification is of particular interest, because they could be modifiable, while the genetic predisposition presents a fixed and basic risk probability of each individual. It should also be noted that the Japanese GWAS on GC was initiated almost 10 years ago. Since then, more powerful platforms for efficient SNP typing including next-generation sequencers have been developed, and more numerous DNA samples have been accumulated for a GWAS by several groups in Japan, including those involved in prospective cohort studies. Other novel GC susceptibility genes and their interactions with non-genetic risk factors can be identified by conducting a GWAS with a large number of the samples and the latest typing platforms, especially in a nested case-control design based on a molecular epidemiological or "genome" cohort. Such a systematic approach will contribute not only to GC prevention but also to the development of new GC therapeutics by unveiling novel molecular pathways involved in GC carcinogenesis and should be one of the urgent agenda items in medical research in light of the overwhelming social burden of cancer death in Japan and the world.

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Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

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

Table S1. Genotype and allele frequencies of two single nucleotide polymorphisms, rs2294008 and rs4072037, in 11 ethnic populations.

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Intraperitoneal delivery of a small interfering RNA targeting *NEDD1* prolongs the survival of scirrhous gastric cancer model mice

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The prognosis of patients with advanced diffuse-type gastric cancer (GC), especially scirrhous gastric cancer (SGC) remains extremely poor. Peritoneal carcinomatosis is a frequent form of metastasis of SGC. With survival rates of patients with peritoneal metastasis at 3 and 5 years being only 9.8% and 0%, respectively, development of a new treatment is urgently crucial. For such development, the establishment of a therapeutic mouse model is required. Among the 11 GC cell lines we examined, HSC-60 showed the most well-preserved expression profiles of the Hedgehog and epithelial-mesenchymal transition pathways found in primary SGCs. After six cycles of harvest of ascitic tumor cells and their orthotopic inoculation in scid mice, a highly metastatic subclone of HSC-60, 60As6 was obtained, by means of which we successfully developed peritoneal metastasis model mice. The mice treated with small interfering (si) RNA targeting *NEDD1*, which encodes a gamma-tubulin ring complex-binding protein, by the atelocollagen-mediated delivery system showed a significantly prolonged survival. Our mouse model could thus be useful for the development of a new therapeutic modality. Intraperitoneal administration of siRNAs of targeted genes such as *NEDD1* could provide a new opportunity in the treatment of the peritoneal metastasis of SGC. (*Cancer Sci* 2013; 104: 214–222)

Gastric cancer (GC) is one of the leading causes of cancer-related death worldwide.[1,2] Histopathological research has long suggested that gastric cancer is not a single disease and recognizes two major categories: intestinal and diffuse.[3] Intestinal-type GC develops through some sequential stages including *Helicobacter pylori* (*H. pylori*)-associated gastritis, intestinal metaplasia (IM), and dysplasia. This type predominates in high-risk geographic areas, such as East Asia, showing a correlation with the prevalence there of *H. pylori* infection among elderly people. Diffuse-type GC, however, is more uniformly distributed geographically, is apparently unrelated to *H. pylori* prevalence and typically develops from *H. pylori*-free, morphologically normal gastric mucosa without atrophic gastritis, or IM. Unlike the decreasing incidence of the intestinal-type, the prevalence of the diffuse-type is reportedly increasing worldwide.[4] Although therapeutic results for GC have recently improved, the prognosis for patients with advanced diffuse-type GC, especially scirrhous gastric cancer (SGC, Borrmann's type IV carcinoma or the linitis plastic type) remains extremely poor. The 5-year overall survival rate of SGC is approximately 10%, and ranges from 18% to 29% even after curative surgery.[5–7] Histopathologically, SGC does not form glands; instead, it causes diffuse infiltration of a broad region of the gastric wall rather than a well-defined mass, resulting in a fibrous-like thickening of the wall. Such pathological features make an early clinical diagnosis of SGC difficult, and in approximately half of the cases, by the time the diagnosis is made, peritoneal dissemination has, unfortunately, already occurred.[5,8] Peritoneal dissemination, known to be a frequent form of metastasis and recurrence of SGC, serves as a major factor determining patient prognosis.[9] Currently, no effective therapy exists for this condition. For SGC patients with peritoneal metastasis, the survival rates at 3 and 5 years are only 9.8% and 0%, respectively, even if the patients received multidisciplinary treatment.[5]

It has been suggested that peritoneal dissemination is a consequence of free cancer cells that are shed from the serosa of the primary lesion and/or may leak out from the lymphatics to the peritoneal cavity; however, no detailed mechanism of peritoneal dissemination has been fully elucidated. In either situation, it is assumed that free cancer cells detached from a primary lesion must have a predilection for the peritoneum. Efficacious control of invisible free cancer cells in the peritoneal cavity should help suppress the progression of carcinomatous peritonitis, and could ultimately yield a survival benefit. Some investigators have reported good, but limited, outcomes with new treatment strategies for peritoneal dissemination, including systemic chemotherapy,[10] intraperitoneal (i.p.) chemotherapy and/or hyperthermia,[11] and peritonectomy.[12] Therefore, to improve patient outcome, the development of a new therapeutic strategy for peritoneal dissemination of SGC is urgently crucial.

In this study, we developed peritoneal metastasis model mice of SGC and an atelocollagen-mediated delivery system for i.p. administration of small interfering (si) RNA, and also reported that the i.p. delivery of an siRNA targeting *NEDD1*, which functions in the metaphase regulation of the cell cycle, was able to regress the tumor and prolong, without toxicity, the survival of the mice.

Materials and Methods

Tissue samples. Gastric cancer and non-cancerous tissues were provided by the National Cancer Center Hospital (Tokyo, Japan) after obtaining informed consent from each patient and approval by the Center's Ethics Committee. Tissue specimens were immediately frozen with liquid nitrogen after surgical extraction, and stored at −80°C until use.

