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Original Article

Hypermethylation of miR-34b/c is associated with early clinical stages and tumor differentiation in Kazakh patients with esophageal squamous cell carcinoma

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Abstract: DNA hypermethylation in tumor suppressor genes has been reported in some cancers. The microRNA-34b/c (miR-34b/c) serves as tumor suppressors in different tumor types. To investigate the methylation status of miR-34b/c in ESCC, MALDI-TOF MS was used to quantitatively analyze the DNA methylation of 16 CpG sites within miR-34b/c in 145 ESCC samples, 60 cancer-adjacent normal (ACN) samples and 39 normal esophageal (NE) samples from the Kazakh population. Our results showed that the overall average methylation levels of miR-34b/c were significantly higher in the ESCC samples than they were in the ACN and NE samples ($P < 0.05$). Furthermore, the methylation levels of CpG_1.2.3, CpG_9.10, CpG_11.12.13, CpG_14, and CpG_15.16 of miR-34b/c were significantly higher in the ESCC tissues than they were in the ACN ($P < 0.05$) and NE tissues ($P < 0.05$). Additionally, the mean methylation levels at CpG_9.10 and CpG_14 were all significantly higher in the ACN samples than they were in the NE samples ($P < 0.01$). Increased methylation levels of CpG_9.10 and CpG_11.12.13 in miR-34b/c predominantly occurred in the early stages (UICC I/II) of ESCC ($P < 0.05$), and the methylation differences (moderately-poorly differentiated > well differentiated) in miR-34b/c CpG_1.2.3 were significant ($P < 0.05$). This is the first study reporting that the hypermethylation of miR-34b/c plays an important role in ESCC and is significantly correlated with the early stages and tumor differentiation of ESCC. The hypermethylation of miR-34b/c may promote the oncogenesis and progression of ESCC, and these findings may provide support for the future development of targeted therapies.

Keywords: miR-34b/c, hypermethylation, esophageal squamous cell carcinoma, MALDI-TOF MS

Introduction

Esophageal carcinoma (EC) is considered the sixth most common cause of cancer-related deaths [1]. China has a high incidence of EC with a high mortality rate. The Kazakh national (ethnic) minority living in Xinjiang (northwest of China) has been reported to be one of the ethnicities with the highest incidence and mortality of ESCC familial aggregation [2-4]. Epidemiological and etiological studies have shown that environmental and genetic factors greatly contribute to esophageal carcinogenesis [5]. DNA methylation is the main pattern of epigenetic modification. Highly methylated CpG islands of a DNA promoter can restrain the relevant transcription of target genes, especially antioncogenes. Interestingly, CpG methylation seems to occur early during tumor progression, and its detection may therefore be applied to tumor diagnosis and prognosis in the future [6].

Recent studies have shown that the expression of microRNA-like protein-coding genes is affected by epigenetic mechanisms such as DNA methylation. MicroRNAs (miRNAs) are a class of 21-25-nt single-stranded non-coding RNAs that can posttranscriptionally regulate gene expression mainly by completely or incompletely binding to the 3'-UTR of mRNA targets [7]. Among the various miRNAs, the miR-34a and miR-34b/c genes represent direct p53 targets and presumably mediate multiple tumor suppressive effects of p53 [8]. Furthermore, they may represent tumor suppressor genes as they are commonly silenced by CpG methylation in tumors [8-10].

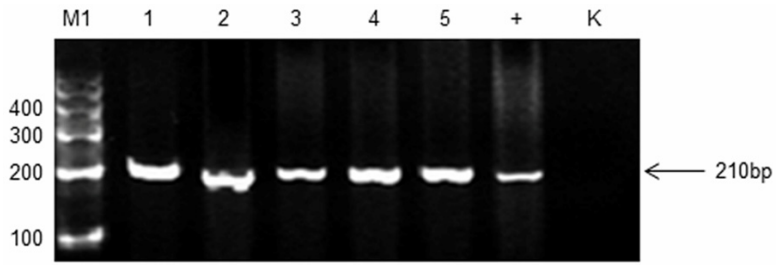

Figure 1. DNA quality evaluation. M1: DNA marker; 1-5: β-globin target fragment samples (210 bp); +: β-globin positive control; k: blank control.

