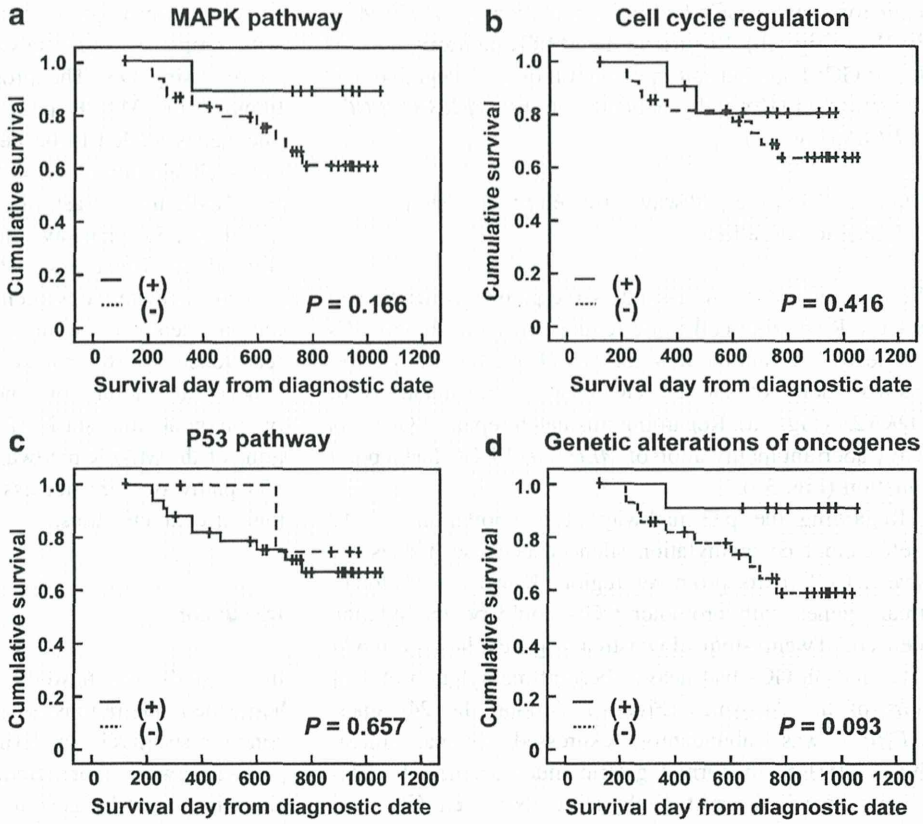

Fig. 4 Associations between a pathway alteration and patient prognosis. Kaplan–Meier curves were drawn using OS. **a** Patients with alterations of the MAPK pathway ($n = 11$) might have better prognosis than those without ($P = 0.116$). **b**, **c** The genetic or/and epigenetic alterations of cell-cycle regulation and the p53 pathway did not show any associations. **d** Patients with genetic alterations of oncogenes ($n = 12$) tended to have better prognosis than those without ($P = 0.093$)

These results showed that epigenetic alterations are deeply involved in gastric carcinogenesis. Aberrant DNA methylation can be restored by the DNA-demethylating drugs 5-azacytidine (azacitidine) and 5-aza-2′-deoxycytidine (decitabine), which are clinically used for patients with myelodysplastic syndromes [34]. Recently, clinical trials using DNA-demethylating drugs for solid tumors have been actively conducted [35], and efficacy was shown in recurrent metastatic non-small cell lung cancer [36]. There is a possibility that these epigenetic drugs are useful for the treatment of GCs.

According to a genome-wide analysis of methylated genes, several hundred to 1,000 genes whose promoter CGIs are aberrantly methylated are accumulated in cancers [37]. Expression levels of most of these genes are absent or very low in normal cells [30]. Most of them are considered not as drivers of carcinogenesis but as passengers. Therefore, we separately analyzed TSS200 CGIs of genes expressed in normal gastric mucosae. These genes are known to frequently include driver genes in carcinogenesis [38]. *DKK3* involved in the WNT pathway and *IGFBP7* involved in the p53 pathway were expressed in normal gastric mucosae and frequently methylated in GCs. It is known that downregulation of *DKK3* is correlated with tumor progression [39], and that *IGFBP7* can inhibit cell growth and induce apoptosis [40]. These results supported that aberrant methylation of *DKK3* and *IGFBP7* was involved in gastric carcinogenesis.