Cell lines and culture. A human scirrhous gastric cancer cell line, HSC-60 was established by a collaborator using the

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procedure as described.[13] A highly peritoneal-seeding cell line, 60As6 was established from HSC-60 using orthotopic tissue implantation into scid mice as briefly follows: the xenografted tumor of HSC-60 cells was transplanted into the gastric wall of a scid mouse. We repeated six cycles of harvest of ascitic tumor cells and the orthotopic inoculation of these cells, in turn, into the animals to establish a highly metastatic 60As6 cell line. These two cell lines were maintained in an RPMI1640 medium supplemented with 10% FCS. In this study, we also used luciferase- or green fluorescence protein (GFP)-expressing transfectants. Another 11 GC-derived cell lines (HSC-39, HSC-43, HSC-44, HSC-58, HSC-59, HSC-60, KATOIII, MKN7, MKN28, MKN74, and HSC-57) were also maintained in the same way. Of them, seven HSC cell lines were established by a collaborator using the procedure as described,[13] and four other cell lines were obtained from American Type Culture Collection.

***In vivo* photon counting analysis.** To establish transfectants expressing the luciferase gene, plasmid vectors carrying the firefly luciferase gene named pLuc/Neo and a transfection reagent, LipofectAMINE 2000 (Invitrogen, Carlsbad, CA, USA) were used in accordance with the manufacturer's instructions. Stable transfectants were selected in geneticin (500 μg/mL; Invitrogen) and bioluminescence was used to screen the transfected clones for luciferase gene expression using the IVIS system (Xenogen, Alameda, CA, USA). *In vivo* photon counting and optical imaging to detect luciferase activity in the mice were conducted on the IVIS system as described previously.[14] Animal protocols were approved by the committee for Ethics of Animal Experimentation and were in accordance with the Guideline for Animal Experiments at the National Cancer Center.

siRNA preparation. The sequence of *NEDD1* siRNA was 5′-CGAAGUGUUAAUGUGAAUGtt-3′ and 3′-ttGCUUCACA AUUACACUUAC-5′ (Ambion, Austin, TX, USA). Non-specific control siRNA duplex and luciferase GL3 siRNA duplex were purchased from Dharmacon (Lafayette, CO, USA). *ELK1* siRNA, 5′-GCUGAGAGAGCAAGGCAAUtt-3′and 5′-AUUGC CUUGCUCUCUCAGCtt-3′ (SI00300146, Qiagen, Valencia, CA, USA) and *MSX2* siRNA, 5′-CCAUUACCUAUAUGCU AAAt t-3′ and 5′-UUUAGCAUAUAGGUAAUGGtt-3′(SI0003 8031, Qiagen) were used. For *in vitro* studies, 5×10^4 cells were seeded per 6-well culture dish. When cells had grown to approximately 80% confluency, a mixture of 3 μg siRNA and 5 μL DharmaFECT (Dharmacon) was added to the medium in each dish.

Therapeutic studies with *NEDD1* siRNA. Intraperitoneal (i.p.) injection of 60As6Luc cells resuspended in 1 mL PBS was conducted in 6-week-old female C.B17/Icr-scid (scid/scid) mice, followed by i.p. inoculation of various siRNA/atelocollagen complexes. Atelocollagen is a highly purified type I collagen of calf dermis with pepsin treatment (Koken, Tokyo, Japan). The siRNA/atelocollagen complexes were prepared as follows: an equal volume of atelocollagen (pH 7.4) and an

