Methylation profiles of *MIR34* gene family in Vietnamese patients suffering from breast and lung cancers

VO THI THUONG LAN[1], HO VAN SON[2], VU LAN TRANG[3], NGUYEN THU TRANG[1], NGUYEN THI PHUONG[1], NGUYEN LINH TOAN[4] and PHAM ANH THUY DUONG[5]

[1]Faculty of Biology, VNU University of Science, Hanoi; [2]Department of Pathophysiology, 175 Hospital, Ho Chi Minh, Vietnam; [3]Laboratoire des Biomolécules, Sorbonne Universités, Université Pierre et Marie Curie University Paris 06, École Normale Supérieure, Paris Sciences et Lettres Research University, Centre National de la Recherche Scientifique, Institut National de la Santé Et de la Recherche Médicale, Assistance Publique Hôpitaux de Paris, 75012 Paris, France; [4]Department of Pathophysiology, Medical University, Ha Dong; [5]Department of Gene Technology, Vinmec Research Institute of Stem Cell and Gene Technology, Hanoi, Vietnam

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Abstract. The three genes encoding small non-coding microRNA (*MIR)34a*, *MIR34b* and *MIR34c* act as tumor-suppressor genes. Their aberrant expressions regulated by DNA methylation have been frequently found in various types of cancer. In the present study, the DNA promoter methylation profiles of the *MIR34* gene family were analyzed using the methylation specific polymerase chain reaction in order to clarify their association with breast and lung cancer, non-cancerous or normal adjacent tissues. The methylation frequency of *MIR34a* was significantly higher in breast cancer (49.37%) compared with normal adjacent tissues (30.38%). The methylation frequency of *MIR34b/c* was 59.49 and 62.03% in breast cancer and normal adjacent tissues, respectively. *MIR34a* methylation showed a significant concordance with that of *MIR34b/c* only in breast cancer tissue. *MIR34a* methylation was significantly associated with cancer and the invasive ductal carcinoma type of breast cancer (P=0.015 and P=0.02, respectively). Methylation frequency of *MIR34a* and *MIR34b/c* was 48.42 and 56.84% in lung cancer, and 47.22 and 51.39% in pulmonary diseases, respectively. No significant association was observed between the methylation status of *MIR34a* and *MIR34b/c*, and the clinicopathological features of lung cancer or with those of non-cancerous pulmonary diseases. Promoter methylation of *MIR34a* and *MIR34b/c* occurs frequently and concomitantly in breast and lung cancer, as well as in pulmonary diseases tissues, but not in breast normal tissues adjacent to tumor. These results of the present study emphasize the involvement of *MIR34* methylation in human diseases, including cancer. Furthermore, *MIR34a* methylation may be a promising marker for a subtype of breast cancer.

Correspondence to: Professor Vo Thi Thuong Lan, Faculty of Biology, VNU University of Science, 334 Nguyen Trai, Hanoi, Vietnam
E-mail: vothithuonglan@hus.edu.vn

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Introduction

DNA methylation at cytosines in CpG islands located in promoters is well known as one of the earliest molecular alteration occurring during carcinogenesis and specific for the malignant state (1). Over the past few years, there have been increasing evidences asserting the role of DNA methylation at promoter of genes encoding small non coding microRNAs (*MIRs*) that act as posttranscriptional regulators of gene expression (2,3). Aberrant expression of microRNAs regulated by DNA methylation is involved in many cellular processes such as DNA repair, cell cycle, apoptosis, through which they promote cell differentiation, proliferation, malignant transformation and tumorigenesis (4,5). For example, the down-regulation of *MIR129-2* by DNA methylation regulates breast cancer cell proliferation and apoptosis (6). Moreover, *MIR449c* expression, which was significantly down-regulated by DNA methylation in osteosarcoma cancer, was negatively correlated with tumor size and tumor stages (7). In addition, integrating DNA methylation data with microRNA expression profile in various types of cancers including lung, colon and breast has been extensively explored by genome wide analysis recently (8,9).