Patients with genetic alterations of oncogenes had a significantly smaller number of lymph nodes with metastasis than those without, and their prognosis tended to be better than those without. Although detailed mechanisms are unknown, it is known that oncogene mutations are associated with the CpG island methylator phenotype (CIMP), and that the prognosis of the CIMP(+) patients tends to be better than that of the CIMP(−) patients in GCs [23].

In conclusion, an integrated profile of genetic and epigenetic alterations of GC-related pathways was obtained using a benchtop next-generation sequencer and a bead array. The profile is expected to be useful for selection of molecular-targeted and epigenetic drugs for individual patients.

Table 2 Associations between genetic/epigenetic alterations and clinicopathological findings

Variable	*N*	Alterations of MAPK pathway		*P* value	Alterations of cell cycle regulation		*P* value	Alterations of p53 pathway		*P* value	Genetic alterations of oncogenes		*P* value
		+	−		+	−		+	−		+	−	
Gender				1.000			0.398			0.567			1.000
Male	34	8	26		9	25		29	5		10	24	
Female	7	1	6		3	4		7	0		2	5	
Age				0.743			0.101			0.436			0.451
Mean ± SD (range)		67.3 ± 11.2 (54–88)	68.8 ± 12.0 (38–88)		73.2 ± 12.2 (54–88)	66.6 ± 11.2 (38–83)		69.0 ± 11.8 (38–88)	64.6 ± 11.6 (47–76)		70.7 ± 11.7 (54–88)	67.6 ± 11.8 (38–84)	
Histological differentiation				0.231			0.494			0.146			0.278
Differentiated	14	5	9		3	11		14	0		6	8	
Undifferentiated	27	4	23		9	18		22	5		6	21	
Depth of tumor				0.088			0.370			0.852			0.230
T1	1	0	1		1	0		1	0		1	0	
T2	9	4	5		2	7		9	0		4	5	
T3	14	4	10		3	11		12	2		4	10	
T4	17	1	16		6	11		14	3		3	14	
Lymph node metastasis				0.070			0.524			0.173			0.021
N0	6	3	3		3	3		6	0		4	2	
N1	7	3	4		1	6		6	1		3	4	
N2	10	1	9		2	8		7	3		0	10	
N3	18	2	16		6	12		17	1		5	13	
Recurrence				0.441			0.305			1.000			0.084
Negative	25	7	18		9	16		22	3		10	15	
Positive	16	2	14		3	13		14	2		2	14	

Depth of tumor and lymph node metastasis are based on the 7th edition tumor-node-metastasis classification of the International Union Against Cancer

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Conflict of interest The authors have declared that no competing interests exist.

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ORIGINAL ARTICLE

DNA Methylation of *MicroRNA-124a* Is a Potential Risk Marker of Colitis-Associated Cancer in Patients with Ulcerative Colitis

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Abstract
Background Colitis-associated cancer (CAC) is the serious complication of ulcerative colitis (UC), and molecular markers to evaluate the individual risk are required. *MicroRNA-124a* (*miR-124a*) is known to have tumor-suppressive function and be methylation-silenced during exposure to chronic inflammation.
Aim We analyzed whether higher methylation levels of *miR-124a* genes correlated with the higher epidemiologic risk of CAC development in UC patients.
Methods Forty UC patients without CAC, four patients with CAC or dysplasia, eight sporadic colorectal cancer (S-CRC) patients, and 12 healthy volunteers (HV) were studied. Methylation status of *miR-124a* genes (*miR-124a-1*, *-2*, and *-3*) was analyzed by methylation-specific polymerase chain reaction (MSP), and methylation levels were quantified by real-time MSP. Expression of cyclin-dependent kinase 6 (CDK6), a target of *miR-124a*, was analyzed by immunohistochemistry.
Results Three *miR-124a* genes were methylated in all neoplastic tissues (CAC, dysplasia, and S-CRC), and CDK6 was highly expressed in those tissues. Regarding disease extent, mean methylation levels of *miR-124a-3* in HV, non-pancolitis, and pancolitis were 2.0, 5.3, and 12.3 %, respectively, and were significantly higher in pancolitis than in HV ($p < 0.01$). Regarding disease duration, mean methylation levels in short-term and long-standing UC patients were 2.5 and 13.2 %, respectively. Long-standing UC patients had significantly higher methylation levels than HV ($p < 0.01$). Moreover, UC patients with both pancolitis and long-standing had 7.4-fold higher methylation levels than those without these risk factors.
Conclusions *MiR-124a* genes are methylated during carcinogenesis in UC patients. The methylation level of *miR-124a-3* is a promising marker for estimating individual risk for CAC.

