

Table 3. Baseline characteristics of study subjects and gastric cancer development in By group

Screening criteria	Group By with HP infection					Group By with HP eradication Total
	Total	Low-titer		High-titer		
		RHG(-)	RHG(+)	RHG(-)	RHG(+)	
Subjects n (%)	150 (100)	68 (45.3)	17 (11.3)	42 (28)	23 (15.3)	149
Follow-up years [mean (SD)]	5.3 (4.0)	5.5 (4.0)	5.5 (4.3)	4.5 (3.9)	6.0 (3.6)	6.2 (3.7) <sup>#</sup>
Person-years	800	378	94	188	140	933
Age [mean (SD)]	52.5 (5.1)	52.8 (4.5)	52.5 (4.0)	52.0 (6.4)	53.0 (5.1)	51.2 (5.3) <sup>#</sup>
Ever-smoking (%)	60.1	64.7	52.9	52.5	65.2	49.0
Alcohol drinking (%)	74	72.1	76.5	71.4	82.6	75.3
HP antibody titer U ml <sup>-1</sup> [mean (SD)]	592.1 (608.0)	231.2 (123.0)	267.6 (120.5)	957.1 (669.0)	1232.3 (668.2)	810.4 (947.0) <sup>#</sup>
PGI ng ml <sup>-1</sup> [mean (SD)]	80.5 (33.6)	77.7 (29.0)	92.6 (31.2)	74.4 (25.0)	90.7 (53.8)	82.8 (24.2)
PGII ng ml <sup>-1</sup> [mean (SD)]	38.9 (17.7)	37.6 (15.8)	45.3 (19.4)	34.8 (14.0)	45.5 (24.8) <sup>+</sup>	39.3 (12.7)
PGI/II [mean (SD)]	2.2 (0.5)	2.2 (0.5)	2.1 (0.4)	2.3 (0.5)	2.0 (0.5)	2.36 (0.43) <sup>#</sup>
Total gastric cancer cases/incidence rate <sup>1</sup>	6/750	0/-	1/1064	1/532	4/2857	0/-
HR (95%CI)			1.59 (0.1-26.11)	1	5.03 (0.53-47.75)	
Intestinal gastric cancer cases/incidence rate <sup>1</sup>	1/125	0/-	0/-	0/-	1/714	0/-
Diffuse gastric cancer cases/incidence rate <sup>1</sup>	5/625	0/-	1/1064	1/532	3/2143	0/-

PG, pepsinogen; HR, hazard ratio; RHG, rugal hyperplastic gastritis; HP, *Helicobacter pylori*.

HR adjusted for age, smoking habit and alcohol drinking was evaluated in each group according to Cox proportional hazards modeling.

<sup>+</sup>*p* < 0.05 compared with RHG(-). <sup>#</sup>*p* < 0.05 compared with Group By without HP eradication.

<sup>1</sup>per 100,000 person-years.

between enlarged gastric folds on barium X-ray and gastric cancer in a case-and-control setting have suggested the possibility that enlarged fold gastritis is associated with high-risk of cancer.<sup>28-30</sup> The present study is the first to report results after a long-term follow-up of subjects with RHG, clearly demonstrating that *H. pylori*-infected subjects with RHG are indeed at higher risk of cancer.

Serum levels of PG and *H. pylori* antibody were significantly more elevated in *H. pylori*-infected subjects with RHG than in subjects without such gastritis, consistent with the highly active *H. pylori*-induced inflammatory process that is reported to be involved in the genesis of RHG.<sup>22,23</sup> The percentage of RHG was highest in Group By with a high-titer, representing the subgroup considered to show the most active gastritis among the groups defined by the two serological tests. Moreover, stratification of each serologically defined group based on endoscopic findings of RHG revealed that the activity of gastritis in Group By with high-titer antibody together with RHG was probably the highest of all the groups in the present cohort according to the two serological tests. In the overall cohort, gastric cancer development, particularly that of diffuse-type cancer, appeared to correlate closely with the activity of gastritis, and was highest in this serologically and endoscopically defined subgroup, with an incidence rate of 2,857/100,000 person-years. All cancers that developed in

this subgroup were located in the corpus of the stomach, and 75% of these cancers were diffuse-type, showing quite a high incidence rate of 2,143/100,000 person-years; compared with the aforementioned incidence for diffuse-type cancer in Japan<sup>21</sup> or in Western countries,<sup>20</sup> the rate in the high-risk group was about 50- or 400-fold higher, respectively. These results strongly suggest that highly active inflammation in the corpus mucosa with RHG represents a major driving force for the carcinogenic pathway in these cancers, consistent with the previous finding that individuals with corpus-predominant gastritis are at high risk for stomach cancer.<sup>10</sup> That is, extensive increases in the mediators of inflammatory responses such as cytokines, reactive oxygen species, nitric oxide, prostaglandins and growth factors can induce genetic and epigenetic changes in the corpus mucosa, which will lead to neoplastic transformation and subsequent progression, and finally to the establishment of cancer with high malignant potential. Indeed, mounting evidence indicates that *H. pylori* infection frequently induces alterations in DNA methylation of various gene promoter regions in the stomach mucosa, including enlarged folds,<sup>39-41</sup> and that inflammation-induced DNA methylation is deeply involved in the development of diffuse-type gastric cancer.<sup>42-44</sup> In the present study, only a small proportion (<5%) of *H. pylori*-infected subjects developed this type of active gastritis. Various cofactors thus

appear to be involved in the establishment of this highly active inflammation of stomach. Because almost all *H. pylori* isolates from East Asia, including Japan, possess the Cag A gene,<sup>45</sup> the differences in cancer risk among the subgroups in this study are highly unlikely to be caused by differences in Cag A expression in the infecting bacteria. Further analyses of other bacterial factors together with host genetic and environmental factors modulating the response to *H. pylori* infection are required.

In line with the above-described active inflammation-based carcinogenesis, the improvement of active gastritis on eradication of *H. pylori* reduced the cancer incidence to zero among 149 Group B $\gamma$  subjects during the study period. These results strongly indicate that *H. pylori* eradication has the potential to prevent gastric cancer development in high-risk subjects with highly active inflammation in nonatrophic stomach, consistent with the current available data that *H. pylori* eradication could reduce the cancer risk, especially in subjects without extensive CAG.<sup>46,47</sup> Because other subjects at high-risk of cancer with extensive CAG, which constitutes a main route of stomach carcinogenesis, remain at high risk even after eradication,<sup>46,47</sup> different mechanisms are highly likely to be involved in carcinogenesis in the nonatrophic stomach. Recent evidence indicates that *H. pylori* eradication not only leads to marked reductions in inflammatory cell infiltration, inflammatory cytokine production and oxidative stress in the stomach mucosa, but also induces the regression of aberrant methylation in various gene regions including the CDH1 (*E-cadherin*) promoter in the *H. pylori*-infected stomach mucosa of both humans and experimental animals, if implemented before the establishment of permanent epigenetic changes, which marks the point of no return.<sup>39-41,48,49</sup> All of these findings together indicate the possibility that most of these high-risk subjects are in the stage before the point of no return in terms of the process of *H. pylori*-induced stomach carcinogenesis.

Some limitations in the present study methods must be considered. First, all study subjects were asymptomatic, cancer-susceptible aged factory workers who were self-referred for EGD. As such, selection bias was unavoidable, as subjects may have been more health-conscious or had undisclosed reasons for suspecting the presence of upper gastrointestinal disease. These subjects may have had overall prevalences and risk profiles for gastric cancer differing from those of the general working population. The reported prevalence of enlarged gastric folds among asymptomatic middle-aged Japanese men who underwent gastric cancer screening by barium X-ray in the early 1990s was about 11%, representing a level similar to that in our subjects.<sup>24</sup> Considering the recent trends toward rapid decreases in the prevalence of *H. pylori* infection, our study subjects appear to have had a higher risk of gastric cancer than the general population. Second, RHG was diagnosed based on endoscopic findings of enlarged folds or hyperrugosity, not on histopathology of the

stomach mucosa, according to the Sydney system.<sup>26</sup> Normal folds in a partially contracted stomach can sometimes be interpreted as being thickened. Although endoscopists endeavor to avoid such misinterpretations, the diagnosis of RHG must still be considered somewhat subjective. The risk of developing cancer from RHG might therefore have been underestimated. Third, although the present study prospectively investigated the possibility of gastric cancer control in Group B $\gamma$  subjects using *H. pylori* eradication, eradication therapy was not allocated in a randomized manner. However, randomization was neither feasible nor ethical, as a considerable number of eligible subjects were unwilling to remain untreated for long periods, particularly given the knowledge that *H. pylori* represents a major risk factor for the development of gastric cancer.

The present results strongly indicate that mucosal inflammation plays a pivotal role in carcinogenesis for the *H. pylori*-infected nonatrophic stomach, and that higher activity is associated with a higher risk of cancer, particularly diffuse-type cancer with higher malignant potential. In addition, the combination of two serological tests, for PG and *H. pylori* antibody, together with an endoscopic finding of RHG can identify a group at high risk of cancer (that is, a group of subjects with highly active *H. pylori*-associated gastritis in the nonatrophic stomach) that has not been fully described until now. In the present study, ~57% of total cancers and 50% of diffuse-type cancers developed from this serologically and endoscopically defined high-risk group, which represented only 4.6% (23/496) of the study cohort. Careful endoscopic follow-up of these subjects thus appears warranted from the perspective of early detection of cancer. Furthermore, *H. pylori* eradication markedly reduced the risk of cancer development in these subjects. Our previous long-term follow-up study revealed that eradication was effective in controlling cancer development among PG test-negative subjects with nonatrophic stomach (Group B subjects), while most PG test-positive subjects with CAG have passed beyond the point of no return in the process of stomach carcinogenesis.<sup>47</sup> However, the cost-effectiveness of eradication targeting middle-aged Group B subjects, who constitute a major proportion of the 60 million people infected with *H. pylori* in Japan,<sup>11,50</sup> is low, as the NNT for 1 year to prevent a single cancer development is around 2,000.<sup>47</sup> Based on the present results, strategies for cancer control by *H. pylori* eradication appear more cost-effective when targeting Group B $\gamma$  subjects (NNT = 133), particularly those with high *H. pylori* antibody titer together with RHG (NNT = 35). We thus believe that the cancer high-risk group identified by the two serological tests together with endoscopic RHG represents a good target for *H. pylori* eradication.