Among the huge number of microRNA genes, the *MIR34* gene family, which consists of three genes *MIR34a*, *MIR34b* and *MIR34c,* has been the focus of numerous studies in cancer research. All the three *MIR34* genes are transcriptionally regulated by p53 protein, a regulator of cell cycle and apoptosis; therefore, they act as tumor suppressor genes by targeting many oncogenes related to proliferation, apoptosis and invasion (10,11). Numerous studies have demonstrated the direct link between dysregulations concerning *MIR34* family and epigenetic and genetic mechanisms in cancers.

For instance, *MIR34a* targets the proto-oncogene c-SRC to attenuate tumor growth in triple-negative breast cancer (12), or programmed death ligand 1 (PDL1) to modulate the tumor immune response in non small cell lung cancer (13). Stahlhut and Slack revealed that combinatorial action of *MIR34a* and microRNA let-7 effectively synergizes with erlotinib to suppress non-small cell lung cancer cell proliferation (14). *MIR34b* and *MIR34c*, which share a common primary transcript, function as metastasis suppressors in lung adenocarcinoma (15,16). A recent study on circulating *MIR34s* in 173 patients with triple-negative breast cancer indicated that *MIR34a, MIR34b* and *MIR34c* expression was respectively correlated with tumor grade (P=0.038), lymph node positivity (P=0.027) and distant metastasis (P<0.001) (17). Importantly, the aberrant low expression of *MIR34s* was associated with promoter methylation. Indeed, data integration from 104 studies on microRNAs revealed that *MIR34s* are silenced by DNA methylation in the highest number of cancer types (18). Epigenetic inactivation of the *MIR34a* was found in ovarian, colorectal, lung, kidney and breast cancer cell lines (19,20). The presence of *MIR34b/c* promoter methylation was significantly associated with the absence of its transcripts and with metastasis formation in primary tumors of colon, lung, head and neck, melanomas and breast cancers (21). Siemens *et al* (22) showed that *MIR34a* methylation is involved in the up-regulation of c-Met, Snail, and β-catenin proteins, which was associated with the metastasis distance of colon cancer cells to the liver. Patients after surgery of lung cancer with aberrant methylation of *MIR34b/c* had a high probability of recurrence and poor prognosis (P=0.026) (23). Furthermore, molecular events from negative surgical margin have been extremely investigated because of their predictive and prognostic values for tumor progression, local recurrence, metastasis and overall survival (24). Multi-platform analyses of DNA defects, epigenetics, and gene expression in cancer-adjacent tissues have extensively been performed recently to provide integrative data to the clinic (25,26). DNA methylation that has occurred at the primary tumor is believed to progressively spread outwards to surrounding tissues (27,28). Increased DNA methylation level in ductal carcinoma *in situ* is related with future development of invasive breast cancer and with the cancer metastasis distance (29,30). Currently, *MIR34* methylation profile in normal adjacent and tumor tissues, and its correlation with clinicopathological features have been frequently reported to gastrointestinal cancer. For instance, *MIR34* methylation occurs in colorectal normal adjacent (43.9%) and tumor tissues (79.3%) and correlated with positive lymph node (P=0.01) (31). Similarly, *MIR34b/c* methylation was considerably different between adjacent (22.7%) and tumors (70%) in gastric cancer (32). However, differences in *MIR34* methylation profile in either non-cancerous or normal adjacent tissues as compared with breast and lung cancer tissues have been rarely described so far while both types of these cancers are the first and second common types of cancer and leading cause of cancer death all over the world (33). Therefore, investigating the methylation profile of the *MIR34* genes in both types of cancer vs. normal adjacent tissues and non-cancerous tissues and moreover their association with the clinicopathological features would provide a comprehensive evaluation on the synergy of these potential methylation biomarkers for cancer diagnosis.

In this study, we investigated the methylation status at the promoter of the genes encoding *MIR34a/b/c* in breast cancer vs. normal adjacent tissues, as well as in non-cancerous lung diseases vs. lung cancer, with samples coming from Vietnamese patients. The objective is to evaluate the methylation profiles of these genes both individually and in an integrative manner in order to establish new integrative methylation biomarkers for cancer detection. Furthermore, the comparison of the methylation profiles of these genes in cancer vs. non-cancerous or normal adjacent tissues has highlighted the epigenetically concomitant changes of these genes in tissues that are physiologically different such as breast and lung.