Keywords Ulcerative colitis · MicroRNA · DNA methylation · Colitis-associated cancer

Abbreviations

UC	Ulcerative colitis
CAC	Colitis-associated cancer
ER	Estrogen receptor
miRNA	MicroRNAs
CDK6	Cyclin-dependent kinase 6
S-CRC	Sporadic colorectal cancer
MSP	Methylation-specific polymerase chain reaction
PCR	Polymerase chain reaction

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Introduction

Patients with long-standing ulcerative colitis (UC) have a high risk for the development of colorectal cancer. The standardized incidence risk of colorectal cancer has been reported to be 1.7-fold for patients with proctitis, 2.8-fold for those with left-sided colitis, and 14.8-fold for those with pancolitis [1]. A meta-analysis revealed that the cumulative risk of colorectal cancer in UC patients was 2 % at 10 years, 8 % at 20 years, and 18 % at 30 years from disease onset [2]. These epidemiological findings show that the extent and duration of disease are major risk factors for the development of colitis-associated cancer (CAC). Therefore, surveillance colonoscopy with multiple-step biopsies every 1–2 years has been recommended for UC patients with long-standing and extensive disease, although the risk of CAC markedly differs among individuals [3, 4]. Recent studies have recommended that surveillance intervals should be tailored to individual risk [5, 6]. To focus on patients with high risk for the development of CAC, useful molecular markers to evaluate the individual risk are required.

Aberrant DNA methylation of promoter CpG islands permanently inactivates tumor suppressor genes and is deeply involved in carcinogenesis, similar to mutations and chromosomal abnormalities [7]. The epigenetic alteration is induced by chronic inflammation and is accumulated in non-cancerous tissues exposed to chronic inflammation. Moreover, methylation levels of specific genes in those tissues have been closely associated with cancer risks, such as for *Helicobacter pylori*-associated gastric cancer [8]. Indeed, several tumor suppressor genes and passenger genes, such as *hMLH1*, *p16*, *p14*, *CDH1*, *RUNX3*, *MINT*, and estrogen receptor (*ER*), have been reported to be methylation-silenced in CAC and in the colorectal mucosa of UC patients [9–16]. These findings indicate that analysis of quantitative methylation levels might estimate an individual risk for the development of CAC. To evaluate a novel risk marker for CAC, it should be clarified that methylation levels in UC patients are correlated with definitive epidemiological risk.

MicroRNAs (miRNA) are a class of small noncoding RNAs that might have oncogenic or tumor-suppressive activities resulting from the translational repression or cleavage of target mRNA [17]. The tumor-suppressive activity of *microRNA-124a* (*miR-124a*) has been shown to down-regulate oncogenic cyclin-dependent kinase 6 (CDK6) [18–20]. The transcription of *miR-124a* is controlled by DNA methylation of the promoter regions of three *miR-124a* isoforms (*miR-124a-1, -2*, and *-3*) in colon, gastric, and liver cancer, as well as in leukemia [18–21]. We previously found that aberrant methylation of *miR-124a* was induced in non-cancerous gastric mucosa infected with *H. pylori* during persistent inflammation-associated carcinogenesis [21]. In contrast, microRNA silencing in CAC and in the colorectal mucosa of UC patients remains unclear.

In the present study, we aimed to clarify whether DNA methylation of *miR-124a* could be one marker for estimating an individual risk for the development of CAC. We evaluated the quantitative methylation levels in the colorectal mucosa of UC patients and analyzed the correlation with methylation levels and the epidemiological risk factors for the development of CAC, as well as the extent and duration of disease.

Methods

Cell Lines and Human Tissue Samples

Two human colon cancer cell lines, DLD-1 and HCT-15, were obtained from Human Science (Osaka, Japan) and the Cell Resource Center for Biomedical Research at Tohoku University (Sendai, Japan), respectively. These cell lines were treated with 5-aza-2′-deoxycitidine (5-aza-dC; Sigma-Aldrich, St Louis, MO, USA). Cells were seeded on day 0, media was added with freshly prepared 2 μM 5-aza-dC on days 1 and 3, and cells were harvested on day 4.