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ORIGINAL ARTICLE

## Inflammation-induced repression of tumor suppressor miR-7 in gastric tumor cells

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Inflammation has an important role in cancer development through various mechanisms. It has been shown that dysregulation of microRNAs (miRNAs) that function as oncogenes or tumor suppressors contributes to tumorigenesis. However, the relationship between inflammation and cancer-related miRNA expression in tumorigenesis has not yet been fully understood. Using *K19-C2mE* and *Gan* mouse models that develop gastritis and gastritis-associated tumors, respectively, we found that 21 miRNAs were upregulated, and that 29 miRNAs were downregulated in gastric tumors in an inflammation-dependent manner. Among these miRNAs, the expression of miR-7, a possible tumor suppressor, significantly decreased in both gastritis and gastric tumors. Moreover, the expression of miR-7 in human gastric cancer was inversely correlated with the levels of interleukin-1 $\beta$  and tumor necrosis factor- $\alpha$ , suggesting that miR-7 downregulation is related to the severity of inflammatory responses. In the normal mouse stomach, miR-7 expression was at a basal level in undifferentiated gastric epithelial cells, and was induced during differentiation. Moreover, transfection of a miR-7 precursor into gastric cancer cells suppressed cell proliferation and soft agar colony formation. These results suggest that suppression of miR-7 expression is important for maintaining the undifferentiated status of gastric epithelial cells, and thus contributes to gastric tumorigenesis. Although epigenetic changes were not found in the CpG islands around miR-7-1 of gastritis and gastric tumor cells, we found that activated macrophage-derived small molecule(s) (<3 kDa) are responsible for miR-7 repression in gastric cancer cells. Furthermore, the miR-7 expression level significantly decreased in the inflamed gastric mucosa of *Helicobacter*-infected mice, whereas it increased in the stomach of germfree *K19-C2mE* and *Gan* mice wherein inflammatory responses were suppressed.

Taken together, these results indicate that downregulation of tumor suppressor miR-7 is a novel mechanism by which the inflammatory response promotes gastric tumorigenesis.

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**Keywords:** miR-7; gastric cancer; inflammation; macrophages

### Introduction

It has been established that inflammatory responses contribute to cancer development through various mechanisms (Coussens and Werb, 2002). The expression of cyclooxygenase-2 (COX-2), a rate-limiting enzyme for prostaglandin biosynthesis, has an important role in both inflammation and cancer (Wang and DuBois, 2010). Using genetic mouse models, we previously demonstrated that induction of COX-2 and its downstream product, prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), is required for gastrointestinal tumorigenesis (Sonoshita *et al.*, 2001; Oshima *et al.*, 2006). The COX-2/PGE<sub>2</sub> pathway, together with a bacterial infection, induces inflammatory responses in the stomach through the recruitment of macrophages, which promotes gastric tumorigenesis (Oshima *et al.*, 2011). However, it remains to be fully elucidated precisely how such inflammatory responses contribute to the promotion of gastric tumors.

MicroRNAs (miRNAs) are a class of single-stranded small noncoding RNAs that regulate gene expression by post-transcriptional interference of specific mRNAs (Ambros, 2004; Bartel, 2004). Through their regulation of cancer-related gene expression, miRNAs can function as either oncogenes or tumor suppressors (Esquela-Kerscher and Slack, 2006; Ventura and Jacks, 2009). Dysregulation of miRNAs in cancer has been shown to be associated with genomic/epigenetic alterations or transcriptional/post-transcriptional mechanisms (Di Leva and Croce, 2010). Moreover, expression of several miRNAs, including oncogenic miRNAs, has been shown to be induced by inflammatory

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responses (Sonkoly and Pivarcsi, 2011). For example, miR-155 is induced in macrophages by nuclear factor- $\kappa$ B, interferon- $\beta$  or Toll-like receptor signaling (O'Connell *et al.*, 2007; Tili *et al.*, 2007), whereas miR-21 is induced by Stat3, a transcription factor activated by interleukin-6 (IL-6) (Iliopoulos *et al.*, 2010). On the other hand, the mechanism responsible for the downregulation of tumor-suppressor miRNA expression in the inflammatory microenvironment has not been fully understood.

Herein, we examined the expression of miRNAs in mouse models of gastritis and gastric tumors, which were developed in *K19-C2mE* and *Gan* mice, respectively (Oshima *et al.*, 2004, 2006). We found the expression level of miR-7 to significantly decrease in gastric tumors in an inflammation-dependent manner. It has been shown that miR-7 has a tumor-suppressor role in several cancers, including glioblastoma, breast cancer and lung cancer (Kefas *et al.*, 2008; Reddy *et al.*, 2008; Webster *et al.*, 2009; Jiang *et al.*, 2010; Saydam *et al.*, 2011). In this manuscript, we demonstrate that miR-7 is induced during the differentiation of normal gastric epithelial cells, and it also has a tumor-suppressor role in the stomach. These results suggest that downregulation of tumor suppressor miR-7 is one of the tumor-promoting mechanisms underlying the role of inflammation in gastric tumorigenesis.

## Results

### *Inflammation-dependent dysregulation of miRNAs in gastric tumors*

To examine whether miRNA expression is dysregulated in gastric tumors by inflammatory responses, we examined the miRNA expression profiles in wild-type mouse stomachs, *K19-C2mE* mouse gastritis and *Gan* mouse gastric tumors by a microarray analysis. In *Gan* mouse gastric tumors, 50 miRNAs were upregulated (>2.0-fold), whereas 42 miRNAs were downregulated (<0.5-fold) compared with the wild-type mouse stomach level (Figure 1a and Supplementary Table 1). Notably, 21 and 29 miRNAs showed upregulation or downregulation, respectively, in both gastritis and gastric tumors. Therefore, it is possible that dysregulation of these miRNAs is caused by inflammatory responses.

We confirmed the results of the microarray analysis by real-time reverse transcriptase-polymerase chain reaction (RT-PCR). In all, 10 miRNAs randomly selected from the upregulated and downregulated miRNAs (Figure 1a, boxed) showed the same dysregulation pattern in both gastritis and gastric tumors (Figure 1b). Importantly, miR-155 and miR-21, which function as oncogenes (Volinia *et al.*, 2006) were upregulated, whereas miR-145 and miR-7, which function as tumor suppressors (Kefas *et al.*, 2008; Sachdeva *et al.*, 2009), were downregulated in both gastritis and gastric tumors (Figures 1a and b). This suggests that inflammation can induce not only upregulation of oncogenic miRNAs but also downregulation of tumor-suppressor miRNAs.

We next picked up 74 miRNAs that were not dysregulated in *K19-C2mE* gastritis compared with the wild-type normal stomach (Supplementary Table 2). Among them, three miRNAs were upregulated in *Gan* mouse tumors compared with *K19-C2mE* gastritis tissue samples (>2.0-fold), whereas three miRNA were downregulated (<0.5-fold) (Figure 1c). It is possible that expression of these miRNAs is dysregulated by carcinogenesis-specific mechanisms.

### *Induction of miR-7 during differentiation of gastric epithelial cells*

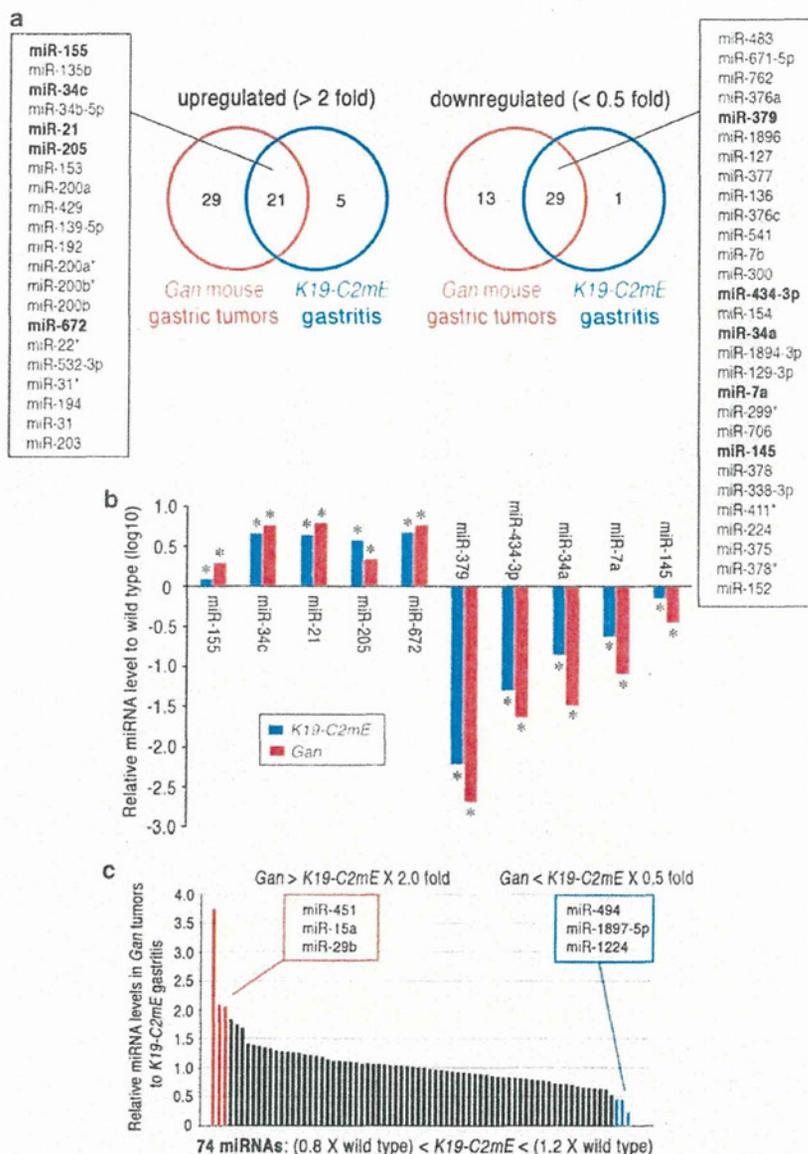
We further examined the expression of miR-7, because its role(s) in the normal stomach and gastric cancer have never been examined. Gastric glands were isolated from the stomachs of the respective mouse models, and the miR-7 expression was examined by real-time RT-PCR. Notably, miR-7 levels were significantly lower in epithelial cells of *K19-C2mE* gastritis tissues and *Gan* mouse tumors compared with the wild-type mouse stomach (Figure 2a), indicating that miR-7 is predominantly expressed in epithelial cells in an inflammation-dependent manner.

When primary cultured gastric epithelial cells were passaged and maintained for 6 days, the cell morphology appeared to be differentiated, with enlarged and mucin-containing cytoplasm (Figure 2b). Consistently, the expression of differentiation markers, *Muc6* and *Muc5AC*, was elevated on day 6, whereas the expression of the Wnt target gene, *Sox9*, decreased (Figure 2c). These results indicate that cultured gastric epithelial cells underwent differentiation through passage and 6-day culture. Importantly, the miR-7 expression level increased significantly on day 6 to 6.5-fold, compared with the level observed on day 2.