We previously found that hypermethylation of miR-34a in Kazakh ESCC is associated with the downregulation of miR-34a expression and lymph node metastasis [11]. The miR-34b/c gene, which resides in chromosome 11q23.1 and belongs to the miR-34 family, is reportedly a tumor suppressor gene [12]. A growing number of studies support the theory that the abnormal expression of miR-34b/c by promoter hypermethylation may contribute to the development of tumors, such as lung cancer [13, 14], ovarian cancers [15], and prostate cancer [16]. We have also found that the hypermethylation of miR-34b/c is correlated with the late clinical stage in patients with soft tissue sarcomas [17]. However, only one study has reported that the hypermethylation ratio of miR-34b/c in ESCC is significantly higher than it is in normal tissues [18]. The hypermethylation level of the miR-34b/c individual CpG unit and the exact function of hypermethylation of miR-34b/c in ESCC remain unclear.

Here, we aimed to identify whether the hypermethylation of miR-34b/c preferentially occurs in Kazakh ESCC patients and correlates with the oncogenesis and progression of ESCC. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) was performed to address this question, and the methylation level of individual CpG units in 281 base pair regions containing 22 CpG sites within 8 CpG units at the miR-34b/c promoter regions with a total of 244 Kazakh samples was quantitatively evaluated.

Materials and methods

Patients and tissue samples

A total of 145 ESCC tissues from Kazakh patients were randomly collected by multistage cluster sampling. These patients did not receive radiotherapy or chemotherapy before surgery. Additionally, 60 ACN samples and 39 NE samples were collected as controls. All patients were recruited from the First Affiliated Hospital of Shihezi University and the People's Hospital of Xinjiang Uygur Autonomous Region from 1984 to 2010. All ESCC specimens obtained after surgery were embedded in paraffin, and subsequently sectioned into 5-mm slices. The diagnosis of ESCC was confirmed by 2 pathologists according to the WHO histological tumor classification criteria; 33 cases of well-differentiated ESCC and 112 cases of poorly-differentiated ESCC were noted. The ACN samples, which were sampled from more than 5 cm away from the cancer region, were confirmed to be free of cancer tissue. Differentiation grade, TNM stage, and lymph node status were determined in accordance with the UICC/AJCC TNM classification (Seventh Edition).

DNA isolation and bisulfate conversion

Genomic DNA was respectively extracted from 5-10-μm-thick tissue sections by proteinase K digestion and a tissue DNA extraction kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's protocol. All purified genomic DNA was successfully amplified by PCR with human β-actin primers, For: 5'-CAGACACCATGGTGCACCTGAC-3' and Rev: 5'-CCAATAGGCAGAGAGTCAGTG-3', indicating that the DNA was of a quality and quantity suitable for use in detecting the profile of miR-34b/c methylation (**Figure 1**). Genomic DNA was treated with bisulfite through an EZ DNA Methylation KitTM according to the manufacturer's guidelines (Zymo Research, Orange, CA, USA).

Quantitative analysis of DNA methylation

The sequence of the CpG island was identified by the UCSC genome browser (http://genome.ucsc.edu/) (chr11:110888379-110889102, **Figure 2**). We designed primer sets for the methylation analysis of the miR-34b/c promoter region using EpiDesigner software (http://epidesigner.com). For the PCR reaction, each reverse primer had a T7 promoter tag (31 bp)