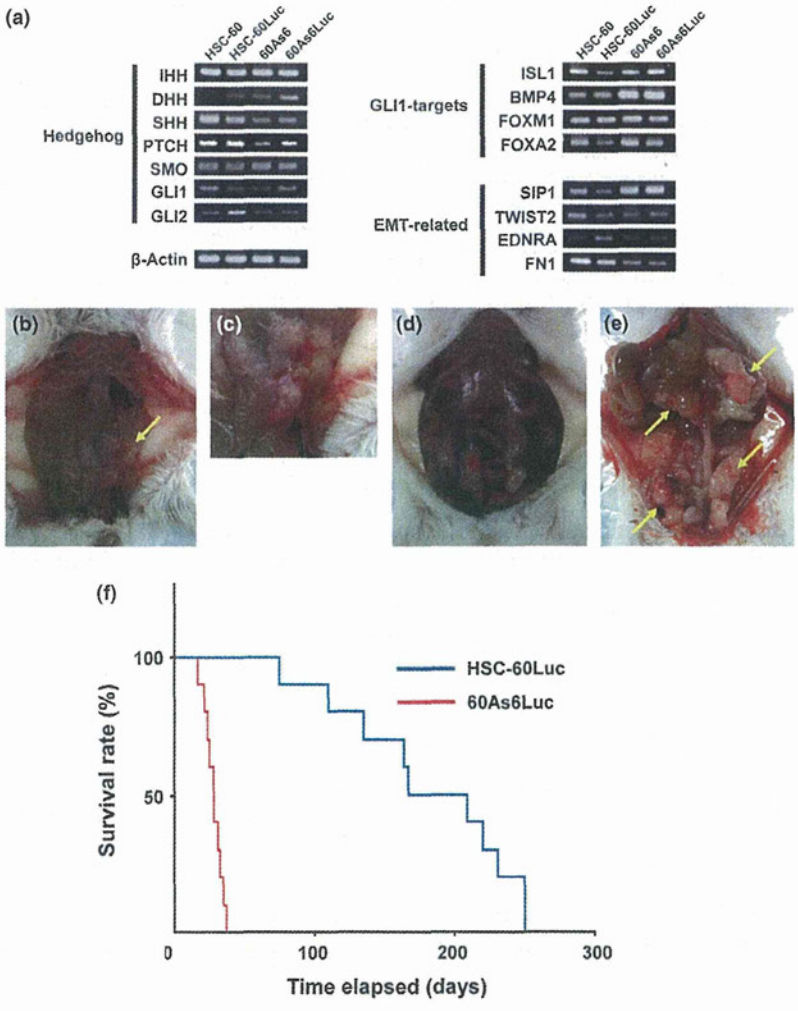

Fig. 1. Characteristics of HSC-60 and 60As6 cells. (a) mRNA expression of hedgehog- and epithelial-mesenchymal transition (EMT)-related gene in HSC-60 and 60As6. Shown are results of reverse transcription-polymerase chain reaction (RT-PCR) of hedgehog ligands (*IHH*, *DHH*, and *SHH*), a receptor (*PTCH*), a modulator (*SMO*), two primary target transcriptional factors (*GLI1* and *GLI2*), four authentic GLI1-targets (*ISL1*, *BMP4*, *FOXM1*, and *FOXA2*), two EMT regulators (*SIP1* and *TWIST2*), two EMT-related molecules (*FN1* and *EDNRA*), and a control (*β-Actin*). (b, c) Macroscopic appearance of the peritoneal dissemination and survival of scid mice after intraperitoneal injection of HSC-60Luc and 60As6Luc. A few peritoneal nodules are observed 15 weeks after i.p injection of HSC-60Luc. Yellow arrow: tumor nodule. (d, e) Carcinomatous peritonitis forming multiple tumor nodules observed 2 weeks after i.p. injection of 60As6. Abdominal distension because of bloody ascites was evident. Yellow arrow: tumor nodule. (f) Survival of HSC-60Luc- and 60As6Luc-tumor-bearing mice. Each median survival time is 28 days and 167 days, respectively. $n = 10$; $P < 0.0001$.

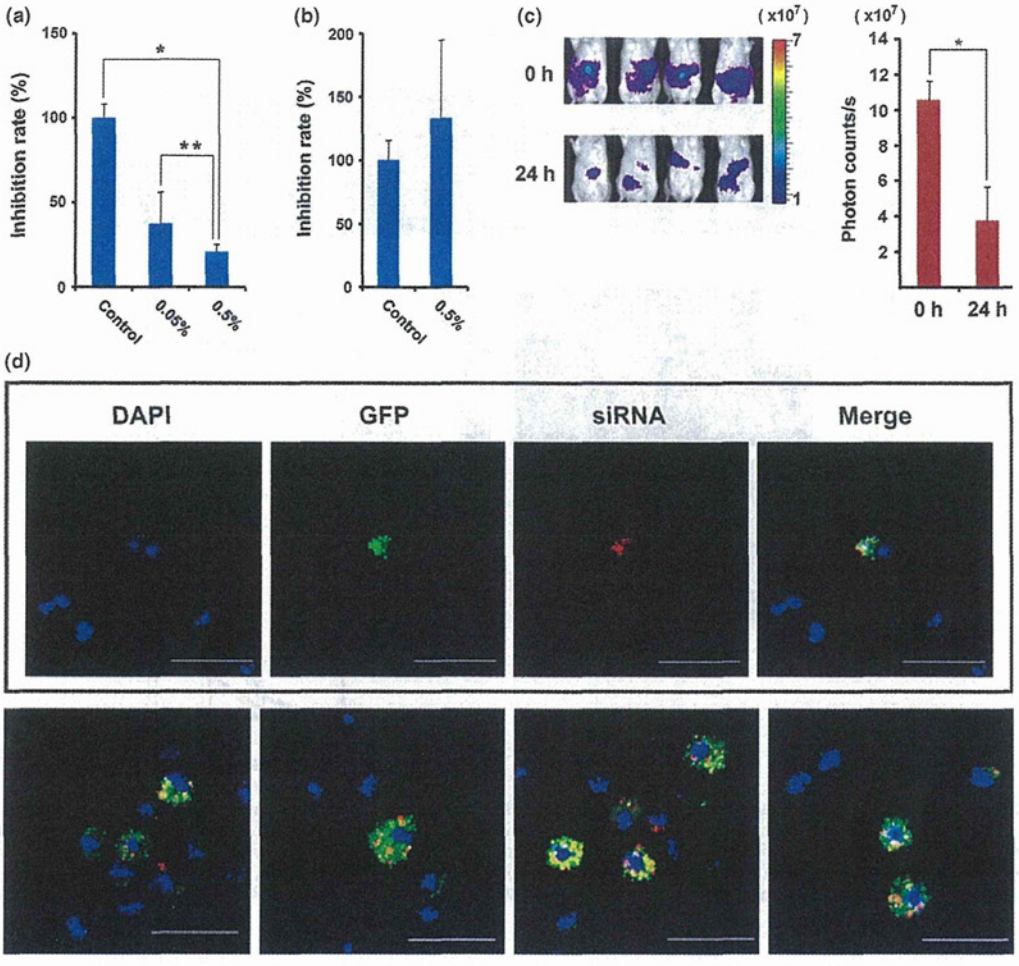

Fig. 2. Evaluation of an atelocollagen-mediated siRNA delivery system by measuring the luciferase activity after i.p injection of luciferase siRNA. (a) Inhibition rates of photon counts 48 h after injection are compared between 0.05% and 0.5% atelocollagen/luciferase siRNA/DharmaFECT1. $*P = 0.006$; $**P = 0.012$. (b) Inhibition rates of photon counts 48 h after injection are compared between 0.5% atelocollagen/control siRNA and 0.5% atelocollagen/luciferase siRNA. No significant inhibition is observed in a luciferase siRNA complex containing no DharmaFECT1. (c) Reduction of luciferase activity 24 h after injection with 0.5% atelocollagen/luciferase siRNA/DharmaFECT1 is visualized in four representative mice (left). Significance of the reduction is also shown (right). $*P < 0.0001$. Color bar indicates $\times 10^7$ photon/s. (d) Delivery evidence of siRNA to cancer cells in the peritoneal cavity. Most green fluorescent protein (GFP)-expressing 60As6 cells (green) incorporate fluorescence-labeled siRNA (red). Bar, 50 μm.

siRNA solution were mixed by rotation at 4°C for 20 min. Before i.p. inoculation, 10 μL of DharmaFECT1 was added to the complex. The final mixture was 1 mL containing 50 μg siRNA, 10 μL DharmaFECT1 and 0.5% atelocollagen. For obtaining delivery evidence of siRNA to cancer cells in the peritoneal cavity, we used a fluorescence-labeled human siGLO LaminA/C Control siRNA (Thermo Fisher Scientific, Rockford, IL, USA).