We next examined the miR-7 level in the stomach during development. The expression level of miR-7 in the stomach increased significantly in 14-day-old and adult mice, to >6-fold of that in E15 embryos (Figure 2d). Conversely, expression of *CD44*, one of the Wnt target genes, decreased significantly during development. We confirmed by an immunohistochemistry that most epithelial cells in the gastric mucosa were Ki-67 positive on days 0 and 7, whereas proliferating cells were limited to the gland neck on day 14 and in adult mice (Figure 2e). Accordingly, the ratio of undifferentiated epithelial cells decreased during development. Taken together, these results indicate that miR-7 expression is induced in gastric epithelial cells during differentiation.

### *Tumor-suppressor role of miR-7 in gastric cancer development*

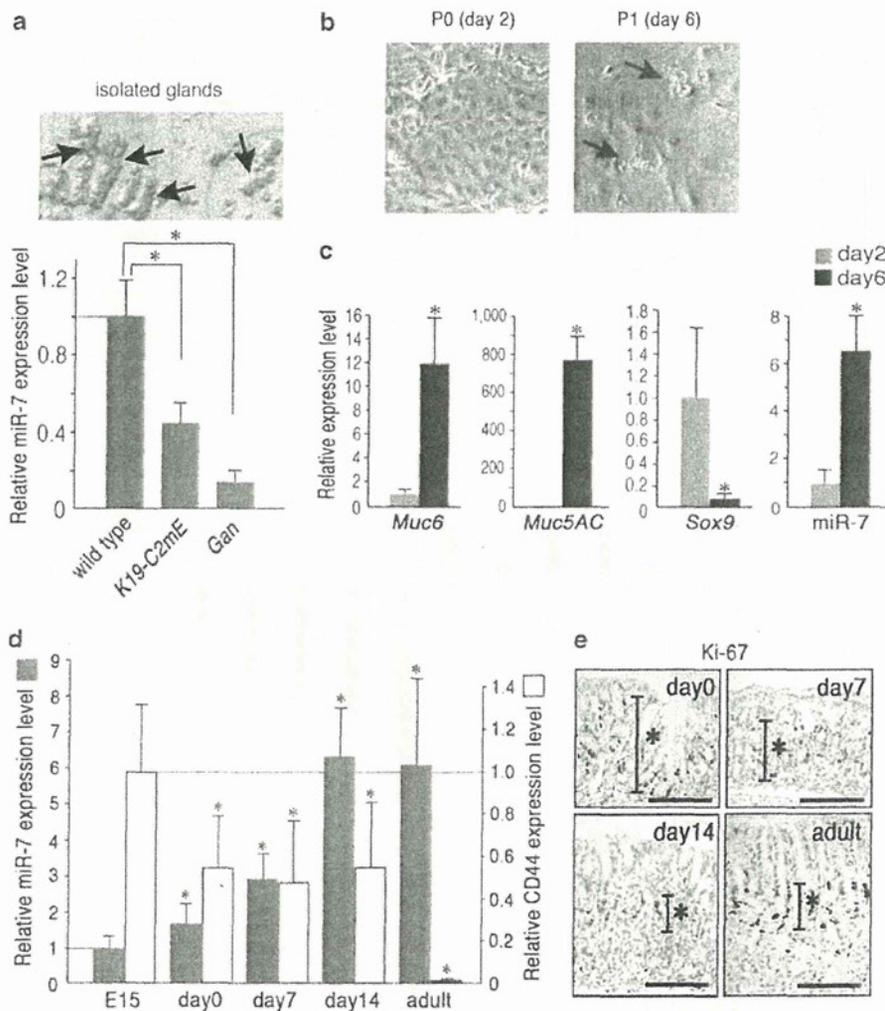
We next examined miR-7 levels in human gastric cancers by real-time RT-PCR. The expression of miR-7 was downregulated in 18 out of 28 human gastric cancer tissue samples (64%) compared with paired non-tumor stomach tissue samples (Figure 3a), suggesting that miR-7 has a tumor-suppressor role in a subpopulation of gastric cancers. We next examined the expression



**Figure 1** Inflammation-dependent dysregulation of miRNA expression in mouse gastric tumors. (a) Venn diagrams of the miRNAs that were upregulated (>2.0) and downregulated (<0.5) in *Gan* mouse gastric tumors and/or *K19-C2mE* mouse gastritis samples as determined by the microarray analysis are shown. The miRNAs listed in boxes were upregulated (left) or downregulated (right) in both gastric tumor and gastritis tissue samples. (b) The relative expression levels of selected miRNAs (indicated in bold in the list) for *K19-C2mE* mouse gastritis (blue bars) and *Gan* mouse gastric tumors (red bars) compared with the wild-type levels examined by real-time RT-PCR are shown as the log10 ratios. \* $P < 0.05$  versus the wild-type level. The expression levels of miRNAs were normalized to the Sno202 level. (c) The miRNA levels in *Gan* mouse tumors relative to those in *K19-C2mE* gastritis tissues examined by the microarray analysis are shown. Red and blue bars indicate upregulated (>2.0) and downregulated (<0.5) miRNAs, respectively, in *Gan* mouse tumors.

level of IL-1 $\beta$  and tumor necrosis factor (TNF)- $\alpha$ , major proinflammatory cytokines, and compared them with the miR-7 level. Importantly, expression levels of miR-7 were inversely correlated with those of IL-1 $\beta$  or TNF- $\alpha$ , suggesting that the downregulation of miR-7 is related to the severity of inflammatory responses (Figure 3b). We also found that miR-7 was markedly downregulated in four out of nine gastric cancer cell lines (Figure 3c).

To examine the tumor-suppressor role of miR-7 in gastric tumorigenesis, we transfected the precursor of miR-7, pre-miR-7, into AZ-521 and Kato-III gastric cancer cells and examined their proliferation and soft agar colony formation. We confirmed that pre-miR-7 transfection into reporter vector-transfected cells resulted in a significant decrease in luciferase activity, indicating an increase of mature miR-7 level (Supplementary Figure 1). Transfection of pre-miR-7



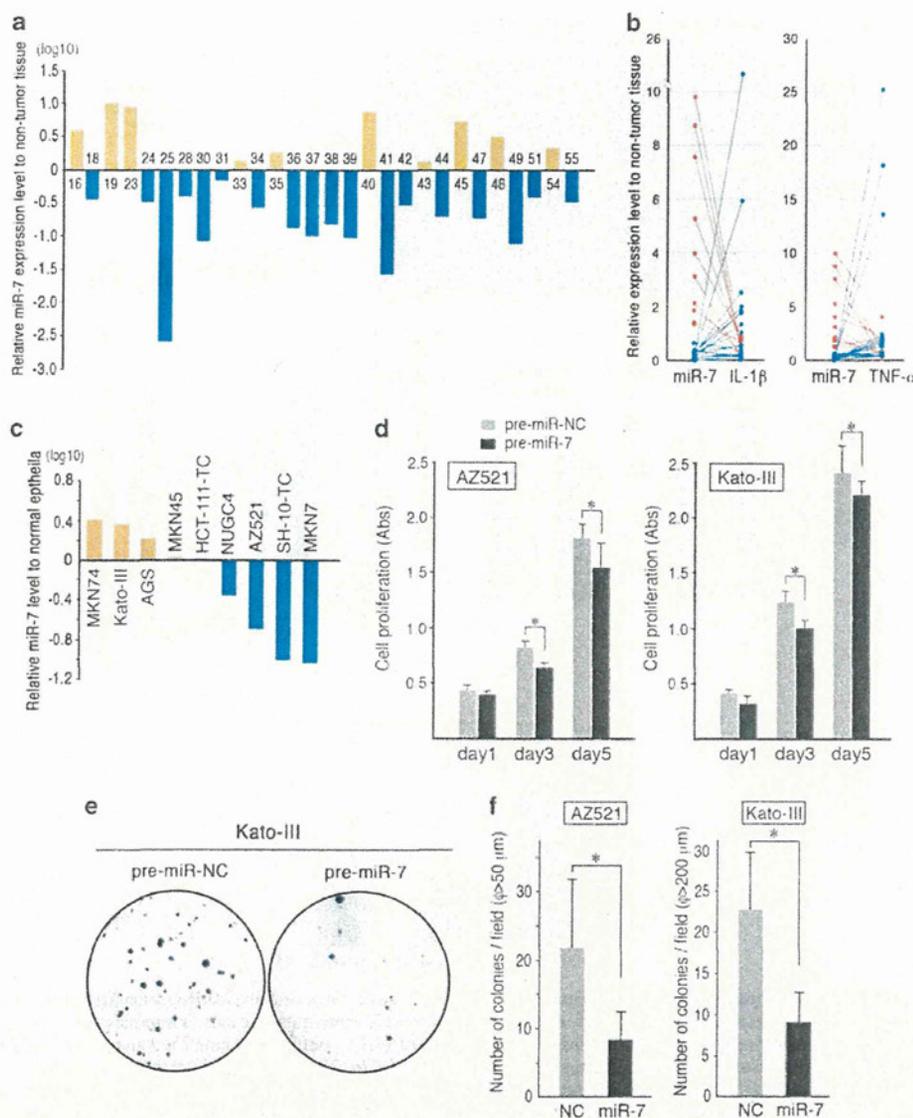
**Figure 2** The induction of miR-7 expression in differentiated gastric epithelial cells. (a) A representative photograph of isolated gastric glands from wild-type mice (top, arrows). The expression levels of miR-7 in the isolated gastric glands of *K19-C2mE* and *Gan* mice relative to the wild-type level are shown (mean  $\pm$  s.d.) (bottom). \* $P < 0.05$ . (b) Representative photographs of primary cultured gastric epithelial cells on day 2 (passage 0: P0) and on day 6 (passage 1: P1) (original magnification,  $\times 100$ ). Arrows in P1 indicate mucin-containing enlarged cells on day 6. (c) The levels of *Muc6*, *Muc5AC* and *Sox9* mRNA and miR-7 in the primary cultured gastric epithelial cells on day 6 (closed bars) relative to the levels on day 2 (gray bars) are shown (mean  $\pm$  s.d.). \* $P < 0.05$  versus the day 2 level. (d) The expression levels of miR-7 (gray bars) and *CD44* (open bars) in the stomach at the indicated ages relative to the levels in E15 embryos are shown (mean  $\pm$  s.d.). \* $P < 0.05$  versus the E15 level. The expression levels of miR-7 were normalized to the *Sno202* level. (e) Representative photographs of Ki-67 immunostaining in the glandular stomach of mice at the indicated ages. Asterisks indicate proliferative zones. Scale bars indicate 50  $\mu$ m.

significantly decreased cell proliferation in both cell lines compared with control vector-transfected cells (Figure 3d). Moreover, pre-miR-7 transfection significantly suppressed soft agar colony formation in both cell lines (Figures 3e and f). These results strongly suggest that miR-7 has a tumor-suppressor role in gastric cancer development.