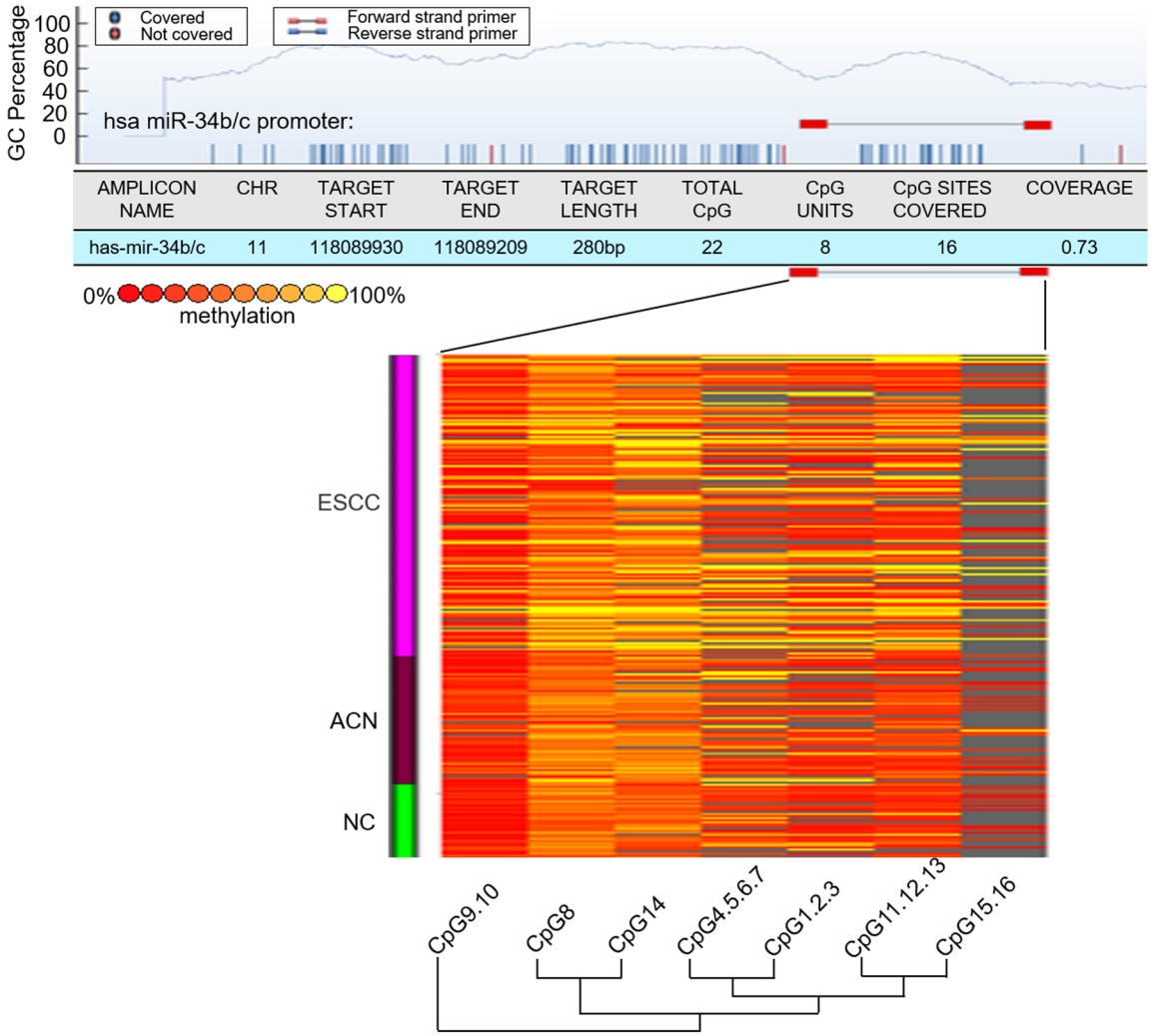

Figure 2. Genomic structure of the distribution of miR-34b/c CpG dinucleotides and hierarchical cluster analysis of CpG units' methylation profiles of the miR-34b/c promoter region in esophageal squamous cell carcinoma (ESCC, n = 145), cancer-adjacent normal samples (ACN, n = 60), and normal esophageal samples (NE, n = 39). The red-filled box coverage area indicated the amplified region of miR-34b/c CpG island-specific primers. Each vertex indicates an individual CpG site. Columns display the clustering of CpG units, which are a single CpG site or a combination of CpG sites. Each row represents a sample. The color gradient between red and yellow indicates methylation of each miR-34b/c CpG unit in each sample ranging from 0% to 100%. Gray represents technically inadequate or missing data.

and the forward primer contained a 10-bp tag to adjust for differences in melting temperature (**Table 1**). DNA methylation of miR-34b/c was quantitatively analyzed by the MassARRAY platform (SEQUENOM) as previously described [11, 19].

MALDI-TOF-MS measurements

The MALDI-TOF MS (Sequenom, San Diego, USA) is an instrument designed for high-throughput detection and quantitative analysis of methylation at a single CpG site in a target fragment (CpG island). Mass spectra were recorded using MALDI-TOF MS and the methylation data of individual units were generated by EpiTyper v1.0.5 software (Sequenom).