Laser microdissection, RNA extraction and RT-PCR. In the gastric corpus, the epithelium consists of three tubular units from surface to base: a pit region containing mucus-secreting pit cells, an isthmus/neck region containing stem cells, and a gland region containing chief and parietal cells.[15] We prepared each region (pit, neck, and gland) as follows: the cryostat sections (8 μm) of frozen tissues were microdissected with a Pixcell II LCM system (Arcturus Engineering, Mountain View, CA, USA). Total RNA was isolated by suspending the cells in an ISOGEN lysis buffer (Nippon Gene, Toyama, Japan) followed by precipitation with isopropanol. The mRNA was amplified by an efficient method of high-fidelity mRNA amplification.[16,17] Other normal and gastric cancer tissues were provided by our hospital between 2003 and 2004 after obtaining informed consent from each patient and approval by the Institutional Ethics Committee. Tissue specimens were snap-frozen in liquid nitrogen, and stored at −80°C until use. Total RNA was isolated by suspending the cells in an ISOGEN lysis buffer followed by precipitation with isopropanol. As described in our previous report,[18] semi-quantitative RT-PCR within linear range by performing 25–35 cycles for *IHH, DHH, SHH, GLI1, GLI2, PTCH, SMO, SIP1, TWIST2, ISL1, BMP4, FOXM1, FOXA2, FN1, EDNRA,* and *ACTB* (*β-Actin*) was carried out. For *NEDD1*, 5′-TTCTGTCACTGCT GGAGTTG-3′ and 5′-TGTGTTGCCAGAAACTTCCC-3′ were used as primers.

Western blot analysis. The proteins (20 μg) were separated on a 10% SDS-polyacrylamide gel and transferred to an Immobilon-P membrane (Millipore, Billerica, MA, USA). The blots were incubated overnight with mouse monoclonal anti-human Nedd1 antibody (Abcam, Cambridge, MA, USA).

Statistical analysis. All data were expressed as the mean ± SE, and analyzed using the unpaired *t*-test. Survival curves were calculated according to the Kaplan–Meier method. Differences

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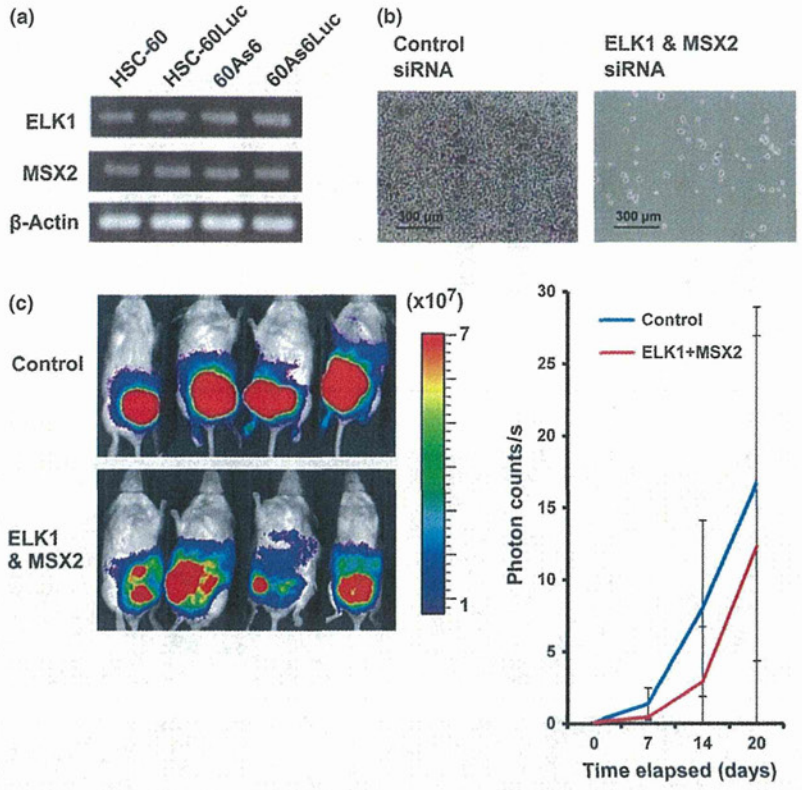

Fig. 3. The expression of cancer-specific hedgehog targets *ELK1* and *MSX2* in HSC-60 and 60As6 and the effect of silencing of *ELK1and MSX2* on *in vitro* and *in vivo* cell growth of 60As6. (a) RT-PCR of *ELK1* and *MSX2* in HSC-60 and 60As6. (b) Cell growth inhibition of 60As6 cells 2 days after double transfection of *ELK1* and *MSX2* siRNA. (c) Each of four mice treated with *ELK1 and MSX2* siRNA or control siRNA is visualized 27 days after inoculation of 60As6Luc by the IVIS system (left). Color bar indicates $\times 10^7$ photon/s. Results of the time course experiments on tumor growth inhibition by the double treatment are shown (right). Although small effects on the growth inhibition are observed, no significance is shown.

between survival curves were examined with the log rank test. The accepted level of significance was $P < 0.05$. SPSS (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses.

Results

Establishment of a highly metastatic cell line 60As6 from a parental cell line HSC-60. We previously reported that the hedgehog signal is more active in diffuse-type gastric cancer (GC) including scirrhous GC (SGC) than in the intestinal-type GC and recently reported that crossroad between hedgehog and epithelial-mesenchymal transition (EMT) signals is present in the diffuse-type.[17,18] Among 11 GC-derived cell lines (HSC-39, HSC-43, HSC-44, HSC-58, HSC-59, HSC-60, KATOIII, MKN7, MKN28, MKN74, and HSC-57), HSC-60 was found to most-closely mimic the diffuse-type GC phenotype in mRNA expression of hedgehog- and EMT-related genes.[18] However, HSC-60 cells often formed only a single tumor nodule in the peritoneal cavity and no ascites in scid mice despite intraperitoneal (i.p.) implantation of many cells (more than 1×10^6 cells) (data not shown). Therefore, we established a highly peritoneal-seeding cell line, 60As6, from this parental cell line, HSC-60, by six cycles of isolating ascitic tumor cells and orthotopic inoculation of these cells as described in our previous report,[13] and we next obtained transfectants (HSC-60Luc and 60As6Luc) containing the luciferase gene for *in vivo* imaging in animal experiments. Reverse transcription-PCR showed that the expression of the above-mentioned hedgehog and EMT signaling genes in HSC-60 cells was maintained in the 60As6 cells (Fig. 1a). The doubling time of these two cell lines was comparable (30 h in HSC-60 and 31 h in 60As6). The peritoneal dissemination and the survival rates of scid mice after i.p. implantation of HSC-60 and 60As6 are shown (Fig. 1b–f). None of the HSC-60-tumor-bearing mice developed ascites (Fig. 1b,c), and the median mice survival time was 167 days after implantation of 5×10^6 HSC-60Luc cells (Fig. 1f). On the other hand, implantation of 5×10^6 60As6Luc cells resulted in the formation of remarkably bloody ascites approximately 14 days later (Fig. 1d), and the median survival time was 28 days (Fig. 1f). In the 60As6-tumor-bearing mice, peritoneal dissemination was often seen in the omentum, mesenterium, parietal peritoneum, diaphragm, and so on (Fig. 1e).