Biopsy samples were collected from colorectum of 40 UC patients without CAC between 2010 and 2012. All patients had confirmed diagnosis of UC based on their clinical symptoms, endoscopic, and histological findings. Patients were followed up endoscopically after the analysis of methylation once a year. They were divided into short-term or long-standing group according to disease duration of under or over 8 years (Table 1). In fourteen UC patients with pancolitis, multiple biopsies were taken from six segments (rectum, sigmoid colon, descending colon, transverse colon, ascending colon, and cecum) of the colorectum. As a control, biopsy samples were collected from rectum of 12 healthy volunteers (HV) (ten men and two women; median age, 35 years; range 31–68) and tumor tissues of eight sporadic colorectal cancer (S-CRC) patients (six men and two women; median age, 66 years; range 55–88). Additionally, CAC or dysplasia tissues were collected from four UC patients (four men; median age, 63 years; range 43–67) who underwent tumor resection. All specimens were collected at Toyama University Hospital, and informed consent was obtained from all patients and HV.

Colorectal mucosae and S-CRC tissues were frozen in RNA *later* Tissue Collection (Ambion, Carlsbad, CA, USA) immediately after biopsy and stored at −80 °C. High-molecular-weight DNA was extracted by the phenol/

Table 1 Clinical characteristics of patients with UC

	$n = 40$
Gender	
Male/female	21/19
Age (years)	
Median (range)	42 (19–71)
Disease duration (years)	
Median (range)	11.9 (0–28)
Short-term (<8 years)	12
Long-standing (≥8 years)	28
Age at diagnosis (years)	
Median (range)	28 (7–69)
Extent of disease	
Pancolitis	27
Proctitis	8
Left-sided colitis	4
Right-sided colitis	1
Daily medication at entry	
Mesalazine (1.5–4.5 g/day)	37
Steroids (5–15 mg/day)	6
Azathioprine (25–50 mg/day)	2
None	2
Others	4
Past use of steroids	
Yes/no	22/18

chloroform method. Sections of CAC and dysplasia tissues were deparaffinized in xylene and rehydrated before extraction of genomic DNA.

Sodium Bisulfite Modification, Methylation-Specific Polymerase Chain Reaction (MSP), and Quantitative Real-Time MSP (qMSP)

Bisulfite modification was performed using 1 μg of *Bam*HI-digested genomic DNA as previously described [22]. For MSP, the primer sequences designed just upstream of transcription start sites of *miR-124a* (*miR-124a-1*, *-2,* and *-3*), *p16,* and *ER* have been described previously [11, 21, 23], and conditions were redetermined using the fully methylated and unmethylated DNA (Supplementary Table 1). qMSP was performed by real-time polymerase chain reaction (PCR) using SYBR Green I (BioWhittaker Molecular Applications, Rockland, ME, USA) and an iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA). The specific annealing temperature in the presence of SYBR Green I was redetermined using the fully methylated and unmethylated DNA (Supplementary Table 1). The number of molecules in a sample was determined by comparing its amplification with those of standard DNA containing exact numbers of molecules (10^1–10^6 molecules). Based on the numbers of M molecules and U molecules for a genomic region, a methylation level of the region was calculated as a fraction of M molecules in the total number of DNA molecules (number of M molecules + number of U molecules).

Quantitative Real-Time Reverse Transcription (RT)-PCR

Total RNA was isolated using a mirVana miRNA isolation Kit (Ambion). For quantitative RT-PCR, cDNA was synthesized from 10 ng of total RNA using TaqMan MicroRNA-specific primers and a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed using a 7300 real-time PCR System (Applied Biosystems). Expression levels of *miR-124a* were normalized to that of a small nuclear RNA *RNU6B* transcript.