Analysis of HPV16 infection by semi-nested PCR

A semi-nested PCR was conducted in 2 steps to detect HPV16E infection as previously described [20].

Statistical analysis

The Mann-Whitney U test and Kruskal-Wallis H test in 2 groups or more were performed to compare the miR-34b/c methylation levels

Table 1. Sequences of PCR primers used in this study

Gene Size (bp)	Primer	Product
β-globin	For: 5'-CAGACACCATGGTGCACCTGAC-3'	210
	Rev: 5'-CCAATAGGCAGAGAGAGTCAGTG-3'	
hsa-miR-34b/c	tag-FW: 5'-aggaagagagGTTATATGGATGGAGGGGTTTTAT-3'	321
	T7-RV: 5'cagtaatacgactcactatagggagaaggctACTACCATCAAAACAAAACACAACA-3'	

"FW": Forward, "RV": Reverse.

among the ESCC, ACN and NE samples, and clinicopathological parameters. All *P* values were two-sided, and the significance level was $P < 0.05$. Hierarchical clustering analysis was used to describe the methylation level of miR-34b/c CpG units among the ESCC, ACN and NE samples and to identify sites with a statistically significant difference by using Cluster 3.0 software and TreeView software. The statistical analyses were performed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA).

Results

Hypermethylation of the miR-34b/c promoter region in ESCC

We quantitatively analyzed the DNA methylation patterns of promoters within the miR-34b/c CpG island by using the MassARRAY system. This system was used to assess the methylation profile of miR-34b/c in all the specimens collected from the ESCC (n = 145), ACN (n = 60), and NE (n = 39) samples. The amplicon detected in the promoter regions of miR-34b/c was 280 base pairs in length and contained 22 CpG sites that could be divided into 8 CpG units. Among these CpG units, 1 CpG unit (CpG_Unit8) yielded unsuccessful measurements. The final dataset consisted of 7 CpG units (1510 sites in 244 analyzed samples, with a coverage rate of 88.4%), and the individual CpG unit methylation level of miR-34b/c that distinguished ESCC from ACN samples and NE samples is depicted in the cluster diagram (**Figure 2**). The CpG methylation levels of the samples could be identified based on color (which varied from red to yellow, indicating a methylation range of 0% to 100%, respectively) for each miR-34b/c CpG unit in each sample. The patterns observed in the cluster analyses show that the methylation status of the ESCC tissues was notably different from those of the ACN and NE samples.

We analyzed the methylation level of each CpG unit in the miR-34b/c promoter by GraphPad Prism 5 (**Figure 3**). The results showed that ACN samples and NE samples are characterized by consistent low methylation levels, and that ESCC samples display more variable methylation patterns. We further evaluated whether the methylation level of individual CpG islands of miR-34b/c differed among the ESCC, ACN and NE samples (**Figure 4**). We found that 5 CpG units (except for CpG_4.5.6.7 and CpG_8) were more highly methylated in ESCC samples compared to ACN and NE samples. Using a nonparametric test (**Table 2**), we found that apart from CpG_4.5.6.7 and CpG_8, the mean methylation levels at CpG_1.2.3, CpG_9.10, CpG_11.12.13, CpG_14, and CpG_15.16 were all significantly higher in patients with ESCC (mean methylation = 26.12%, 17.52%, 26.12%, 32.52%, and 28.33%, respectively) than those in the ACN samples (mean methylation = 18.76%, 11.02%, 15.53%, 29.35%, and 13.60%, respectively) and NE samples (mean methylation = 15.48%, 6.07%, 13.33%, 21.55%, and 6.82%, respectively; all *P* values less than 0.01). Additionally, the mean methylation levels at CpG_9.10 and CpG_14 were all significantly higher in the ACN samples than those in the NE samples ($P < 0.01$). However, no statistical differences were observed in the methylation patterns between the ESCC and ACN samples in the miR-34b/c promoter ($P > 0.05$).