#### Repression of miR-7 in gastric cancer cells by macrophage-derived factor(s)

We detected primary (pri)-miR-7-1, pri-miR-7-2 and pri-miR-7b in the mouse normal stomach by real-time RT-PCR (Supplementary Figure 2), suggesting that mature miR-7 is processed from all these primary miR-7

in the normal gastric mucosa. MiR-7-1 is located in the intron of the *Hnnpk* gene, and a CpG island is found in the promoter region of *Hnnpk* (Supplementary Figure 3a). On the other hand, we could not determine CpG islands that regulate the transcription of miR-7-2 and miR-7b. We thus examined DNA methylation in the CpG islands in the *Hnnpk* promoter region. Notably, DNA methylation levels in *K19-C2mE* gastritis and *Gan* mouse tumor tissues were not increased compared with the wild-type mouse stomach (Supplementary Figure 3a). Consistently, DNA methylation was not detected in the promoter region of the *HNRNPK* gene in human gastric cancer tissues (Figure 4a). We also examined the trimethylation of histone H3 at lysine 27 (H3K27me3) in

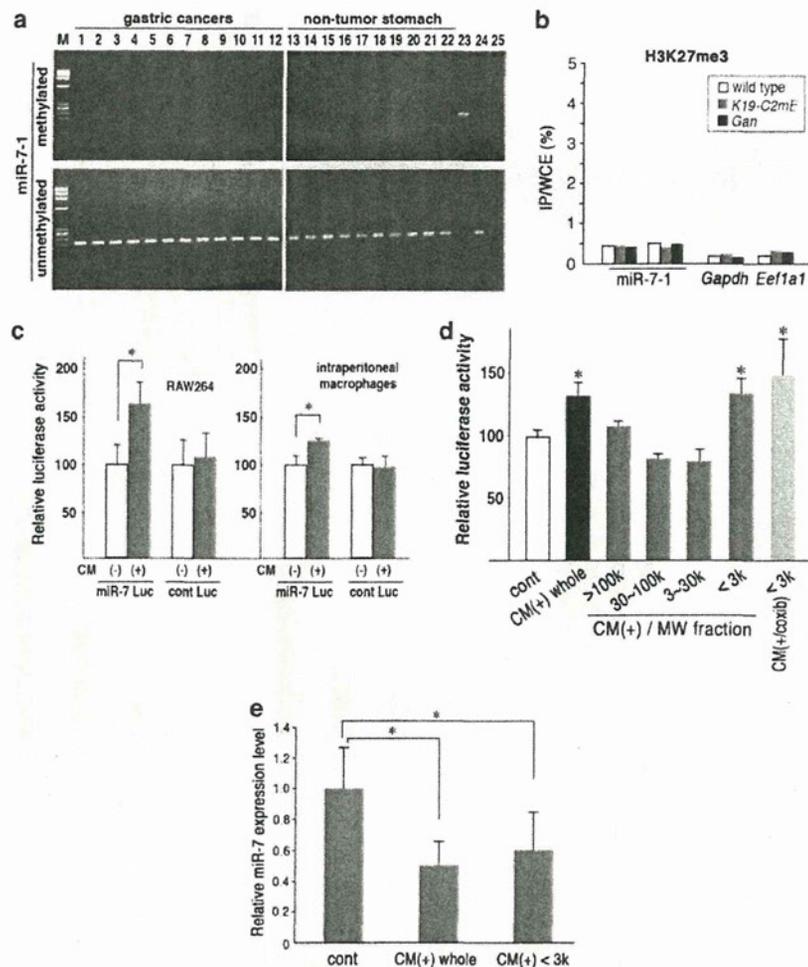


**Figure 3** The tumor-suppressor roles of miR-7 in gastric cancer cells. (a) The relative miR-7 expression levels in the human gastric cancer tissue samples to the level of non-tumor stomach tissue samples are shown as the log<sub>10</sub> ratios. The indicated numbers correspond to the patient ID in the clinicopathological data (Supplementary Table 3). (b) Comparison of the relative expression levels of miR-7 and IL-1 $\beta$  (left) or TNF- $\alpha$  (right) in gastric cancer tissues to the non-tumor stomach tissue levels in each patient is shown. Red and blue lines indicate that the expression of miR-7 was increased (>1.0) and decreased (<1.0) in the gastric cancers, respectively. (c) The relative expression levels of miR-7 in gastric cancer cell lines compared with the mean level in the human normal gastric epithelial cells are shown as the log<sub>10</sub> ratios. The expression levels of miR-7 were normalized to those of U44. (d) The proliferation of control (gray bars) and pre-miR-7-transfected (closed bars) AZ521 cells and Kato-III cells at the indicated culture days are shown (mean  $\pm$  s.d.). \* $P$ <0.05. (e) Representative photographs of soft agar colonies in 6-well plates showing the pre-miR-NC- (left) and pre-miR-7-transfected (right) Kato-III cells. (f) The mean numbers of soft agar colonies larger than the indicated diameters in each well of 6-well plate of control (gray bars) and pre-miR-7-transfected (closed bars) AZ521 cells and Kato-III cells are shown (mean  $\pm$  s.d.). \* $P$ <0.05.

the upstream CpG islands of *Hnnpk*. However, the H3K27me<sub>3</sub> level was not increased in mouse gastritis and gastric tumors compared with the wild-type stomach (Figure 4b). These results indicate that DNA methylation and trimethylation of H3K27 are not involved in the downregulation of miR-7-1. Moreover, the genomic region including miR-7-1 was not deleted in human gastric cancer cells (Supplementary Figure 3b),

suggesting that miR-7 downregulation in gastric cancer is not caused by genomic deletion.

We next examined whether activated macrophages have a role in the downregulation of miR-7, because the major source of proinflammatory cytokines in gastric tumors are macrophages (Oshima *et al.*, 2004, 2011). To monitor miR-7 activity, reporter vector-transfected cells were used. We confirmed that luciferase activity



**Figure 4** The mechanism responsible for the downregulation of miR-7 in gastric tumor cells. (a) Representative results of methylated (top) or unmethylated (bottom) status-specific PCR for miR-7-1. Lanes 1–12, human gastric cancer samples; lanes 13–22, non-tumor stomach samples; lane 23, methylated DNA control; lane 24, unmethylated DNA control; and lane 25, water control. (b) The results of ChIP-PCR analyses of miR-7-1 and the housekeeping genes, *Gapdh* and *Eef1a1*, for H3K27me3 in the gastric mucosa of respective genotype mice. The percentages of immunoprecipitated (IP)/whole-cell extracts (WCE) are shown for each primer set. (c) The luciferase activities of miR-7 Luc- or control Luc-transfected AZ521 reporter cells stimulated with CM(+) (gray bars) relative to those with CM(-) (open bars) are shown (mean  $\pm$  s.d.). \* $P < 0.05$ . The conditioned medium was prepared from RAW264 cells (left) or intraperitoneal macrophages (right). (d) The luciferase activities of miR-7 Luc-transfected AZ521 reporter cells stimulated with whole CM(+) (closed bars), CM(+) fractionated to the indicated molecular sizes (gray bars), or fractionated CM(+) to  $< 3$  kDa collected from celecoxib-treated and LPS-stimulated macrophages (light gray bar) relative to the control level (open bar) are shown (mean  $\pm$  s.d.). \* $P < 0.05$  versus the control level. (e) The relative miR-7 expression levels examined by real-time RT-PCR in AZ521 cells stimulated with whole CM(+) or fractionated CM(+)  $< 3$  kDa relative to the control level are shown (mean  $\pm$  s.d.). \* $P < 0.05$  versus the control level. The expression levels of miR-7 were normalized to the U44 level.

was increased significantly when the miR-7 inhibitor was transfected into reporter vector-transfected Kato-III cells (Supplementary Figure 1c), indicating that the luciferase reporter system was working. Reporter cells were then treated with the conditioned medium of lipopolysaccharide-stimulated RAW264 cells (CM(+)) or unstimulated RAW264 cells (CM(-)). Importantly, the luciferase activity increased significantly when cells were stimulated with CM(+), whereas the luciferase activity was not changed in control vector-transfected cells (Figure 4c). Similar results were obtained when CM(+) and CM(-) were prepared using mouse intraperitoneal macrophages. These results indicate that

activated macrophage-derived factor(s) caused miR-7 downregulation in gastric cancer cells.

To identify macrophage-derived factor(s) that suppress miR-7 expression, reporter cells were stimulated with TNF- $\alpha$ , IL-1 $\beta$ , IL-6 or PGE $_2$ . However, none of these factors caused an increase in luciferase activity (Supplementary Figure 4). We thus fractionated CM(+) by ultrafiltration, and separated by molecular weight. Interestingly, a CM(+) fraction of  $< 3$  kDa significantly increased the luciferase activity to a similar level as that induced by whole CM(+), whereas the other CM(+) fractions did not (Figure 4d). Moreover, the luciferase activity was still increased when CM(+)

was prepared under co-treatment of RAW264 cells with lipopolysaccharide and a COX-2 inhibitor, celecoxib. We confirmed the decreased level of miR-7 by real-time RT-PCR in CM(+)- or CM(+) fraction <3 kDa-treated AZ521 cells (Figure 4e). These results indicate that small molecule(s) (<3 kDa) derived from activated macrophages are responsible for miR-7 repression in gastric cancer cells, and that such small molecule(s) are expressed in activated macrophages in a COX-2/PGE<sub>2</sub>-independent manner.

*Downregulation of miR-7 in the stomach by inflammatory responses*

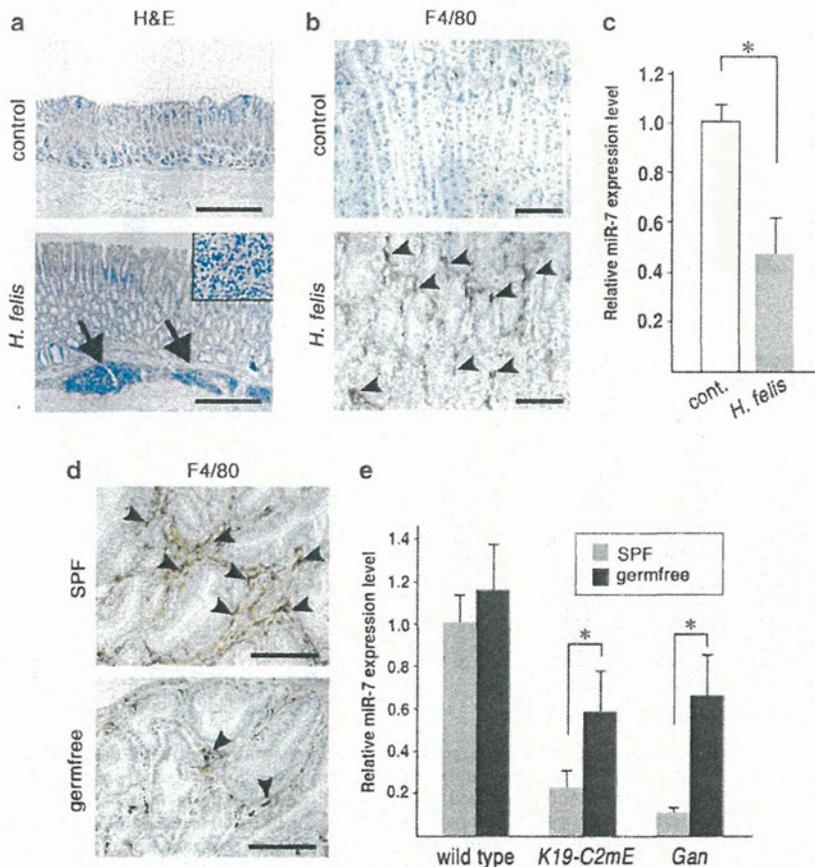
We next examined whether inflammatory responses are responsible for miR-7 downregulation in the stomach using different mouse models. The stomachs of wild-type mice were infected with *Helicobacter felis*, and submucosal inflammatory infiltration and mucosal macrophage accumulation were confirmed at 20 weeks after the infection (Figures 5a and b). Notably, the

miR-7 expression level was significantly decreased in the *H. felis*-infected inflamed gastric mucosa (Figure 5c).