Materials and methods

Sample collection. Surgically resected specimens from breast carcinomas and matched adjacent tissues were collected from 79 breast cancer patients having undergone mastectomy at the Department of Pathology, National Cancer Hospital K (Hanoi, Vietnam) between 2012 and 2013. The corresponding adjacent tissue samples were selected 3-5 cm away from the site at which the primary tumor was obtained. Breast tumor and corresponding adjacent tissues were snap-frozen in liquid nitrogen immediately after resection and examination by pathologists, and stored at -80˚C until further used. Formalin-fixed, paraffin-embedded (FFPE) tissue specimens were collected from 95 lung cancer patients and 72 patients suffering from non-cancerous lung diseases (whose classification was examined by pathologists) at the Department of Pathology, 175 Hospital (Ho Chi Minh, Vietnam) during 2016. Informed consent was obtained from patients in written form and the study was approved by the guidelines of the VNU University of Science ethical committee in Vietnam (106-YS.06-2015.07).

Genomic DNAs extraction and bisulfite modification. Genomic DNAs were extracted from freshly frozen breast or FFPE lung tissues by using the QIAampDNA Mini kit or QIAamp DNA FFPE Tissue kit (Qiagen, Inc., Valencia, CA, USA), and treated with sodium bisulfite by using the EpiTect Bisulfite kit (Qiagen, Inc.). During the treatment, the unmethylated cytosines of the genomic DNAs were converted into uracils, but the methylated cytosines remained unchanged (34). Polymerase chain reaction (PCR) realized on native DNA using primer sets specific to methylated sequences was performed to confirm the accuracy of the primer sets. PCR realized on the bisulfite-treated DNA using primer sets specific to unmethylated sequences of the *β-globin* gene was performed to determine the efficiency of bisulfite conversion (35).

Methylation specific PCR (MSP). The methylation status of the investigated genes was evaluated by using MSP to amplify bisulfite treated DNA with primers that distinguish methylated (M) and unmethylated (U) DNA (36). *MIR34a* gene locates on chromosome 1 and is transcribed from the minus strand. The *MIR34a* gene structure is described by Tarasov *et al* (37) and the CpG island in its promoter is indicated by Lodygin *et al* (19). *MIR34b/c* gene locates on chromosome 16 and is transcribed

Table I. MSP primers for analysis of *MIR34a*, *MIR34b/c* methylation.

Primers	Sequence (5'-3')	Amplicon size (bp)	MSP conditions
MIR34a EF570049.1			
miR-34a-meF	TTTTGGGTAGGCGCGTTTCGC	Round 1: 147	94˚C 5 min, 40 cycles of (94˚C 30 sec, 60˚C 10 sec, 72˚C 15 sec), 72˚C 5 min
miR-34a-meR	CCAATCCCGCCGAACACGAAA		
miR-34a-meF	TTTTGGGTAGGCGCGTTTCGC	Round 2: 100	94˚C 5 min, 40 cycles of (94˚C 30 sec, 63˚C 10 sec, 72˚C 15 sec), 72˚C 5 min
miR-34a-meR1	GCCCCCGCCTAAACTAACG		
miR -34a-unF	GGTGGTGTTTTGTGATTTAGT GGTGGT	143	94˚C 5 min, 40 cycles of (94˚C 30 sec, 63˚C 10 sec, 72˚C 15 sec), 72˚C 5 min
miR-34-unR	CAAAACCAATCCCACCAAACA CAAAATC		
MIR34b/c BC021736			
miR-34b/c-meF	TCGTTTCGTTTCGCGTTCGTT	93	94˚C 1 min, 40 cycles of (94˚C 30 sec, 66˚C 30 sec, 72˚C 30 sec), 72˚C 5 min
miR-34b/c-meR	GCCGCTCTAAACGACCGAAT		
miR-34b/c-unF	TTGTGGGGTTTTAAGGATGGTT GGTTGTTT	160	94˚C 1 min, 40 cycles of (94˚C 30 sec, 66˚C 30 sec, 72˚C 30 sec), 72˚C 5 min
miR-34b/c-unR	CCCTTCACCTCCTCAACCCAAAC		

miR, microRNA; MSP, methylation specific polymerase chain reaction; F, forward; R, reverse; un, unmethylated sequences; me, methylated sequences.