Development of an siRNA delivery system into peritoneal metastatic tumor cells. The atelocollagen-mediated gene delivery system was originally developed by a collaborator.[19] In mice, this delivery system has been reported to be useful for gene delivery into some body sites including metastatic tumors and also for systemic gene delivery;[20] however, its application into a peritoneal metastatic tumor has not been reported. Previous studies indicated that a low concentration (0.05%) of atelocollagen was effective for systemic siRNA delivery, whereas a high concentration (0.5%) was useful for an intratumor siRNA delivery,[20] and also indicated that a transfection reagent, DharmaFECT1 accelerated an atelocollagen-mediated siRNA delivery.[20] We first investigated an optimal atelocollagen concentration for an i.p. siRNA delivery into tumor cells by measuring luciferase activity. Those between a 50 μg luciferase

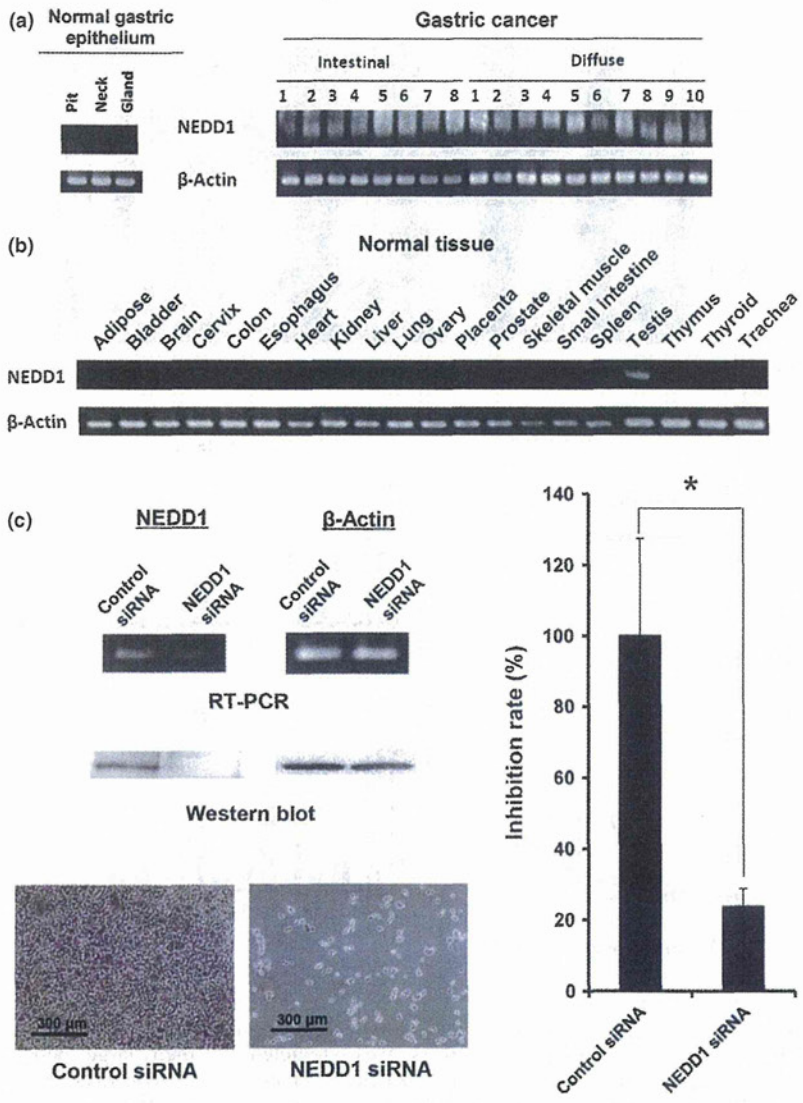

Fig. 4. The expression and silencing of *NEDD1*. (a) *NEDD1* mRNA expression in three regions of normal gastric mucosa (pit, neck, and gland) and 18 primary GCs containing eight intestinal-type and 10 diffuse-type. (b) *NEDD1* mRNA expression in 20 other organs of the human body. (c) Reverse transcription-polymerase chain reaction (RT-PCR) and western blot results for *NEDD1* gene silencing (upper left) and representative photos for growth inhibition (lower left) of 60As6 cells 2 days after treatment of *NEDD1* siRNA are shown. The cell growth inhibition rate 5 days after siRNA transfection is also shown (*P = 0.029) (right).

GL3 siRNA/0.05%atelocollagen/DharmaFECT1 complex and a 50 μg luciferase GL3 siRNA/0.5%atelocollagen/DharmaFECT1 complex 48 h after i.p. injection were compared in scid mice that had 1×10^6 60As6Luc cells introduced into the peritoneal cavity. Both of the two complexes clearly reduced the luciferase activity compared with an untransfected control (Fig. 2a), and the 0.5% atelocollagen complex rather than the 0.05% complex reduced it significantly (Fig. 2a). However, a 50 μg luciferase GL3 siRNA/0.5%atelocollagen only complex did not inhibit luciferase activity (Fig. 2b). The reduction of the luciferase activity 24 h after injection of a 50 μg luciferase GL3 siRNA/0.5%atelocollagen/DharmaFECT1 complex was visualized in four scid mice (Fig. 2c, right), and the significant reduction of the photon counts was shown (Fig. 2c, left).