Hypermethylated miR-34b/c in ESCC is correlated with the early stage and tumor differentiation of ESCC

The relationship between the quantitative methylation patterns of every CpG unit within the miR-34b/c promoter and the clinical-pathologic features of the 145 Kazakh patients with ESCC was further evaluated (**Table 3**). The CpG_9.10 and CpG_11.12.13 methylation lev-

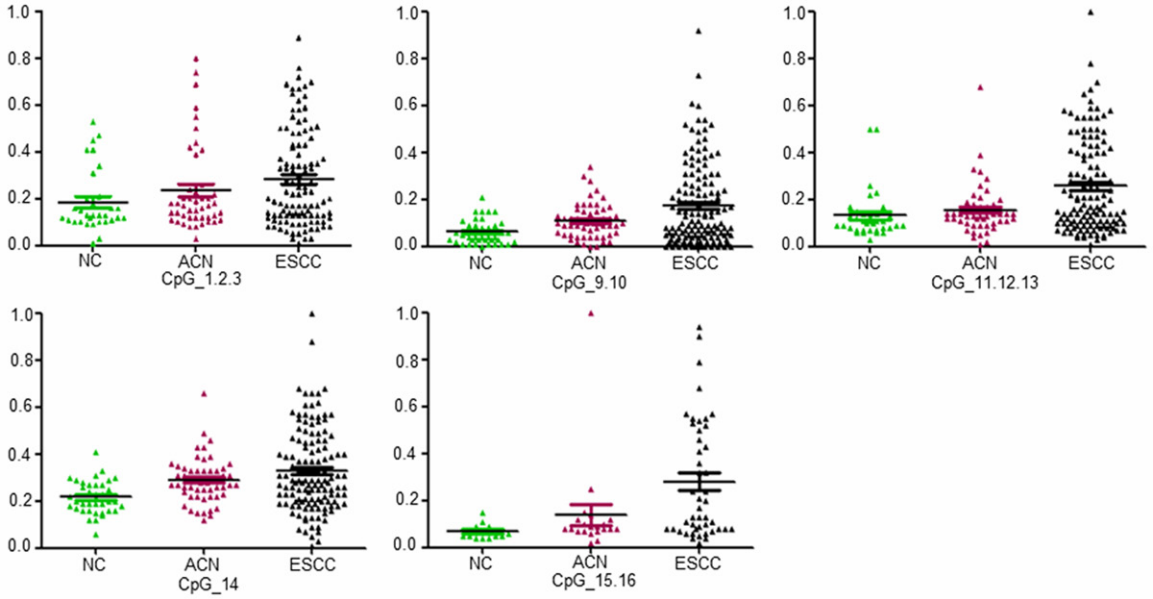

Figure 3. Analysis of the significantly differentially methylated CpG units of miR-34b/c. The average methylation levels of 5 CpG units in miR-34b/c promoter are increased in ESCC compared to those in ACN samples and NE samples. Green represents the CpG methylation rate distribution of NE, red represents the CpG methylation rate distribution of ACN samples, and black represents the CpG methylation rate distribution of ESCC. The middle line represents the average rate of methylation, and the horizontal lines on both sides of the middle represent the standard error of the average methylation rate.

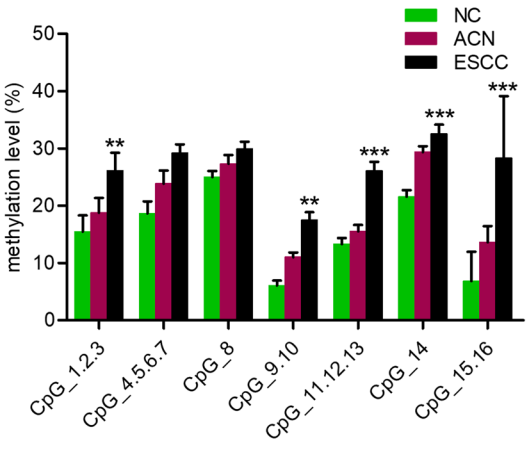

Figure 4. Evaluation of CpG methylation in the miR-34b/c promoter (Kruskal-Wallis H test). The median methylation levels of 16 informative CpG units of the miR-34b/c promotor are increased in ESCC compared to those in ACN samples and NE samples. $^{**}P < 0.01$, $^{***}P < 0.001$.