from the plus strand. The CpG island of *MIR34b/c* promoter locates in the upstream sequence of the *BTG4* gene as described by Toyota *et al* (38). Based on these reports, we look for the sequences corresponding to *MIR34a*, *MIR34b/c* promoters and designed the MSP primer sets used in our study. The primers for detecting the methylation status of *MIR34s* were designed based on the primer designing tool for MSP method (http://www.urogene.org/methprimer/index1.html). The primer sequences, MSP conditions and amplicon lengths are shown in Table I. Bisulfite treated DNAs were subjected to single or nested PCR depending on the particular targeted genes. The MSP products were resolved by electrophoresis in 8% polyacrylamide gel, then stained with ethidium bromide and imaged with the UVP, LLC (Upland, CA, USA). DNA extracted from lymphocytes of healthy volunteers and treated with bisulfite was used as a positive control for unmethylation of the targeted genes in numerous reports in which the MSP method was also applied (23,36). Water with no DNA template was included in each PCR reaction as a control for contamination. All MSP reactions were performed in duplicate. The methylation status was confirmed by direct sequencing of the MSP products for a subset of samples from each assay.

Statistical analysis. Chi-square test was used to determine the difference in *MIR34s* methylation levels between cancer and non-cancerous/normal adjacent tissues. The Kappa statistic was used to assess the agreement between two dichotomous variables such as the concordance between the methylation status of genes analyzed two by two in a given tissue type or the concordance between the methylation status of individual gene in tumor and adjacent normal tissues. Chi-square test and Fisher's exact test was used to examine the association of the methylation status of individual or combined genes with clinicoathological characteristics. For all statistical analyses, a $P<0.05$ was considered to indicate a statistically significant difference. All analyses were done by using the STATA program version 12 (StataCorp LP, College Station, TX, USA; https://www.stata.com/).

Results

In order to assess the methylation status of *MIR34* genes, we first set up an MSP assay and verified its specificity, since we have previously shown that false positive results from the MSP assay could be due to the MSP primers amplifying nonspecifically untreated genomic DNAs (35). Native DNAs were subjected to MSP analysis with the primer sets specific to the methylated status of the targeted sequences *MIR34a* and *MIR34b/c*. No MSP products were amplified from untreated DNAs extracted from lymphocytes of healthy donors (Fig. 1), confirming that the MSP primers were specific for the methylated targets and that false positive results have been avoided.

Genomic DNAs were subsequently treated with bisulfite and then subjected to the MSP assays. Representative results of the MSP reactions were illustrated in Fig. 1. The MSP products specific to methylated sequences of the *MIR34* promoters were directly sequenced. The results showed that all cytosines in the CpG sites remained cytosines and the cytosines alone were converted to thymidines (data not shown), indicating the completely conversion of genomic DNA by bisulfite treatment.

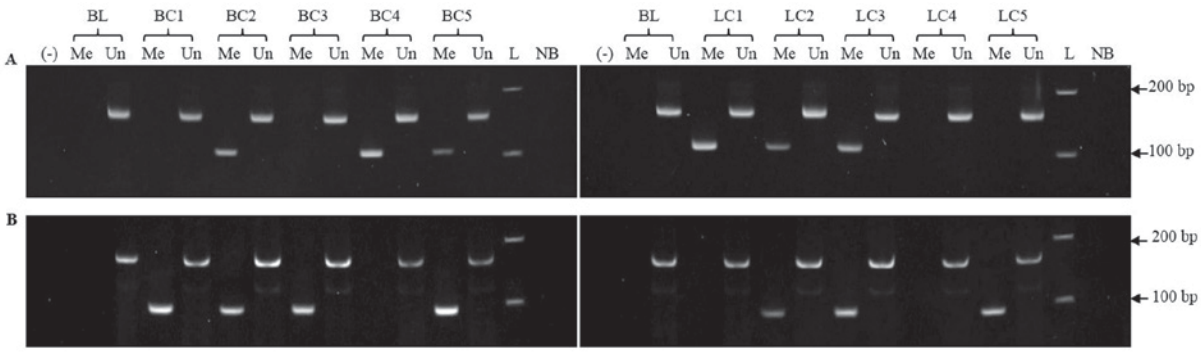

Figure 1. Representative analysis of methylation specific polymerase chain reaction products showing the detection of unmethylated sequences (Un) and methylated ones (Me) of the (A) *MIR34a*, (B) *MIR34b/c* genes from breast tumor (BC) and lung tumor (LC) tissues. BL, Treated bisulfite DNA isolated from blood lymphocytes of healthy volunteers; NB, Untreated bisulfite DNA isolated from blood lymphocytes of healthy volunteers; (-), Negative control without DNA templates; L, DNA ladders. MSP product sizes of the methylated sequences of *MIR34a* and *MIR34b/c* are 100 and 93 bp, respectively.