By this method, we also obtained delivery evidence of fluorescence-labeled siRNA to tumor cells in scid mice that had 1×10^6 GFP-expressing 60As6 cells introduced into the peritoneal cavity. As shown in Figure 2(d), most GFP-expressing 60As6 cells (89.6%, 69/77 cells in eight fields), which were recovered from the peritoneal cavity 72 h after injection, incorporated fluorescence-labeled siRNA. These results demonstrated that siRNA was able to be delivered into peritoneal metastatic tumor cells by using both 0.5% atelocollagen and DharmaFECT1.

***In vitro* and *in vivo* effects of siRNA treatment of two diffuse-type GC-specific hedgehog targets, *ELK1* and *MSX2* on highly metastatic 60As6 cells.** As mentioned in the first part of the Results, we previously reported hedgehog signal activation in diffuse-type GC including a scirrhous-type,[17] and identified two cancer-specific hedgehog targets, *ELK1* and *MSX2*.[18] Treatment of each siRNA of *ELK1* and *MSX2* induced growth inhibition (53% and 41% respectively), of HSC-60 cells. Reverse transcription-PCR confirmed that expression of *ELK1* and *MSX2* in HSC-60 cells was maintained in 60As6 cells (Fig. 3a). Double transfection of these two siRNA strongly inhibited *in vitro* cell growth of 60As6 (Fig. 3b) as well as HSC-60.[18]

Before starting *in vivo* tumor growth inhibition studies, we accessed the tumor formation ability of 60As6 cells in scid mice. Tumor formation rates in serial i.p. injection of 1×10^4, 1×10^5, 5×10^5, 1×10^6, and 5×10^6 cells into 10–27 mice showed 0% (0/10), 76% (13/17), 100% (20/20), 100% (27/27, and 100% (15/15), respectively. By i.p. injection of more than 5×10^5 cells, all of the subject mice formed multiple tumors. In 1×10^6 or 5×10^6 cells, tumors were formed

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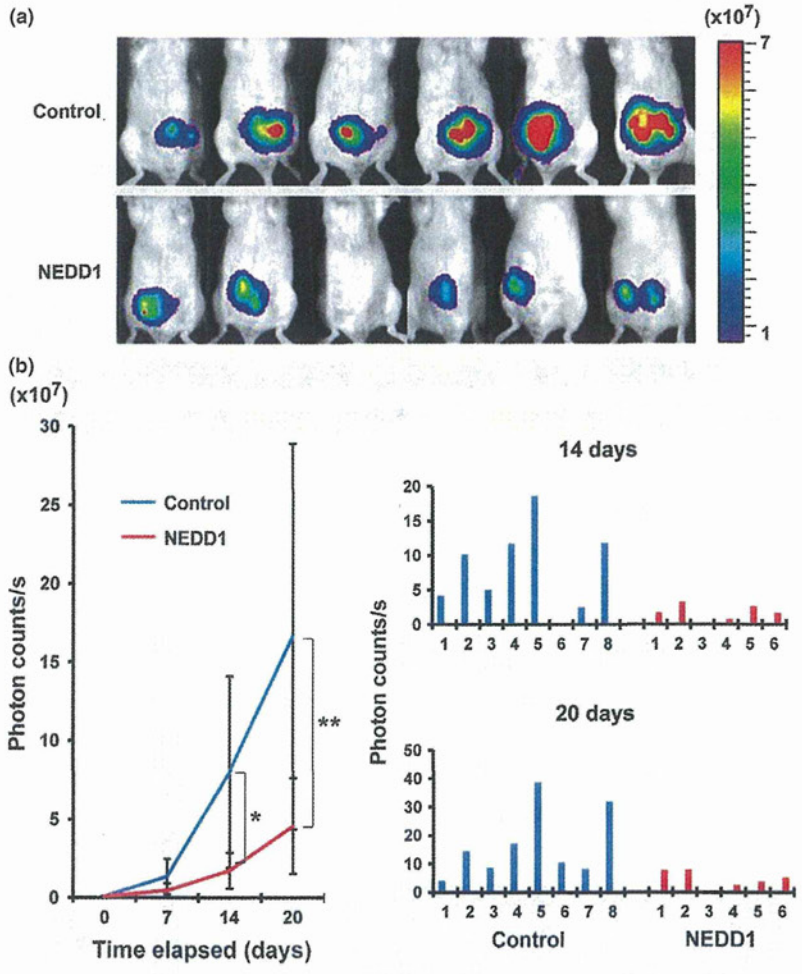

Fig. 5. Inhibition of tumor cell growth in the peritoneal cavity by atelocollagen-mediated *NEDD1* siRNA delivery. (a) Each of six mice treated with *NEDD1* siRNA or vehicle alone as control is visualized at 21 days after inoculation of 60As6Luc. Color bar indicates $\times 10^7$ photon/s. (b) Results of quantitative photon-counting analysis three times a week for 3 weeks are shown after inoculation of 60As6Luc with or without the *NEDD1* siRNA treatment. This experiment was repeated three times, and similar results were observed (*P = 0.04; **P = 0.034) (left). Photon counts 14 and 20 days after inoculation in each mouse are also indicated (right), because results among animal experiments often are variable.

within 2 weeks, and the tumor-bearing mice died rapidly (median survival time: approximately 30 days). These cases were thought to be quite different from peritoneal recurrence in humans, which is known to develop from occult or minimal tumor cells.[21] On the other hand, by injection of 5×10^5 cells, tumors were formed within 4 weeks, and a median survival time was approximately 50 days (data not shown). To extend our *in vitro* studies (Fig. 3b), 10 scid mice were inoculated with 5×10^5 60As6Luc cells, and each five of those 10 mice was injected with a 25 μg *ELK1 and* 25 μg *MSX2* siRNA/0.5% atelocollagen/DharmaFECT1 complex or a 0.5% atelocollagen/DharmaFECT1 complex as control five times every 3 days for 15 days. After exclusion of one pair of un-inoculated mice, optical imaging of the luciferase activity by use of the IVIS system at 27 days after administration was shown (Fig. 3c, left). Results of the time course experiments (at 0, 7, 14, 20 days) of tumor growth inhibition were shown by the quantification of photon counts (Fig. 3c, right). Although small effects on tumor growth inhibition by the double treatment of *ELK1* and *MSX2* siRNAs were observed, no significance was shown. Accordingly, no significant difference on mouse survival was found (data not shown). Therefore we next searched for other powerful targets for prolonging survival by intraperitoneal delivery of a single siRNA.