els of miR-34b/c in tumor stage I/II tissues were significantly higher than those in stage III/IV tissues (18.71% vs. 13.77%, $P = 0.049$; 28.2% vs. 19.08%, $P = 0.008$, respectively; two-tailed Mann-Whitney U-test, **Figure 5**). The results also showed that the CpG_1.2.3 methylation levels of miR-34b/c correlated with pathological grade given that the methylation levels in poorly-differentiated tumor tissues were markedly greater than those in well-differentiated tumor tissues (27.8% vs. 14.15%, $P = 0.003$; two-tailed Mann-Whitney U-test, **Figure 5**). However, the CpG unit methylation of miR-34b/c promoter showed no association with gender, age, tumor location or gross pathologic classification in ESCC.

Although the average methylation rates of CpG_1.2.3, CpG_9.10, CpG_11.12.13, CpG_14, and CpG_15.16 in miR-34b/c in lymph node metastasis tumor tissues were greater than those in tumor tissue without lymph node metastasis, the difference was not statistically significant (**Table 4**). Considering that our previous reports exhibited a strong positive association between HPV16 infection and ESCC in Kazakh patients [20], we explored the relationship between HPV16 infection and miR-34b/c methylation in ESCC (47 cases in the positive infection group and 98 cases in the negative infection group). The data revealed that the methylation levels of CpG_1.2.3, CpG_9.10, CpG_11.12.13, and CpG_15.16 were higher in ESCC with HPV16 infection than those in ESCC without HPV16 infection, but the difference was not statistically significant (**Table 5**).

Table 2. Comparison of the methylation rate of miR-34b/c gene CpG island in Kazakh ESCC, ACN samples and NE samples

CpG Unit	CpG Site	NE/ACN/ESCC[a]		NE/ESCC[b]		NE/ACN[b]		ESCC/ACN[b]	
		χ^2	*P*	Z	*P*	Z	*P*	Z	*P*
Unit1	CpG_1.2.3	10.650	0.006*	-2.916	0.004*	-2.215	0.027	-1.578	0.115
Unit2	CpG_4.5.6.7	6.148	0.058	-2.296	0.022	-1.379	0.168	-1.061	0.289
Unit3	CpG_8	1.320	0.517	-0.935	0.350	-1.158	0.247	-0.259	0.796
Unit4	CpG_9.10	15.026	0.001*	-3.476	0.001*	-3.525	0.000***	-0.948	0.343
Unit5	CpG_11.12.13	16.374	0.000***	-3.501	0.000***	-2.161	0.031	-2.470	0.014
Unit6	CpG_14	16.959	0.000***	-3.882	0.000***	-3.810	0.000***	-0.791	0.429
Unit7	CpG_15.16	15.294	0.000***	-3.526	0.000***	-2.065	0.039	-2.263	0.024

Note: [a]Kruskal-Wallis H test (two-sided) and [b]Mann-Whitney U test (two-sided). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Table 3. Correlations of miR-34b/c promoter methylation and various clinicopathological parameters in ESCC patients

CpG site	Clinical parameters													
	Gender (Z/P)[a]		Age (Z/P)[a]		Tumor location (x^2/P)[b]		Tumor gross type (x^2/P)[b]		Differentiation (Z/P)[a]		Lymphatic metastasis (Z/P)[a]		TNM stage (Z/P)[a]	
CpG_1.2.3	-0.144	0.886	-0.554	-3.016	0.873	0.646	3.200	0.202	-3.016	0.003**	-0.837	0.402	-0.644	1.000
CpG_9.10	-0.680	0.497	-0.228	-1.565	4.178	0.124	0.206	0.902	-1.565	0.117	-1.293	0.196	-1.969	0.049*
CpG_11.12.13	-1.708	0.088	-0.005	-1.105	3.326	0.190	4.291	0.117	-1.105	0.269	-1.783	0.075	-2.641	0.008**
CpG_14	-0.237	0.813	-0.977	-1.715	2.154	0.341	0.015	0.992	-1.715	0.086	-1.235	0.217	-0.953	0.341
CpG_15.16	-0.552	0.581	-1.287	-0.880	1.349	0.509	3.489	0.175	-0.880	0.379	-0.304	0.761	-0.672	0.501

Note: [a]Mann-Whitney U test (two-sided) and [b]Kruskal-Wallis H test (two-sided). *$P < 0.05$, **$P < 0.01$.