We recently showed that inflammatory responses and macrophage infiltration were suppressed in *K19-C2mE* mouse gastritis and *Gan* mouse tumors when mice were maintained under germfree conditions (Figure 5d and Oshima *et al.*, 2011). Notably, miR-7 expression levels were increased significantly in germfree *K19-C2mE* and *Gan* mice compared with the levels of mice maintained in a specific pathogen free (SPF) facility (Figure 5e). These *in vivo* experiments suggest that inflammatory responses are responsible for the miR-7 downregulation in the stomach, although further genetic studies are required to examine the role of macrophages in miR-7 downregulation.

*Inflammation-dependent upregulation of miR-7 target genes in gastric tumors*

The epidermal growth factor receptor (*EGFR*) mRNA is one of the miR-7 target genes (Kefas *et al.*, 2008;

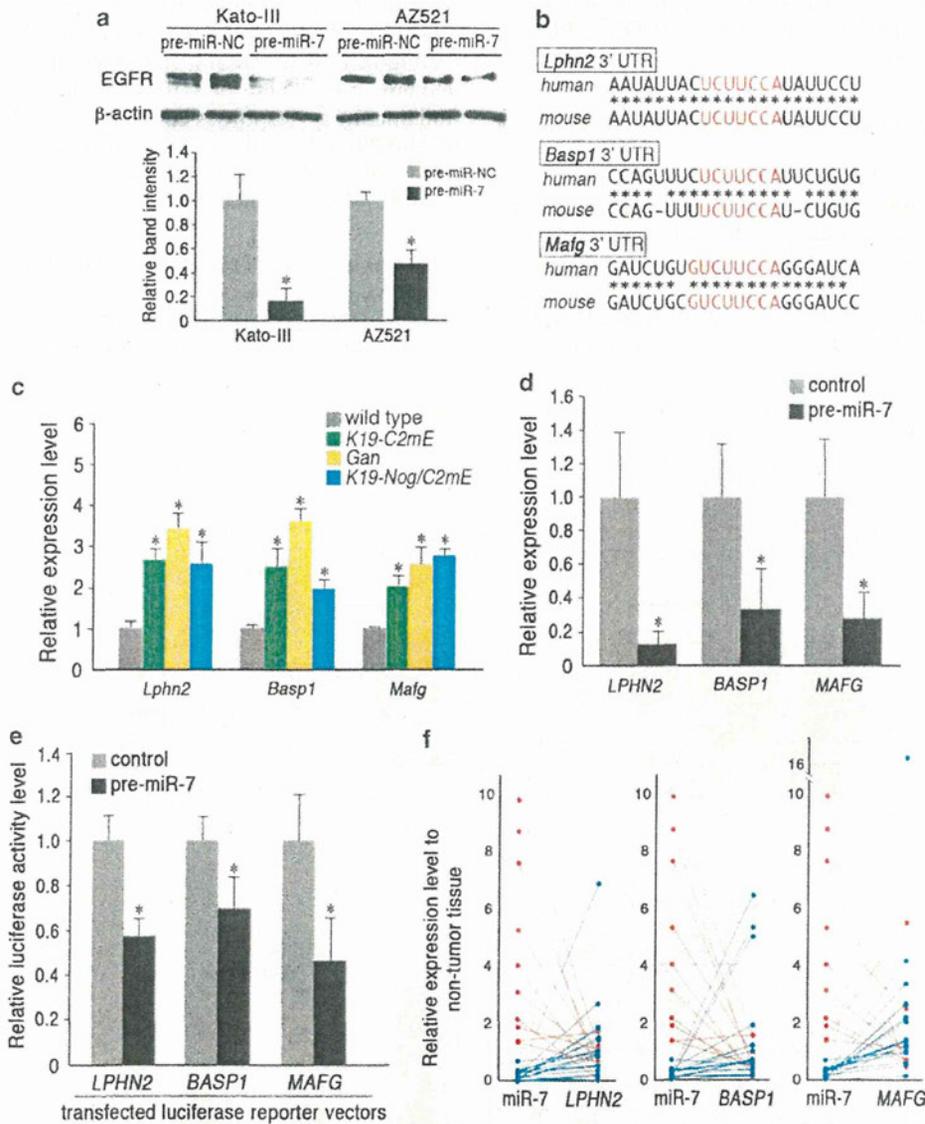


**Figure 5** Inflammation-induced miR-7 repression in the mouse stomachs. (a) Histology of the wild-type mouse normal glandular stomach (top) and *H. felis*-infected inflamed glandular stomach (bottom). Arrows and inset indicate submucosal inflammatory cell infiltration. Scale bars indicate 0.5 mm. (b) Immunostaining of F4/80 in the normal glandular stomach (top) and *H. felis*-infected glandular stomach (bottom). Arrowheads indicate macrophages. Scale bars indicate 100  $\mu$ m. (c) The miR-7 expression level of *H. felis*-infected gastric mucosa (gray bar) relative to that of the control stomach (open bar) is shown (mean  $\pm$  s.d.). \**P*<0.05. (d) Immunostaining of F4/80 in a SPF control *Gan* mouse tumor (top) and a germfree *Gan* mouse tumor (bottom). Arrowheads indicate macrophages. Scale bars indicate 100  $\mu$ m. (e) The expression levels of miR-7 in SPF (gray bars) and germfree (closed bars) *K19-C2mE* mouse gastritis and *Gan* mouse gastric tumors relative to the SPF wild-type stomach levels are shown (mean  $\pm$  s.d.). \**P*<0.05. The expression levels of miR-7 were normalized to the Sno202 level.

Webster *et al.*, 2009). We thus examined *EGFR* expression levels in pre-miR-7-transfected Kato-III and AZ521 gastric cancer cells. As expected, the *EGFR* expression level was decreased significantly by pre-miR-7 transfection in both cell lines (Figure 6a),

suggesting that suppression of *EGFR* expression is one of the tumor-suppressor mechanisms of miR-7 against gastric cancer development.

To identify novel miR-7 target genes that are upregulated in the inflammatory microenvironment,



**Figure 6** Inflammation-dependent upregulation of miR-7 target genes. (a) Representative results of the western blotting analysis of *EGFR* in Kato-III cells (top left) and AZ521 cells (top right) transfected with pre-miR-NC and pre-miR-7. The results for two independently prepared samples are shown.  $\beta$ -Actin was used as an internal control. The relative band intensities of the western blotting results are shown in the bar graph (mean  $\pm$  s.d.) (bottom). \* $P < 0.05$  versus the control level. (b) Alignment of the miR-7 target sequences in the 3'-UTR of *Lphn2*, *Basp1* and *Mafg* in human and mouse mRNAs. The seed match sequences for miR-7 are indicated in red. (c) The expression levels of the indicated genes in *K19-C2mE* mouse gastritis (green bars), *Gan* mouse gastric tumors (yellow bars) and *K19-Nog/C2mE* mouse gastric hamartomas (blue bars) relative to the wild-type levels (gray bars) are shown (mean  $\pm$  s.d.). \* $P < 0.05$  versus the wild-type level. The results were extracted from the microarray data set (Gene Expression Omnibus (GEO), accession GSE16902). (d) The expression levels of the indicated genes examined by real-time RT-PCR in pre-miR-7-transfected Kato-III cells (closed bar) relative to those of the control cells (gray bar) are shown (mean  $\pm$  s.d.). \* $P < 0.05$  versus control level. (e) The relative luciferase activity levels of pre-miR-7-transfected reporter cells (closed bars) to the levels of control pre-miR-NC-transfected reporter cells (gray bars) are shown. The target genes for the respective luciferase reporter vectors are indicated. \* $P < 0.05$  versus the control level. (f) Comparison of the relative expression levels of miR-7 and *LPHN2* (left), *BASP1* (center) or *MAFG* (right) in human gastric cancers with non-tumor stomach levels is shown. Red and blue lines indicate that miR-7 was increased ( $> 1.0$ ) and decreased ( $< 1.0$ ) in gastric cancers, respectively. The expression levels of miR-7 were normalized to the U44 level.

we searched for putative miR-7 target genes from the upregulated gene set in both *K19-C2mE* mouse gastritis and *Gan* mouse gastric tumors using the results of the microarray analyses (Itadani *et al.*, 2009). We found that the *Lphn2*, *Baspl* and *Mafg* genes have conserved miR-7 target sequences in their 3' untranslated region in both mouse and human mRNAs (Figure 6b). Notably, the expression of these genes was significantly increased not only in *K19-C2mE* mouse gastritis and *Gan* mouse tumors but also in *K19-Nog/C2mE* mouse gastric hamartomas (Figure 6c). The gastric mucosa and tumors in these strains are inflamed as a result of induction of the COX-2/PGE<sub>2</sub> pathway (Oshima *et al.*, 2004, 2006, 2009), suggesting that downregulation of miR-7 in inflammatory lesions is involved in upregulation of these genes. Notably, transfection of pre-miR-7 into Kato-III cells resulted in a significant decrease of *LPHN2*, *BASPI* and *MAFG* expressions (Figure 6d). Moreover, we constructed luciferase reporter plasmids that contained the 3' untranslated region fragment of the putative miR-7 target genes, and transfected these vectors into Kato-III cells. Consistent with the real-time RT-PCR results, luciferase activities of reporter vector-transfected cells decreased significantly when cells were co-transfected with pre-miR-7 (Figure 6e). Taken together, these results indicate that these three genes are miR-7 targets.