***NEDD1* siRNA inhibited *in vitro* cell growth of a highly metastatic 60As6 cell line.** Taxanes, which bind microtubules and inhibit tumor cell division in the metaphase of the cell cycle, are anti-tumor reagents for GC patients with peritoneal recurrence, because a significant pharmacokinetic advantage associated with i.p. delivery was predicted by their large bulky structure and known hepatic metabolism.[22] However, this drug has many adverse reactions including bone marrow suppression, alopecia, and neuropathy.[23] Therefore, we investigated target genes, which are involved in the metaphase regulation, from our previously reported GC-related genes.[18] In the previous study, we obtained gene expression profiles of 18 intestinal-type GCs and 12 diffuse-type GCs, and six non-cancerous tissues, and approximately 60 genes were found to be expressed aberrantly in more than 80% of GCs. Among them, only the *NEDD1* gene was known to be involved in the metaphase regulation. Therefore, we selected this gene as a new target for evaluating the therapeutic effect of an atelocollagen-mediated siRNA delivery on the peritoneal metastasis model mice established here. The *NEDD1* gene encodes a protein that binds to the gamma-tubulin ring complex, a multiprotein complex at the centrosome and at the mitotic spindle that mediates the nucleation of microtubules.[24] First, we showed *NEDD1* mRNA expression in three regions (pit, neck, and gland) of normal gastric mucosa prepared by laser-captured microdissection[17,18] in 18 primary gastric cancer tissues (Fig. 4a) and in 20 normal organs of the human body (Fig. 3b). *NEDD1* mRNA was found to be highly expressed in all primary lesions of gastric cancer by RT-PCR, while no or low expression was observed in normal organs except for the testis. The expression pattern of *NEDD1* was similar to that of the testis-tumor antigen gene. Next, we examined whether

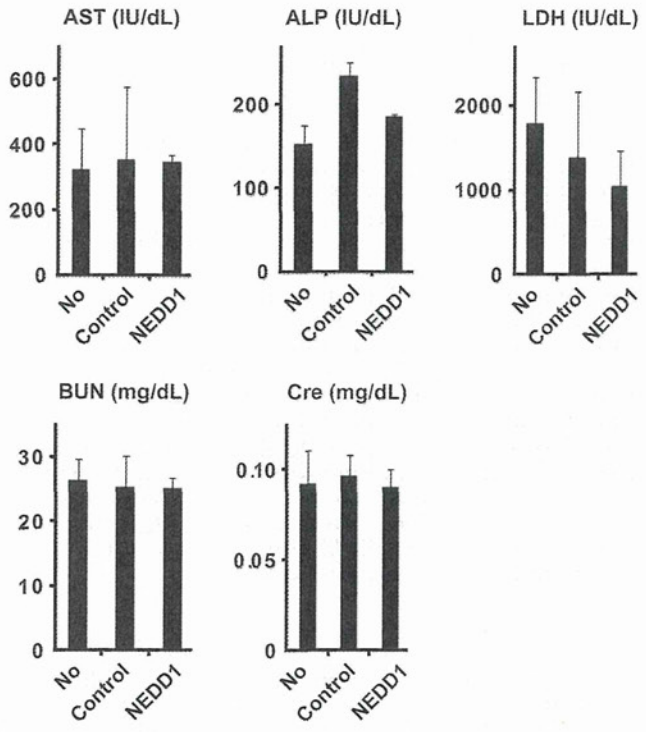

Fig. 6. Evaluation of selected serum chemistries 3 weeks after i.p. administration of *NEDD1* siRNA. No significant difference in the activity of aspartate aminotransferase (AST), lactate dehydrogenase (LDH) and alkaline phosphatase (ALP) and in levels of blood urea nitrogen (BUN) and Cre is observed among the no-treatment group (No), control siRNA-treated group (Control) and *NEDD1* siRNA-treated group (NEDD1) (n = 9).

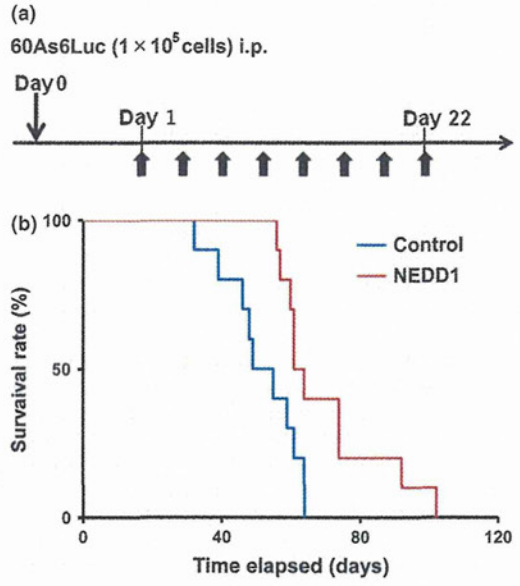

Fig. 7. Effect on survival of mice treated with *NEDD1* siRNA. (a) Schedule of i.p. administration of *NEDD1* siRNA 1 day after inoculation of 1×10^5 60As6Luc cells. (b) The i.p. administration of *NEDD1* siRNA significantly prolongs mice survival (P = 0.012).

NEDD1 knockdown inhibits the growth of highly metastatic 60As6 cells *in vitro*. RT-PCR and Western blot analyses revealed that Nedd1 protein expression was diminished efficiently by treatment of the *NEDD1* siRNA (Fig. 4c, upper left). In accordance with a decrease of *NEDD1* mRNA, 60As6 cell growth was eminently inhibited by treatment of *NEDD1* siRNA compared with the control siRNA (Fig. 4c, right). Representative photos of the cells are also shown (Fig. 4c, lower left).

***In vivo* inhibition of peritoneal metastasis in the mouse xenograft model by i.p. administration of *NEDD1* siRNA.** To extend our *in vitro* studies (Fig. 4), 12 scid mice were inoculated with 5×10^5 60As6Luc cells, and each of six of those 12 mice was injected with a 50 μg *NEDD1*siRNA/0.5% atelocollagen /DharmaFECT1 complex or a 0.5% atelocollagen/DharmaFECT1 complex as control five times every 3 days for 15 days. Optical imaging to detect luciferase activity in the mice was performed by using the IVIS system to evaluate tumor progression three times a week for 3 weeks. Quantitative photon-counting analysis of disseminated 60As6 cells revealed effective and significant inhibition of the tumor growth in the mice treated with *NEDD1* siRNA (Fig. 5).