Immunohistochemistry

mmunohistochemical analysis of CDK6 was performed with formalin-fixed, paraffin-embedded tissue sections. The sections were deparaffinized in xylene and rehydrated. Endogenous peroxidase activity was blocked for 10 min with 3 % H_2O_2. After washing with phosphate-buffered saline (PBS), the sections were treated with 5 % goat serum albumin (Dako, Tokyo, Japan) in PBS for 30 min. Then, the sections were allowed to react with goat anti-CDK6 (SC-177; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h at room temperature. The antibody was diluted to 1:40. The sections were washed, incubated with the secondary antibody (DC Envision + System HRP, Dako) for 30 min and washed in PBS. The immunoreactive products were visualized using 3,3′-diaminobenzidine (Dojindo, Kumamoto, Japan). All sections were processed under the same conditions. Epithelial cells that showed brown staining in the nucleus or cytoplasm were considered CDK6-positive.

Statistical Analysis

Differences in methylation levels of *miR-124a* among colorectal segments were analyzed by the Kruskal–Wallis test, and differences in mean methylation levels of *miR-124a* in colorectal mucosa were analyzed by Welch's *t* test. Correlations between disease duration and methylation levels and between methylation levels of each of the *miR-124a* genes were analyzed by Spearman's rank correlation coefficient. All analyses were performed using JMP software (SAS Institute Inc., Cary, NC, USA), and the results were considered significant at two-sided *p* values of <0.05.

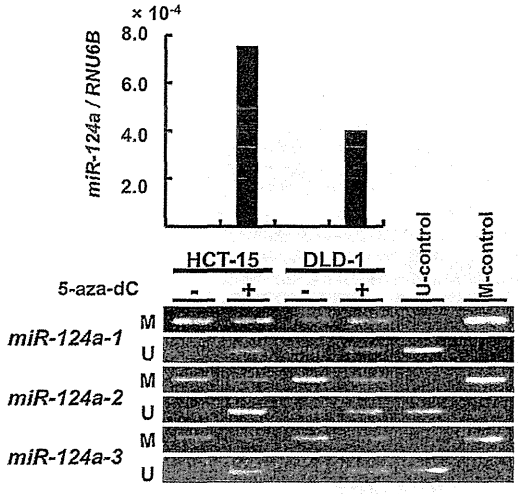

Fig. 1 Expression levels and methylation statuses of *miR-124a* in colon cancer cell lines. Expression levels of *miR-124a* were analyzed by quantitative RT-PCR and normalized to *RNU6B* expression. Methylation statuses of *miR-124a-1*, *-2*, and *-3* were analyzed by MSP. M-control, genomic DNA treated with SssI methylase; U-control, DNA amplified with GenomiPhi; and M and U, primer sets specific to methylated and unmethylated DNA, respectively. After treatment with 5-aza-dC, *miR-124a* was re-expressed in HCT-15 in association with demethylation, and *miR-124a* expression was restored with an increase in unmethylated DNA molecules in DLD-1

Results

Epigenetic Inactivation of *miR-124a* in Colon Cancer Cell Lines

To assess the role of DNA methylation in the silencing of *miR-124a*, we analyzed the methylation status and the expression in colon cancer cell lines (Fig.). It was found that *miR-124a-1*, *miR-124a-2*, and *miR-124a-3* were completely methylated in HCT-15 and partially methylated in DLD-1. In HCT-15, *miR-124a* was consistently unexpressed, with simultaneous methylation of its three *miR-124a* isoforms. Re-expression of *miR-124a* and the appearance of unmethylated *miR-124a-1*, *miR-124a-2*, and *miR-124a-3* were observed after treatment with a demethylating agent, 5-aza-dC. In DLD-1, *miR-124a* expression was also restored in association with an increase in unmethylated *miR-124a-1*, *miR-124a-2*, and *miR-124a-3* after treatment with 5-aza-dC. These data showed that *miR-124a* was inactivated by DNA methylation in colon cancer cell lines.

Presence of *miR-124a* Methylation in CAC and Dysplasia Tissues

We next analyzed the methylation status of *miR-124a* in CAC, dysplasia, and S-CRC tissues (Fig. a). All three *miR-124a* isoforms were methylated in all CAC, dysplasia, and S-CRC tissues, and *miR-124a-1* and *miR-124a-3* were completely unmethylated in normal rectal mucosa.

We further examined CDK6 protein expression in these tissues (Fig. b). CDK6 was highly expressed in all CAC, dysplasia, and S-CRC cells with methylation of all three *miR-124a* isoforms and was not expressed in normal rectal epithelial cells. These results showed that CDK6 is one of the targets of *miR-124a* and that epigenetic inactivation of *miR-124a* results in CDK6 up-regulation.