Finally, we examined the expressions of *LPHN2*, *BASPI* and *MAFG* in human gastric cancers by real-time RT-PCR and compared their expressions with miR-7 expression levels. We found that expression levels of miR-7 and *LPHN2*, *BASPI* and *MAFG* were inversely correlated (Figure 6f). Accordingly, it is possible that inflammation causes induction of these genes in human gastric cancers through downregulation of miR-7, which may contribute to gastric tumorigenesis, although this will need to be investigated in future studies.

## Discussion

It has been shown that miR-155 and miR-21, which function as oncogenes, are induced by inflammatory pathways, providing a link between inflammation and cancer (O'Connell *et al.*, 2007; Tili *et al.*, 2007; Iliopoulos *et al.*, 2010). Consistently, we found inflammation-dependent induction of miR-155 and miR-21 in mouse gastric tumors. On the other hand, miR-145 and miR-7, which function as tumor suppressors, are downregulated in both mouse gastritis and gastric tumor tissues. Moreover, we found that miR-7 levels are inversely correlated with the levels of proinflammatory cytokines, suggesting that the severity of inflammatory response is related to miR-7 downregulation. Therefore, it is possible that inflammation can promote tumorigenesis both by the upregulation of oncogenic miRNAs and by the downregulation of tumor-suppressor miRNAs, possibly through different mechanisms. Such alterations of cancer-related miRNA expression likely link inflammation and cancer.

It has been reported that miR-7 has a tumor-suppressor role in various cancers including brain tumors, breast cancer and lung cancer (Kefas *et al.*, 2008; Reddy *et al.*, 2008; Webster *et al.*, 2009), and we herein showed that miR-7 also functions as a tumor suppressor in gastric cancer. Interestingly, in the normal stomach, miR-7 expression is induced during the differentiation of gastric epithelial cells, suggesting a role of miR-7 in the regulation of epithelial cell differentiation. It has also been reported that miR-7 expression is induced during differentiation of intestinal epithelial cells (Nguyen *et al.*, 2010) and cortical neurons (Chen *et al.*, 2010). These results collectively suggest that miR-7 has a role in regulating cell differentiation in various organs. Therefore, it is conceivable that suppression of miR-7 expression is required for maintenance of the undifferentiated status of stem or progenitor cells in these organs.

These results suggest the possibility that inflammation suppresses epithelial cell differentiation through repression of miR-7. It is not surprising that the inflammatory response suppresses cell differentiation and enhances proliferation when regeneration is required in injured tissues. It has also been shown that a disruption of Toll-like receptor signaling causes an impairment of tissue repair by intestinal epithelial cells (Rakoff-Nahoum *et al.*, 2004). Moreover, activated macrophages are important niche components for intestinal epithelial progenitors in regenerative responses (Pull *et al.*, 2005). Therefore, it is conceivable that downregulation of miR-7 leads to suppression of differentiation, inducing the proliferation of undifferentiated epithelial cells in the inflammatory microenvironment.

Although several target genes of miR-7 have been identified, it is still unclear how miR-7 regulates differentiation. *EGFR* is one of the important miR-7 target genes, and is at least partially responsible for its tumor-suppressor role (Kefas *et al.*, 2008). Moreover, p21-activated kinase 1, Raf1, and the insulin-like growth factor 1 receptor have also been identified as miR-7 target genes that are upregulated in cancer cells (Reddy *et al.*, 2008; Webster *et al.*, 2009; Jiang *et al.*, 2010). Although most of these gene products contribute to cancer cell proliferation, we believe that miR-7 inhibits expression of other factors that have a role in the maintenance of the undifferentiated status of epithelial cells. In this study, we identified three novel miR-7 target genes that are upregulated in gastric cancers in an inflammation-dependent manner. *LPHN2* is a G protein-coupled receptor that binds  $\alpha$ -latrotoxin (Ichchenko *et al.*, 1999), whereas *BASPI* is implicated in neurite outgrowth (Korshunova *et al.*, 2008). *MAFG* is one of the small Maf proteins that is important for antioxidant responses (Katsuoka *et al.*, 2005). Although it remains to be investigated, it is of interest to examine whether these molecules have any role in the differentiation or tumorigenesis of gastric epithelial cells.

We examined the mechanisms responsible for miR-7 downregulation in gastric tumor cells. *Helicobacter pylori* infection induces chronic gastritis, resulting in induction of DNA methylation (Niwa *et al.*, 2010). The

expression of miR-34b/c is suppressed by DNA methylation in *H. pylori*-associated gastric cancer cells (Suzuki *et al.*, 2010). Moreover, H3K27me3 leads to tumor-suppressor gene silencing in cancer (Kondo *et al.*, 2008). However, we showed that downregulation of miR-7 is not caused by genomic deletion nor by epigenetic mechanisms, but through stimulation by macrophage-derived factor(s). Several mechanisms for the regulation of miR-7 expression have been reported. For example, miR-7 transcription is directly induced by HoxD10 (Reddy *et al.*, 2008) or c-Myc (Chou *et al.*, 2010). Splicing factor SF2/ASF binds the pri-miR-7 to enhance its cleavage by Drosha (Wu *et al.*, 2010). Accordingly, it is conceivable that macrophage-derived molecule(s) directly downregulate miR-7 expression or indirectly suppress miR-7 expression through modulation of these regulation systems. The identification of responsible macrophage-derived molecule(s) will provide a novel mechanism by which macrophages promote tumorigenesis.

In conclusion, we showed that inflammation simultaneously induces upregulation of oncogenic miRNAs and downregulation of tumor-suppressor miRNAs, which promote tumorigenesis. The expression of miR-7 is induced during differentiation of gastric epithelial cells, suggesting a role for miR-7 in the regulation of epithelial cell differentiation. Accordingly, it is possible that the downregulation of miR-7 contributes to suppression of differentiation, resulting in the promotion of gastric tumorigenesis. Moreover, small molecule(s) expressed by activated macrophages are responsible for miR-7 repression, providing a link between inflammation and cancer. Therefore, miR-7 may be useful for devising a new preventive or therapeutic strategy against gastric cancer through the induction of cancer cell differentiation.

## Materials and methods

### Mouse models

Construction of *K19-C2mE* and *Gan* (*K19-Wnt1/C2mE*) mice was described previously (Oshima *et al.*, 2004, 2006). In brief, *K19-C2mE* mice express *Ptgs2* and *Ptges* in gastric epithelial cells, whereas *Gan* mice express *Ptgs2*, *Ptges* and *Wnt1*. For expression analyses, *K19-C2mE* mouse gastritis and *Gan* mouse gastric tumor samples, and wild-type mouse stomach tissues were obtained at 30–40 weeks of age. Germfree mouse colonies were constructed as described previously (Oshima *et al.*, 2011), and the histology and miR-7 expression were examined at 55 weeks of age ( $n=5$ ). *H. felis* (American Type Culture Collection 49179, ATCC, Manassas, VA, USA) were inoculated at  $10^8$  per mouse into wild-type mice at 6–8 weeks of age, and the histology and miRNA expression were examined at 20 weeks after infection ( $n=6$ ). All animal experiments were carried out according to a protocol approved by the Committee on Animal Experimentation of the Kanazawa University.

### Microarray analysis

Total RNA was extracted from mouse stomachs ( $n=3$ ) using ISOGEN (Nippon Gene, Tokyo, Japan), pooled with the same

genotype mouse RNAs, labeled with Cy3 and hybridized to Mouse miRNA microarray Rel. 12.0 (Agilent Technologies, Santa Clara, CA, USA). The raw data were normalized using the GeneSpring GX software program (Agilent Technologies), and expression levels of miRNAs in gastritis and gastric tumor tissues were compared with those in the wild-type mouse stomach. Transcripts with low signals (less than threefold of the background level) were not used for further analyses. The expression of miRNAs was further examined by real-time RT-PCR using RNA samples independently prepared from a different set of wild-type, *K19-C2mE* and *Gan* mice ( $n=5$  for each genotype).

The results of cDNA microarray data sets of *K19-C2mE*, *Gan* and *K19-Nog/C2mE* mice were deposited into the Gene Expression Omnibus, as accession number GSE16902 (Itadani *et al.*, 2009), and were searched for the presence of novel miR-7 target genes using the TargetScan 5.1 program (MIT, Cambridge, MA, USA) (<http://www.targetscan.org>).

### Real-time RT-PCR

Paired gastric cancer and non-tumor stomach tissue samples were obtained from 28 patients during surgery at the Kanazawa University Hospital, Japan. Fresh frozen tissues were used for expression analyses. Clinicopathological data of patients are shown in Supplementary Table 3. Human normal gastric epithelial cells were prepared by isolating the gastric glands from normal stomach tissues ( $n=4$ ) as described previously (Cheng *et al.*, 1984). Approval for this project was obtained from the Kanazawa University Medical Ethics Committee, and written informed consent was obtained before specimen collection. Mouse stomachs were obtained from E15 wild-type mouse embryos, and day 0, day 7, day 14 and adult mice ( $n=3$  for each). Total RNAs were extracted from tissues or cells using ISOGEN (Nippon Gene), and cDNAs for miRNAs and mRNAs were constructed using QuantiMir RT kit (System Bioscience, Mountain View, CA, USA) and the PrimeScript RT reagent Kit (Takara, Tokyo, Japan), respectively. Real-time RT-PCR was performed using SYBR Premix Ex TaqII (Takara, Tokyo, Japan) and Stratagene Mx3000P (Agilent Technologies). Sno202 and U44 were used as endogenous miRNA controls for mice and humans, respectively, whereas  $\beta$ -actin was used for endogenous mRNA control. The primer sequences for the miRNAs are shown in Supplementary Table 4. The primers for mRNAs were purchased from Takara.

### Cell culture experiments

Mouse glandular stomachs were treated with 0.1% collagenase for 30 min at 37 °C, followed by centrifugation at 20 g for 3 min to isolate gastric glands. For the primary culture, isolated gastric glands were digested with trypsin and seeded on collagen-coated dishes as described previously (Oshima *et al.*, 2004). On day 2, the primary cultured cells were treated with 10 mM EDTA-phosphate-buffered saline and passaged. The primary cultured cells on day 2 (P0) and day 6 (P1) were used for the expression analysis. Human gastric cancer cell lines, AGS (ATCC), AZ521, MKN74, MKN45, NUGC4, HCT-111-TC, SH-10-TC (RIKEN BioResource Center, Tsukuba, Japan), Kato-III and MKN7 (Cell Resource Center for Biomedical Research, Tohoku University, Japan) were used in this study.

The pre-miR miRNA-7 (Pre-miR-7), pre-miR negative control (Pre-miR-NC) and anti-miR-7 inhibitor (Ambion, Austin, TX, USA) were used for transfection. The cell proliferation rate was measured using the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). For the soft agar colony

formation assay, cells were suspended in culture medium with 0.33% agar, and seeded in a 6-well plate. After 21 days of culture, soft agar was stained with Giemsa solution (Wako, Osaka, Japan).