The MSP analysis revealed that methylation of *MIR34* promoters occurred in all analyzed tissues (breast cancer and adjacent tissues as well as in lung cancer and non-cancerous tissues). Among 79 pairs of matched breast cancer and adjacent tissues, *MIR34a* methylation occurred with the frequency of 49.37% in cancer tissue, which is significantly higher than its frequency of 30.38% in normal adjacent tissue (P=0.015). These frequencies for *MIR34b/c* methylation were 59.49 and 62.03%, respectively, with no significant difference. The methylation frequencies of *MIR34a* and *MIR34b/c* in lung cancer (48.42 and 56.84%) were similar to those in non-cancerous lung diseases (47.22 and 51.39%) (Table II).

As assessed by the calculation of the Kappa coefficient, the methylation status of *MIR34a* showed a significant concordance with that of *MIR34b/c* in breast cancer tissue but not in normal adjacent tissues (OR=2.12, 95% CI: 1.04-4.28, P=0.04). On the contrary, a significant concordance of the methylation status of both *MIR34a* and *MIR34b/c* genes was observed in lung cancer as well as in non-cancerous pulmonary diseases (P=0.0001; <0.0001) (Table II). Additionally, the methylation status of each gene promoter was also analyzed in association with clinicopathological features such as the histological tumor type, the tumor grade and the metastasis status (Table III). For breast cancer, the results showed that the methylation frequency of *MIR34a* was significantly associated with tumor type IDC (P=0.02). The univariate logistic analysis indicated that *MIR34a* was more methylated in the IDC type than in the other carcinoma types (OR=6.17, 95% CI: 1.25-30.32, P=0.03). However, no significant differences in the methylation frequency of the *MIR34b/c* gene were associated with clinical features of breast cancer patients such as the tumor grade and the metastasis status, nor with the patient' age. As far as lung cancer and non-cancerous pulmonary diseases are concerned, there was no significant association of the methylation frequency of *MIR34a* and *MIR34b/c* with any clinical features nor with the patient' age or sex.

Discussion

Over the past few years, there have been increasing evidences asserting the role of small non coding microRNA genes in different cellular processes promoting cell differentiation, proliferation, malignant transformation and tumorigenesis (4,5). Among the huge number of microRNA genes, the *MIR34* genes have been extensively focused on because they play a key role as tumor suppressors in cancer (10,11). Currently, clinical trial on cancer therapy based on *MIR34a* has already shown antitumor activity in refractory advanced solid tumor (39). Therefore, investigating the aberrant expression of the *MIR34* family in cancer has been being an attractive subject. The down regulation of all the three members of the *MIR34* family via promoter methylation, the correlation between *MIR34* methylation with cancer type, grade, metastasis and survival, as well as the aberrant expression of *MIR34* targeted genes have been extensively reported in multiple types of cancers including breast and lung cancers (12,17,19,40). However, an integrative comparison of *MIR34* methylation between both type of cancers vs. normal tissues adjacent to breast cancer or non-cancerous pulmonary disease tissues has rarely been described so far, while both types of these cancers are the first and second common types of cancer (33). From a more general standpoint over the literature, the difference in *MIR34s* methylation frequency among non-cancerous, cancer and normal adjacent tissues has been only explored in several types of cancer such as prostate, colon, gastric and skin cancer (19,31,32). In this study, we revealed that *MIR34* methylation was frequently found not only in breast cancer but also in normal tissue adjacent to tumor, with the lowest frequency being around 30% (Table II). Interestingly, *MIR34a* methylation occurred with significantly higher frequency in breast cancer than in adjacent tissues (Table II), and showed a concordance with *MIR34b/c* methylation only in breast cancer tissues (Table III). Concerning lung cancer, we showed here that methylation of *MIR34a* and *MIR34b/c* occurs in non-cancerous lung disease tissues with similar frequency than in lung cancer (Table II), suggesting their role in cancerous and non-cancerous lung disease onset and progression. Indeed, it is worth noting that no *MIR34a* methylation and a tiny frequency of *MIR34b/c* methylation have been detected in normal tissue adjacent to tumor of lung cancer (16,40,41). In addition, the contribution of *MIR* methylation to the pathogenesis of pulmonary fibrosis has been described previously (42). In our study, the concomitant methylation of *MIR34a* and *MIR34b/c* in breast

Table II. Methylation profile of the *MIR34* genes in breast and lung cancers.