Methylation Levels of *miR-124a-1* and *miR-124a-3* in Colorectal Mucosa of UC Patients with Pancolitis Along Colorectal Segments

Since *miR-124a* was methylated in CAC and dysplasia tissues, we further analyzed the methylation levels of *miR-124a-1* and *miR-124a-3* in colorectal mucosa of UC patients with pancolitis. It was not known whether the methylation level of *miR-124a* differs according to site of the colon, although inflammatory changes were extensively present in all parts of the colorectal mucosa in UC patients with pancolitis. Therefore, methylation levels were quantified in each segments of the colorectum of UC patients with pancolitis (Table). Interestingly, aberrant methylation of *miR-124a-1* and *miR-124a-3* was present in various percentages of all segments; however, mean methylation levels did not differ significantly among segments (*miR-124a-1*, $p = 0.45$; *miR-124a-3*, $p = 0.71$). When correlations between the two *miR-124a* genes were examined by correlation coefficients, strong correlations were confirmed ($r = 0.66$, $p < 0.01$, data not shown).

Methylation Levels of *miR-124a-3* in Rectal Mucosa of UC Patients and Association with the Risk of CAC Development

Since the methylation level of *miR-124a-3* correlated with that of *miR-124a-1*, we analyzed the methylation levels of *miR-124a-3* in rectal mucosa from UC patients and HV and evaluated associations with two major risk factors for CAC. Regarding disease extent, the mean methylation levels were 2.0 % in HV, and 5.3 % and 12.3 % in UC patients with non-pancolitis and pancolitis, respectively (Fig. a). The mean methylation levels in patients with pancolitis were significantly higher than in HV ($p < 0.01$) and tended to be higher than in patients with non-pancolitis ($p = 0.06$).

Regarding disease duration, the mean methylation levels in short-term and long-standing groups were 2.5 and 13.2 %, respectively (Fig. b). The mean methylation levels were significantly higher in long-standing group than in HV ($p < 0.01$) and short-term group ($p < 0.01$). The