Evaluation of i.p. administration safety of *NEDD1* siRNA and mice survival rates. To assess the safety of i.p. administration of a *NEDD1*siRNA/atelocollagen/DharmaFECT1 complex in liver and kidney function, we compared the activities of three enzymes (aspartate aminotransferase [AST], lactate dehydrogenase [LDH], alkaline phosphatase [ALP]) and the levels of blood urea nitrogen (BUN) and Cre in the serum of each group (no treatment, control siRNA, and *NEDD1*siRNA group; n = 9) 3 weeks after treatment. No significant toxicity was detected in the mice treated with *NEDD1*siRNA (Fig. 6). In addition, no difference in the activity or glossiness of hair was also observed among the three groups (data not shown). To yield survival benefits, 20 scid mice were inoculated with 1×10^5 60As6Luc cells and, 3 weeks after inoculation, each of 10 of those 20 mice was injected with *NEDD1* siRNA or control siRNA eight times every 3 days for 22 days (Fig. 7a). The survival rates of these 20 mice are shown in Figure 7(b). Although *NEDD1* siRNA administration stopped at 22 days after inoculation of 60As6Luc, mice treated with *NEDD1* siRNA survived longer than the control mice with a significance (*NEDD1* siRNA: the median survival time was 61 ± 2 days; control siRNA: the median survival time was 49 ± 6 days, P = 0.0115).

Discussion

For gene therapy, one of the most dramatic events of the past 5 years in this field has been the discovery of RNA interference (RNAi). The success of cancer therapeutic use of RNAi relies on the development of safe and efficacious delivery systems that introduce siRNA and shRNA expression vectors into target tumor cells. However, such delivery systems into peritoneal metastatic tumor cells have not been established well. The atelocollagen-mediated gene delivery system was originally developed for a adenovirus vector by a collaborator.[19] An atelocollagen is a highly purified type I collagen of calf dermis with pepsin treatment, which allows nuclease resistance, prolonged release of genes and reduction of cellular immune responses. In mice, this delivery system has been reported to be useful for gene delivery into some body sites including metastatic tumors and also for systemic gene delivery.[20] As mentioned in the introduction, diffuse-type GCs including scirrhous GC frequently show peritoneal dissemination even if tumor cells are circulating systemically. Therefore, in this type of GCs, peritoneal metastasis control is urgently crucial for improving the quality of life and patient outcome. Here we provided peritoneal metastasis model mice and an effective delivery system for i.p. administration of

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siRNA. Figure 2 showed that the 0.5% atelocollagen/DharmaFECT1/siRNA complex rather than the 0.05% atelocollagen /DharmaFECT1/siRNA complex reduced luciferase activity and that the DharmaFECT1 free complex did not reduce it. To date, a collaborator usually uses DharmaFECT1 in the atelocollagen-mediated systemic gene delivery by i.v. administration, because this reagent improves it (Takeshita F, unpublished observation, 2010).

In peritoneal metastasis model mice, the i.p. administration of *NEDD1* siRNA was able to inhibit tumor growth and prolong survival even without any side effects (Figs 5,7). If targets such as *NEDD1* function in the cell cycle regulation, the slow gene release arising from protection from nucleases by atelocollagen may provide an advantage for long acting and for reducing the number of administrations. As shown in Figure 7, we administered the *NEDD1* siRNA complex five times every 3 days for 15 days in this study; however, that number may possibly be reduced. In another report for intraperitoneal administration of siRNA targeting nuclear factor-κB with only DharmaFECT1, the siRNA prolonged the survival of mice only by the administration of paclitaxel, whereas the siRNA/DharmaFECT1 complex alone could not succeed.[25] Taken together, the atelocollagen/DharmaFECT1/siRNA complex is very useful for gene delivery to the peritoneal cavity.

In the mouse model used, the number of tumor cells (1×10^5 cells) implanted into the mouse peritoneal cavity was estimated to be still very large compared with the number of tumor cells in the peritoneal cavity in human GC patients with cytology positive, who often showed peritoneal metastasis within 2 years. Therefore, the present i.p. delivery system of siRNA has a great potential for treatment of such GC patients.

Although *in vitro* cell growth inhibition was observed by the double siRNA treatment of *ELK1* and *MSX2* (Fig. 3b), no significant difference on *in vivo* tumor growth and mouse survival was found (Fig. 3c and data not shown). Investigation of other hedgehog components (SMO, GLIs, ISL1, BMP4, FOXM1, and FOXA2) and EMT-regulators (SIP1/ZEB2, TWIST2) remains for a future study, because cross-talk between hedgehog and EMT signals is specific to diffuse-type GC.[18]

Atelocollagen has also been reported to efficiently deliver microRNA.[26] Recently, genome-wide microRNA expression profiles of 353 GC samples have shown that some microRNAs including let-7b, miR-214, and miR-433 are expressed aberrantly and correlate with tumorigenesis, progression, and prognosis of diffuse-type GC.[27] Thus, these microRNAs may be candidates for SGC therapy. In addition, transforming growth factor-β (TGF-β) has been reported to induce apoptosis of a subset of diffuse-type GCs whose receptor is not inactivated.[28,29] Therefore, adenovirus-mediated TGF-β or the downstream targets such as Gasdermin/GasderminA delivery also have great potential for SGC therapy.

In conclusion, we developed a novel i.p. delivery system of siRNA to disseminated tumor cells in the peritoneal cavity that successfully prolongs the survival of model mice. The present mouse model is for an adjuvant therapy after surgical resection. The ability of atelocollagen/DharmaFECT complex is keeping siRNA from nucleases, leading slow gene release and reducing the amount of administration that results in effective eradication of residual tumor cells in the peritoneal cavity.

Thus, considering other potential targets of the diffuse-type GCs, this system is a highly flexible therapeutic platform for the treatment of peritoneal dissemination.

Acknowledgments

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Disclosure Statement

The authors have no conflict of interest.

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