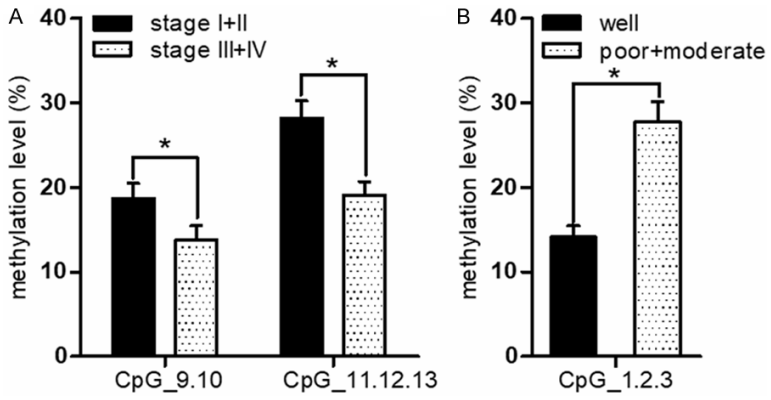

Figure 5. Hypermethylation of miR-34b/c is associated with TNM stage and tumor differentiation in ESCC (Mann-Whitney U-test). A. TNM stage. B. Tumor differentiation. *$P < 0.05$.

Discussion

This is the first study that quantitatively measured the DNA methylation levels of miR-34b/c CpG islands in ESCC, ACN and NC samples from Kazakh individuals using MALDI-TOF-MS. Five significant methylation-specific CpG units were screened, and the methylation status of the ESCC tissues was notably different from those of the ACN and NE samples. Additionally, the mean methylation level was also significantly higher in the ACN samples than those in NE samples. Moreover, the hypermethylation of miR-34b/c was associated with various clinicopathological parameters, highlighting the importance of miR-34b/c in the development of ESCC. What's more, the results are consistent with the tendency for methylation of the miR-34b/c gene found in other epithelial-related tumors (colorectal, breast and ovarian) [21-23].

MiRNAs are important posttranscriptional regulators of proteins in a sequence-specific manner. Studies with large sample sizes have shown that the epigenetic inactivation of miR-34b/c by DNA hypermethylation can be found in some cancers and has been confirmed to be related to tumorigenesis and pathogenesis [10]. However, only one study has shown that the hypermethylation ratio of miR-34b/c in ESCC is higher than the ratio in corresponding

Table 4. Relationship between miR-34b/c methylation and lymphatic metastasis in ESCC patients

CpG Site	With lymphatic metastasis (57 cases)		Without lymphatic metastasis (88 cases)		Z	*P*
	N	$\bar{X} \pm S$	N	$\bar{X} \pm S$		
CpG_1.2.3	46	0.2259 ± 0.2107	74	0.2605 ± 0.2304	-0.837	0.402
CpG_9.10	53	0.1487 ± 0.1605	85	0.1884 ± 0.1856	-1.293	0.196
CpG_11.12.13	47	0.2209 ± 0.1830	80	0.2769 ± 0.2044	-1.783	0.075
CpG_14	51	0.3031 ± 0.1625	8	0.3460 ± 0.1804	-1.235	0.217
CpG_15.16	16	0.2569 ± 0.2069	31	0.2913 ± 0.2741	-0.304	0.761

Note: Mann-Whitney U test; $^{*}P < 0.05$.

Table 5. Correlations between miR-34b/c methylation and HPV16 infection in ESCC

CpG Site	HPV 16 + (47 cases)		HPV 16 - (98 cases)		Z	*P*
	N	$\bar{X} \pm S$	N	$\bar{X} \pm S$		
CpG_1.2.3	41	0.2059 ± 0.1755	79	0.2687 ± 0.2421	-0.537	0.591
CpG_9.10	44	0.1664 ± 0.1624	94	0.1763 ± 0.1839	-0.080	0.936
CpG_11.12.13	42	0.2400 ± 0.1819	85	0.2641 ± 0.2059	-0.315	0.753
CpG_14	43	0.3530 ± 0.1935	91	0.3187 ± 0.1646	-0.687	0.492
CpG_15.16	18	0.2594 ± 0.1926	28	0.2921 ± 0.2849	-0.451	0.652