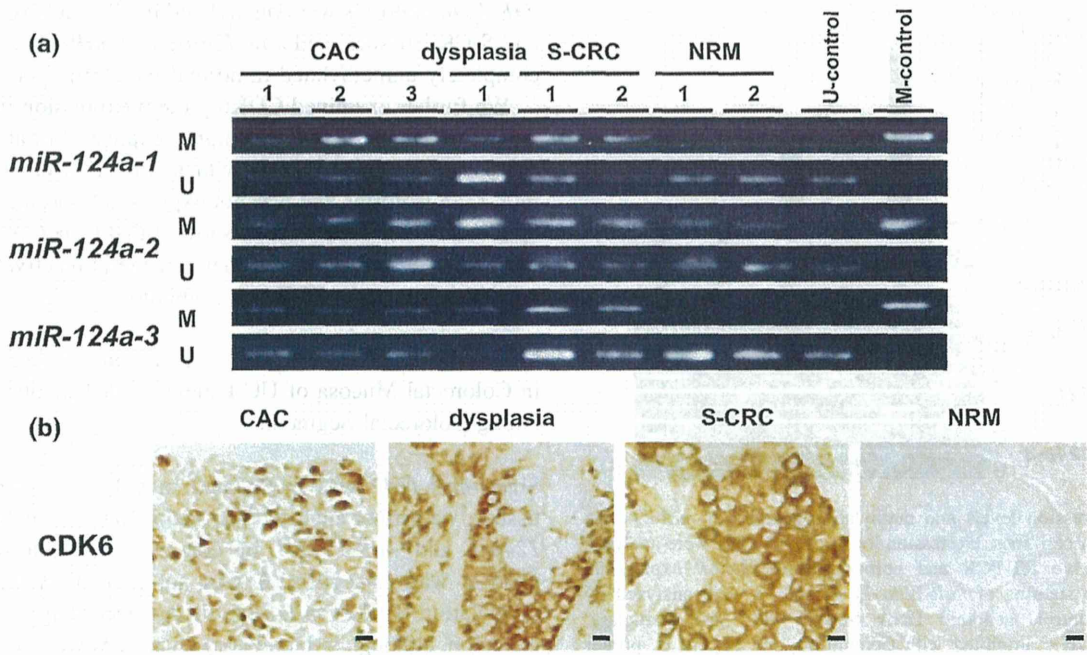

Fig. 2 Presence of *miR-124a* methylation in CAC and dysplasia tissues. **a** The methylation statuses of *miR-124a-1*, *-2*, and *-3* in colitis-associated cancer (*CAC*), dysplasia, sporadic colorectal cancer (*S-CRC*), and normal rectal mucosa (*NRM*) were analyzed by MSP. M-control, genomic DNA treated with SssI methylase; U-control, DNA amplified with GenomiPhi; and M and U, primer sets specific to methylated and unmethylated DNA, respectively. **b** Representative immunohistochemical staining of cyclin-dependent kinase 6 (CDK6) in CAC, dysplasia, S-CRC tissues, and NRM. Magnification of each figure is ×200. The *scale bars* indicate 100 μm

Table 2 Methylation levels of *miR-124a-1* and *miR-124a-3* in colorectal mucosa of 14 patients with pancolitis along colorectal segments

	Rectum	Sigmoid colon	Descending colon	Transverse colon	Ascending colon	Cecum	*p* value
miR-124a-1 (%: mean ± SD)	5.1 ± 7.2	5.0 ± 9.8	1.9 ± 2.0	4.9 ± 8.8	2.7 ± 3.7	2.4 ± 1.7	0.45
miR-124a-3 (%: mean ± SD)	14.1 ± 15.0	12.0 ± 18.0	10.3 ± 9.7	7.8 ± 8.3	4.4 ± 3.3	6.4 ± 6.8	0.71

p values were analyzed by Kruskal–Wallis test

SD standard deviation

methylation levels of *miR-124a-3* correlated with disease duration in all UC patients ($r = 0.50$, $p < 0.01$) (Fig. 3c). The associations between methylation levels of *miR-124a-3* and epidemiological risk factors (extent and duration of disease) were analyzed (Table 3). UC patients with both pancolitis and long-standing duration had 7.4-fold higher methylation levels than patients without these factors. Interestingly, one patient with a very high methylation level was diagnosed with high-grade dysplasia at 14 months during follow-up after methylation analysis (arrowed case in Fig. 3c).

Finally, we evaluated the methylation status of the representative protein-coding genes *p16* and *ER* in the rectal mucosa of UC patients (Fig. 3d). *P16* was methylated in none (0 %) of the patients, and *ER* was methylated in 33 (82.5 %) of 40 patients. In our quantitative assay, Patients with high methylation levels of *miR-124a-3* were completely included in patients with methylation of *ER*.

Discussion

The present study demonstrated that DNA methylation of *miR-124a* genes (*miR-124a-1*, *-2*, and *-3*) was induced in CAC and dysplasia tissues, resulting in expression of CDK6. Moreover, significantly higher methylation levels of *miR-124a-3* were present in the rectal mucosa of UC patients with pancolitis and long-standing duration that were major epidemiological risk factors for CAC development. To our knowledge, this is the first study to demonstrate that quantitative methylation levels of microRNA genes correlate with risk factors for CAC development and