#### Histological and immunohistochemical analyses

Tissues were fixed in 4% paraformaldehyde, embedded and sectioned at 4- $\mu$ m thickness. Sections were stained with hematoxylin and eosin. Antibodies against Ki-67 (DakoCytomation, Carpinteria, CA, USA) and F4/80 (Serotec, Oxford, UK) were used as the primary antibodies. Immunostaining signals were visualized using the Vectastain Elite Kit (Vector Laboratories, Burlingame, CA, USA), and the MOM kit (Vector Laboratories) was used to minimize the background staining signals.

#### Methylation-specific PCR analysis

The methylation status of miR-7-1 in human gastric cancer and non-tumor tissues was examined by methylation-specific PCR analysis as described previously (Yamashita et al., 2008). Methylation-specific PCR was performed with a primer set specific for the methylated or unmethylated sequence (Me or Un set in Supplementary Table 4). DNA methylated by SssI methylase (New England Biolabs, Ipswich, MA, USA) and DNA amplified by a GenomiPhi DNA amplification kit (GE Healthcare, Buckinghamshire, UK) were used as methylated and unmethylated controls, respectively.

#### Chromatin immunoprecipitation analyses

Histone modifications were examined by chromatin immunoprecipitation assay as described previously (Yamashita et al., 2008; Takeshima et al., 2009). In brief, gastric epithelial cells of wild-type, *K19-C2mE* and *Gan* mice ( $n=3$  for each genotype) were cross-linked with 1% formaldehyde, and sheared chromatin by Bioruptor UCD-250 (Cosmo Bio, Tokyo, Japan). The samples were incubated with an antibody against H3K27me3 (07-449, Millipore, Billerica, MA, USA), and genomic DNA samples were used for the quantitative chromatin immunoprecipitation-PCR analyses. The primer set was designed for the upstream CpG islands of miR-7-1 (Supplementary Table 4).

#### Luciferase reporter assay

Constructions of the miR-7 luciferase reporter vector (miR-7 Luc) and control vector (control Luc) are shown in Supplementary Figure 1. Luciferase reporter vectors for the *LPHN2*, *BASPI* and *MAFG* genes were constructed by subcloning 3' untranslated region fragments of the respective human genes into the pGL3 plasmid (Promega, Madison, WI, USA). PCR

primer sequences for cloning 3' untranslated region of the respective genes and amplified fragment lengths are indicated in Supplementary Table 4. The luciferase activity was measured using a Luciferase Assay System (Promega), and the levels were normalized to the total protein levels detected using the Pierce 660 nm Protein Assay (Thermo Scientific, Yokohama, Japan). RAW264 cells (RIKEN BioResource Center) and mouse intraperitoneal macrophages were stimulated with 10 ng/ml lipopolysaccharide (Sigma, St Louis, MO, USA) for 24 h and the conditioned medium was collected as CM(+). The conditioned medium of unstimulated macrophages was collected as CM(-). The conditioned medium of lipopolysaccharide-stimulated and COX-2 inhibitor celecoxib-treated (Pfizer, New York, NY, USA) (10  $\mu$ M) RAW264 cells was collected as CM(+ /coxib). CM(+) was fractionated by ultrafiltration using Centricon Plus-70 (Millipore) to prepare CM(+) > 100 kDa, 30–100 kDa, 3–30 kDa and < 3 kDa. Cells were stimulated with CM at a 50% concentration.

#### Western blotting analysis

Cells were transfected with pre-miR-7 or pre-miR-NC, lysed at 24 h after transfection, and 10  $\mu$ g of protein was separated in 7.5% SDS-polyacrylamide gels. An antibody against EGFR (Cell Signaling Technology, Danvers, MA, USA) was used as the primary antibody. Anti- $\beta$ -actin (Sigma) was used as an internal control. The ECL detection system (GE Healthcare) was used to detect the signals, and band intensities were quantified using the ImageJ application (NIH, Bethesda, MD, USA).

#### Statistical analysis

The data were analyzed by the unpaired *t*-test using the Microsoft Excel software program (Microsoft). A value of  $P < 0.05$  was considered to be statistically significant.

#### Conflict of interest

The authors declare no conflict of interest.

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## Hypomethylation of Alu repetitive elements in esophageal mucosa, and its potential contribution to the epigenetic field for cancerization

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### Abstract

**Background** Aberrant hypermethylation of specific genes is present in esophageal squamous cell carcinomas (ESCCs). Such hypermethylation is also present in normal-appearing esophageal mucosae of ESCC patients and is considered to contribute to the formation of a field for cancerization. On the other hand, the presence of global hypomethylation in ESCCs or in their background esophageal mucosae is unknown.

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**Method** We collected 184 samples of esophageal mucosae (95 normal mucosae from healthy subjects, and 89 non-cancerous background mucosae from ESCC patients) and 93 samples of ESCCs. Methylation levels of repetitive elements (Alu, LINE1) and cancer/testis antigen genes (*NY-ESO-1*, *MAGE-C1*) were measured by bisulfite pyrosequencing and quantitative methylation-specific PCR, respectively.

**Results** Methylation levels of Alu, LINE1, *NY-ESO-1*, and *MAGE-C1* were significantly lower in ESCCs than in their background and normal mucosae. Also, in the background mucosae, a significant decrease of the Alu methylation level compared with the normal mucosae was present. In ESCCs, methylation levels of the two repetitive elements and the two cancer/testis antigen genes were correlated with each other.

**Conclusion** This is the first study to show the presence of global hypomethylation in ESCCs, and even in their non-cancerous background mucosae. Alu hypomethylation might reflect the severity of an epigenetic field for cancerization.

**Keywords** Esophageal squamous cell carcinoma · Hypomethylation · Repetitive element · Cancer/testis antigen · Epigenetics

### Abbreviations

ALDH2 Acetaldehyde dehydrogenase 2  
LINE1 Long interspersed nucleotide element 1  
PCR Polymerase chain reaction  
UICC Union for International Cancer Control

### Introduction

Esophageal squamous cell carcinoma (ESCC) remains the predominant histological type of esophageal cancers in

Asian countries [1–3]. It is known for its high prevalence of multiple occurrences, including synchronous and metachronous occurrence [4, 5]. As for mechanisms of multiple occurrences, it is believed that genetic/epigenetic alterations accumulate in normal-appearing tissues, forming a field for cancerization [6, 7]. We previously demonstrated that aberrant methylation of promoter CpG islands was present in non-cancerous esophageal mucosae of ESCC patients and that methylation of specific genes was associated with smoking history [8] and cancer risk [9]. Because tumor-suppressor genes, such as *CDKN2A*, *CDH1*, *FHIT*, and *RASSF1A*, are inactivated by methylation of promoter CpG islands in ESCCs [10–13], it was considered that accumulation of aberrant methylation in esophageal mucosae was involved in the formation of a field for ESCCs.

In addition to methylation of promoter CpG islands, global hypomethylation is characteristic of tumor cells [14, 15]. Global hypomethylation involves hypomethylation of normally methylated repetitive elements, such as Alu and LINE1 [16], and normally methylated cancer/testis (CT) antigen genes [17, 18]. The hypomethylation of repetitive elements can serve as a surrogate marker for global DNA hypomethylation [19, 20]. “Normally methylated” genes are physiologically methylated and unexpressed in most tissues of adults, except testicular germ cells. In contrast, they are demethylated and expressed in various types of human cancers [21], and specific ones, such as *NY-ESO-1* and *MAGE-C1*, are expected to be useful as potential targets for cancer immunotherapy [22, 23]. The presence of global hypomethylation is associated with increased rates of chromosome recombination and increased incidence of tumor formation [24–26]. Nevertheless, in ESCCs, the presence of global hypomethylation in cancer cells and its potential involvement in the field for cancerization are unknown.

In the current study, we aimed to elucidate whether or not hypomethylation is present in ESCCs, and also in non-cancerous background esophageal mucosae. To this end, we accurately quantified methylation levels of two repetitive elements, Alu and LINE1, and two CT antigen genes, *NY-ESO-1* and *MAGE-C1*, in ESCCs, their background mucosae, and normal esophageal mucosae from healthy volunteers.

## Materials and methods

### Patients and tissue samples

Normal mucosae of 95 healthy volunteers (69 male and 26 female; average age = 58, median age = 59, range = 25–91) and cancerous lesions of 93 ESCC patients (85

male and 8 female; average age = 61, median age = 60, range = 43–85) before any therapeutic intervention (surgery, chemotherapy, or radiation therapy) were collected by endoscopic biopsy at the National Taiwan University Hospital, Taipei, Taiwan. In 89 of the 93 ESCC patients, we collected biopsy samples not only from cancerous lesions but also from non-cancerous background mucosae (82 male and 7 female; average age = 61, median age = 60, range = 43–85). A total of 277 samples for methylation analysis were collected. Additionally, for immunohistochemical staining, surgical specimens of primary ESCCs and their paired non-cancerous background mucosae were collected from 48 patients (41 male and 7 female; average age = 63, range = 41–83) who underwent esophagectomy without any neoadjuvant therapy at the National Cancer Center Hospital, Tokyo, Japan. Non-cancerous background mucosa was defined as the area that was at least 5 cm away from the cancerous margin and appeared normal by iodine staining. All samples were stored at  $-80^{\circ}\text{C}$  after biopsy or resection until the extraction of genomic DNA.

Informed consents and interviews for lifestyle risk factors, including cigarette smoking and alcohol intake, were obtained from all the individuals. The range of follow-up period after endoscopic examination was 461–944 days. Disease stages were classified according to the 7th edition of the TNM classification by UICC.

### Bisulfite pyrosequencing for repetitive elements

Sodium bisulfite modification was performed using 1  $\mu\text{g}$  of *Bam*HI-digested genomic DNA as previously described [27]. The modified DNA was suspended in 40  $\mu\text{l}$  of Tris-EDTA buffer, and an aliquot of 1  $\mu\text{l}$  was used for bisulfite pyrosequencing. The CpG sites that showed most significant hypomethylation in gastric cancer [28] were selected for analysis in this study. All primers for pyrosequencing were the same as those we previously reported [28] and are listed in Supplementary Table 1. The PCR products labeled with biotin were annealed to 0.2  $\mu\text{M}$  pyrosequencing primers, and pyrosequencing was carried out using the PSQ 96 Pyrosequencing System (Qiagen, CA, USA). Methylation levels were obtained using PSQ Assay Design software (Qiagen).

### Quantitative methylation-specific PCR (qMSP)

Real-time MSP was performed with a primer set specific to methylated (M) or unmethylated (U) sequence by using a 2  $\mu\text{l}$  aliquot of the sodium bisulfite-treated DNA, SYBR Green (Bio Whittaker Molecular Applications, MD, USA), and an iCycler Thermal Cycler (Bio-Rad Laboratories, CA, USA). MSP primers and PCR conditions are shown in Supplementary Table 2. DNA methylated by *Sss*I

methylase (New England Biolabs, MA, USA) and DNA amplified twice by a GenomiPhi DNA amplification kit (GE Healthcare Bio-Science, Buckinghamshire, England) were used as fully methylated and unmethylated control DNA, respectively. As in our previous report [8], methylation levels were calculated as the fraction of methylated molecules in the total number of DNA molecules (the number of methylated molecules + the number of unmethylated molecules).