Note: Mann-Whitney U test (two-sided); N stands for the number of analyzed CpG units.

normal tissues [18]. Our results showed that the overall average methylation levels of miR-34b/c were higher in the ESCC samples compared with the ACN and NE samples. Furthermore, the methylation levels of CpG_1.2.3, CpG_9.10, CpG_11.12.13, CpG_14, and CpG_15.16 of miR-34b/c were remarkably higher in the ESCC tissues than those in ACN and NE tissues. Additionally, the mean methylation levels at CpG_9.10 and CpG_14 were all significantly higher in the ACN samples than they were in the NE samples.

Then we investigated possible associations between the hypermethylation of miR-34b/c and the clinical characteristics in ESCC. We first found that the average hypermethylation rates of CpG9.10 and CpG_11.12.13 in miR-34b/c were particularly marked in early-stage ESCC tissues, indicating that DNA methylation of miR-34b/c within CpG9.10 and CpG_11.12.13 may be an important diagnostic marker for early-stage ESCC. The methylation rate of miR-34b/c CpG_1.2.3 was also significantly correlated with moderately-poorly-differentiated ESCC, indicating that DNA methylation of miR-34b/c within CpG_1.2.3 may be an important prognostic factor for this subtype of ESCC. Besides, we need to focus further on the correlation studies of the methylation level of miR-34b/c on survival and response to therapies.

Some studies have demonstrated that miR-34b/c methylation is related to metastasis in some tumors [22], but we failed to find a strong association between hypermethylated miR-34b/c and metastasis. In addition, no significant correlations were found between the methylation status of miR-34b/c and gender, age, or tumor location, but further studies with larger samples should be conducted to confirm our observations. Previous studies have investigated the combined effects of DNA methylation and HPV infection on the risk of ESCC. MiR-203 reportedly influences HPV-encoded epitopes and affects the risk of ESCC among Kazakhs in Xinjiang, China [20]. Our study found no connection between miR-34b/c methylation and HPV infection, indicating that HPV infection and miR-34b/c methylation may function independently to influence the risk of ESCC in the Kazakh population.

DNA hypermethylation of CpG sites in promoters plays an important role in epigenetic modification and regulation, which subsequently results in the silenced expression of cancer-related genes and the inhibition of transcription. Our previous research has confirmed that miR-34a inactivation and the epigenetic silencing of miR-203 are correlated with aberrant CpG methylation in Kazakh patients with ESCC [11, 20]. Additionally, other studies have shown that the hypermethylation of miR-34b/c contributes to the downregulation of protein expression in lung adenocarcinoma [14], colon cancer [24], chronic lymphocytic leukemia [25], and malignant pleural mesothelioma [26]. In

our study, abnormal miR-34b/c methylation occurred in ESCC, and we need further studies to clarify whether the abnormal methylation of miR-34b/c influences the expression level of miR-34b/c in ESCC.

According to previous data, the palindromic sequence in the promoter regions of miR-34b/c match the canonical p53-binding site, so miR-34b/c expression may be induced by P53 in response to DNA damage or cell stress [27]. Additionally, compared with the normal controls, miR-34b/c hypermethylation is significantly higher in colorectal cancer and cell lines, and the silencing of miR-34b/c gene expression by DNA hypermethylation may further lead to the upregulation of a series of downstream target genes (MET, CDK4, SFRS2, C-MYC, CDK6, E2F3, and TGIF2) in the development of colorectal cancer [28]. Thus, whether a relationship exists between p53 mutations and miR-34b/c hypermethylation in ESCC should be considered. Besides, future mechanistic studies are needed to show the downstream targets of miR-34b/c in the regulation of tumor growth in ESCC.

Conclusion

Our study showed that the overall average methylation levels of miR-34b/c were significantly higher in ESCC than those in ACN and NE, and the hypermethylation of miR-34b/c is associated with the early clinical stage and poorly tumor differentiation in ESCC in Kazakh patients. Importantly, these findings showed that hypermethylated miR-34b/c is a potential biomarker of ESCC carcinogenesis and that demethylation treatment of miR-34b/c may be an alternative approach for molecular-targeted prevention and therapy.

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Disclosure of conflict of interest

None.

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