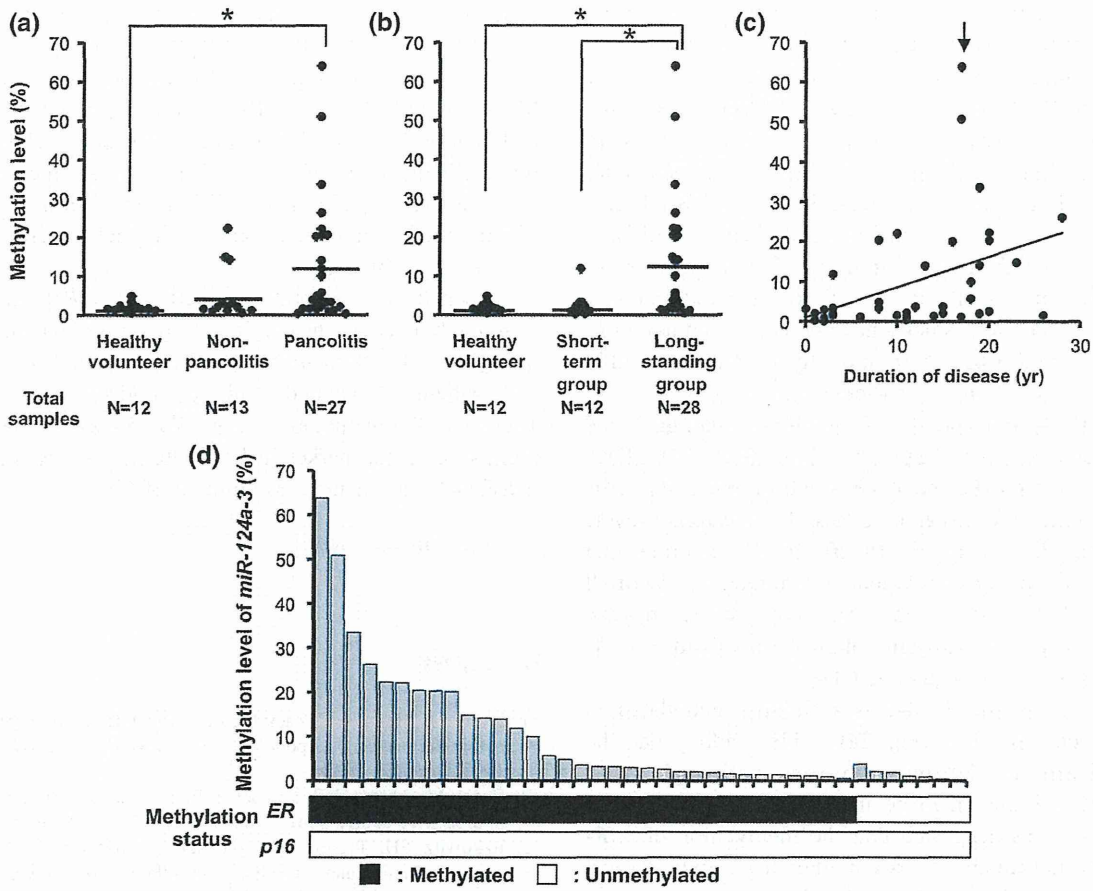

Fig. 3 Association between methylation levels of *miR-124a-3* and risk factors in UC patients. **a** Methylation levels of *miR-124a-3* in rectal mucosa of healthy volunteers (HV) and UC patients according to disease extent. **b** Methylation levels of *miR-124a-3* in rectal mucosa of HV and UC patients according to disease duration. The *horizontal lines* represent the mean methylation level for each group. The mean methylation level was significantly higher in patients with pancolitis than in HV (*t* test; $*p < 0.01$). The mean methylation level was significantly higher in patients with long-standing group than in HV and patients with short-term groups (*t* test; $*p < 0.01$). **c** Correlation between methylation levels of *miR-124a-3* and disease duration (Spearman's rank correlation coefficient, $r = 0.50$, $p < 0.01$). *Arrow* indicates the patient who was given a diagnosis of dysplasia during follow-up. **d** Association between methylation levels of *miR-124a-3* and methylation statuses of *p16* and *ER* in UC patients. Methylation status was analyzed by MSP. Methylated genes; *closed boxes* and unmethylated genes; *open boxes*

Table 3 Association between methylation levels of *miR-124a-3* and risk factors in patients with UC

Methylation level of *miR-124a-3* (%) (mean ± SD)	Extent of disease	
	Non-pancolitis	Pancolitis
Duration of disease		
<8 years	2.1 ± 0.9	2.8 ± 4.1
	$n = 5$	$n = 7$
≥8 years	7.3 ± 8.5	15.6 ± 17.4*
	$n = 8$	$n = 20$

p values were analyzed by *t* test

SD standard deviation

* $p < 0.01$ as compared with methylation levels of *miR-124a-3* in healthy volunteers (2.0 ± 1.1 %)

that they are potential markers for estimating individual risk for CAC development.

The degree of aberrant DNA methylation accumulated in non-cancerous tissues has been correlated with risk for gastric and liver cancer [24–26]. From the standpoint of inflammation-associated cancers, we quantified methylation levels of *miR-124a-3* in the rectal mucosa of UC patients, and patients with high methylation levels were found to have two major epidemiological risk factors and methylation of *ER*. There were various degrees of *miR-124a-3* methylation even in these UC patients with higher risk of CAC, and one patient with a very high methylation level, pancolitis, and long-standing disease was diagnosed with high-grade dysplasia during follow-up. These findings suggest that the risk of CAC development differs