#### Immunohistochemistry of CT antigens

Immunohistochemical staining of NY-ESO-1 and MAGE-C1 antigens was performed using a mouse monoclonal anti-NY-ESO-1 antibody (clone E978, Invitrogen, CA, USA) and anti-MAGE-C1 antibody (clone CT7-33, DAKO, CA, USA) as primary antibodies. Formalin-fixed and paraffin-embedded samples were sliced at 3  $\mu\text{m}$  thickness, deparaffinized, and heated in 10 mM citrate buffer (pH 6.0) for 20 min (NY-ESO-1) and 5 min (MAGE-C1) at 120  $^{\circ}\text{C}$  by autoclave. After blocking with Blocking-One (Nacalai Tesque, Kyoto, Japan), the sections were incubated with a primary antibody at a concentration of 2.5  $\mu\text{g}/\text{ml}$  (NY-ESO-1) or 0.21  $\mu\text{g}/\text{ml}$  (MAGE-C1) at 4  $^{\circ}\text{C}$  overnight. Detection of the primary antibody was performed with the DAKO Envision Plus system at room temperature for 60 min and DAB (Wako, Osaka, Japan) as a chromogen. Slides were counterstained with hematoxylin. As a positive control, we used the normal part of a testis with intact spermatogenesis collected from an adolescent patient with seminoma.

#### Statistical analysis

Differences in mean methylation levels were analyzed by Student's *t* test (when variances were equal) and Welch's *t* test (when variances were unequal). Correlations of methylation levels among individual repetitive elements and promoter CpG islands were analyzed using Pearson's product-moment correlation coefficients. All the analyses were performed using PASW statistics version 18.0 (SPSS Japan Inc., Tokyo, Japan), and the results were considered significant when *p* values <0.05 were obtained by a two-sided test.

## Results

### Hypomethylation of repetitive elements and CT antigen genes in ESCCs, and Alu hypomethylation in background mucosae

Methylation levels of repetitive elements were quantified by bisulfite pyrosequencing in a total of 277 samples. The mean methylation level of Alu was significantly lower in ESCCs

( $42.0 \pm 3.8\%$ , mean  $\pm$  SD) than in non-cancerous background mucosae of cancer patients ( $45.7 \pm 2.3\%$ ) and normal mucosae of healthy volunteers ( $46.5 \pm 2.4\%$ ) ( $p < 0.001$ ) (Fig. 1a). Notably, the mean methylation level of Alu was significantly lower even in the background mucosae than in the normal mucosae ( $p = 0.018$ ). The mean methylation level of LINE1 was significantly lower in ESCCs ( $62.2 \pm 12.1\%$ ) than in the normal and background mucosae ( $79.7 \pm 3.9\%$ ,  $78.8 \pm 6.7\%$ , respectively,  $p < 0.001$ ) (Fig. 1b). Unlike Alu, significant differences between the normal and background mucosae were not observed.

Methylation levels of CT antigen genes were quantified by real-time MSP (Fig. 1c, d). Both *NY-ESO-1* and *MAGE-C1* were almost fully methylated in the normal and background mucosae but demethylated in 22 (23.7%) and 23 (24.7%) samples of ESCCs, respectively, with a threshold value of 95%. The mean methylation levels of *NY-ESO-1* and *MAGE-C1* were significantly lower in ESCCs ( $89.3 \pm 22.4\%$ ,  $94.0 \pm 14.9\%$ , respectively) than in their background ( $99.6 \pm 1.4\%$ ,  $99.8 \pm 0.6\%$ , respectively) and normal mucosae ( $99.7 \pm 0.9\%$ ,  $99.7 \pm 2.2\%$ , respectively) ( $p < 0.001$  each).

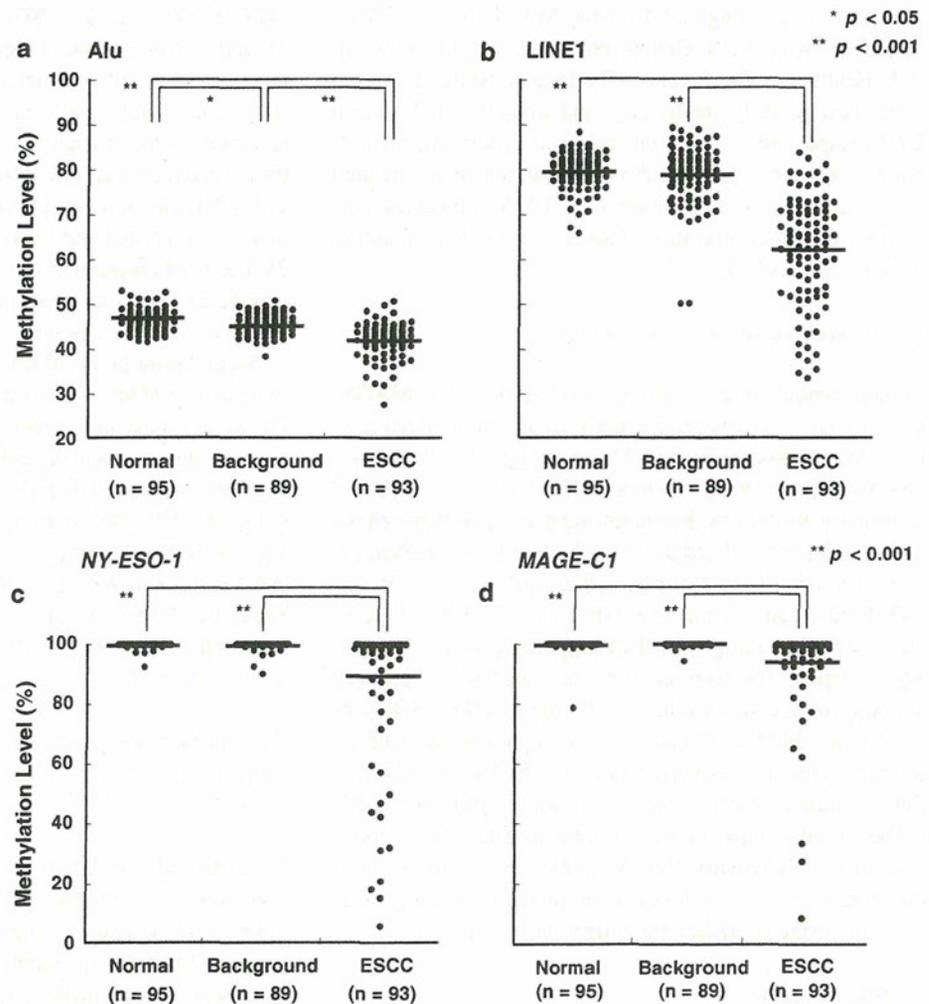
### Correlation among methylation levels of repetitive elements, CT antigen genes, and other promoter CpG islands

We analyzed correlation of methylation levels among two repetitive elements, two CT antigen genes, and four promoter CpG islands in the background mucosae and in ESCCs (Table 1 and Supplementary Fig. 1). As promoter CpG islands hypermethylated in background mucosae, we selected four genes, *HOXA9*, *NEFH*, *UCHL1*, and *MTIM*, whose methylation levels were analyzed in our previous study (Supplementary Fig. 2) [9]. In the background mucosae, methylation levels were significantly correlated among the four genes. Also, significant negative correlations were observed between the hypomethylation of repetitive elements and hypermethylation of the four genes. In ESCCs, significant positive correlation was observed among individual repetitive elements and CT antigen genes. Correlations between Alu and LINE1 hypomethylation and between *NY-ESO-1* and *MAGE-C1* hypomethylation were especially stronger than the other correlations.

### The lack of association between hypomethylation and exposure to risk factors, and between hypomethylation and clinicopathological findings

We first analyzed correlation between the age and degree of hypomethylation of the two repetitive elements. No significant correlation was observed in healthy volunteers

**Fig. 1** Methylation levels of the two repetitive elements and the two CT antigen genes in normal mucosae of healthy subjects ( $n = 95$ ), non-cancerous background mucosae of cancer patients ( $n = 89$ ), and ESCCs ( $n = 93$ ) collected by endoscopic biopsy. Distribution of the methylation levels at a particular CpG site of Alu (a), LINE1 (b), *NY-ESO-1* (c), or *MAGE-C1* (d) is shown, and a horizontal line in a chart represents a mean methylation level for each group. The mean methylation levels of Alu, LINE1, *NY-ESO-1*, and *MAGE-C1* in ESCCs were significantly lower than those in normal and background mucosae. In addition, Alu methylation levels were significantly lower in the background mucosae than in the normal mucosae



(Alu;  $r = -0.181$ ,  $p = 0.080$ , LINE1;  $r = 0.129$ ,  $p = 0.214$ ) or in ESCC patients (Alu;  $r = -0.085$ ,  $p = 0.426$ , LINE1;  $r = 0.010$ ,  $p = 0.925$ ), showing the hypomethylation was not age-dependent. Then, in normal mucosae, background mucosae, and ESCCs, we analyzed association between exposure to risk factors and the degree of hypomethylation of the two repetitive elements and the two CT antigen genes. In any group of samples, methylation levels were not associated with history of cigarette smoking or alcohol intake (Table 2). Even when the samples were classified according to the genetic polymorphisms, whether they had an active ALDH2 allele ( $ALDH2^1/ALDH2^1$  homozygote) or an inactive ALDH2 allele ( $ALDH2^1/ALDH2^2$  heterozygote and  $ALDH2^2/ALDH2^2$  homozygote), methylation levels were not associated with alcohol intake (data not shown).

In ESCCs, we also analyzed association between methylation levels of repetitive elements or CT antigen genes and clinicopathological findings, including depth of tumor, tumor differentiation, lymph node metastasis,

multiplicity of tumor, and tumor recurrence (Table 3). There was no significant association between methylation levels of repetitive elements in ESCCs and clinicopathological findings, and between methylation levels of CT antigen genes and clinicopathological findings.

#### Association between hypomethylation and expression of CT antigen genes

Finally, we aimed to assess association between hypomethylation and expression of CT antigen genes using immunohistochemistry. From 48 surgical specimens, we selected six ESCCs that had various degrees of hypomethylation and their paired non-cancerous background mucosae (Table 4; representative results in Fig. 2 and Supplementary Fig. 3). For *NY-ESO-1*, positive staining was observed in two of four ESCCs with demethylation and none of two background mucosae with demethylation. For *MAGE-C1*, positive staining was observed in three of five ESCCs with demethylation and none of one