	Number of methylated cases (%)			
Tissue	*miRa*	P-value	*miRb/c*	P-value
Breast cancer		0.015[a]		0.745
Tumor (n=79)	39 (49.37) [38.34; 60.39]		47 (59.49) [48.67; 70.32]	
Normal adjacent tissue (n=79)	24 (30.38) [20.24; 40.52]		49 (62.03) [51.32; 72.73]	
Lung cancer		0.878		0.483
Tumor (n=95)	46 (48.42) [38.37; 58.47]		54 (56.84) [46.88; 66.80]	
Pulmonary diseases (n=72)	34 (47.22) [35.69; 58.75]		37 (51.39) [39.84; 62.93]	

[a]Significant (P<0.05). The methylation status was indicated as (+) for methylated and (-) for unmethylated. *miRa*, MIR34a; *miRb/c*, MIR34b/c. Numbers in parentheses, when not preceded by 'n=', indicate the methylation frequency. Numbers in brackets indicate the 95% confidence interval of the methylation frequency. miR, microRNA.

Table III. Association of the methylation status of the *MIR34s* genes with the clinicopathologicalcharacteristics of the 79 breast cancer, 95 lung cancer and 72 non-cancerous pulmonary disease patients.

A, Breast cancer

		miR34a			miR34b/c		
Feature	No. of patients	Un	Me	P-value	Un	Me	P-value
Histological tumor type	79			0.015[b]			0.274
IDC	67	30	37		25	42	
ILC	5	3	2		2	3	
Other	7	7	0		5	2	
Tumor grade	65			0.999			0.382
Grade 1	3	1	2		0	3	
Grade 2	54	24	30		22	32	
Grade 3	8	4	4		2	6	
Metastasis	79			0.934[a]			0.753[a]
No	51	26	25		20	31	
Yes	28	14	14		12	16	
Age, years	79			0.749[a]			0.192[a]
<50	29	14	15		9	20	
≥50	50	26	24		23	27	

B, Lung cancer

		miR34a			miR34b/c		
Feature	No. of patients	Un	Me	P-value	Un	Me	P-value
Histological tumor type	95			0.809			0.786
NSCLC	72	38	34		30	42	
SCLC	1	0	1		0	1	
Other	22	11	11		11	11	

Table III. Continued.

B, Lung cancer

Feature	No. of patients	miR34a		P-value	miR34b/c		P-value
		Un	Me		Un	Me	
Stage	79			0.739			0.311
I	2	1	1		0	2	
II	28	17	11		11	17	
III	49	25	24		25	24	
Sex	94			0.877[a]			0.961[a]
Female	21	21	20		18	23	
Male	28	28	25		23	30	
Age, years	86			0.999			0.999
<50	10	5	5		4	6	
≥50	76	41	35		35	41	
EGFR mutation	44			0.480			0.484
No	11	3	8		4	7	
Yes	33	15	18		16	17	

C, Lung diseases

Feature	No. of patients	miR34a		P-value	miR34b/c		P-value
		Un	Me		Un	Me	
Diagnosis	71			0.768			0.306
Aspergillus	9	3	6		4	5	
Tuberculosis	16	8	8		8	8	
Pneumonia	22	11	11		8	14	
Pulmonary gas pressures	17	10	7		9	8	
Benign tumors	3	2	1		2	1	
Other diseases	4	3	1		4	0	
Sex	72			0.554[a]			0.118[a]
Female	25	12	13		9	16	
Male	47	26	21		26	21	
Age, years	70			0.863			0.863
<50	46	24	22		22	24	
≥50	24	12	12		12	12	

P-values were calculated by the Fisher's test, [a]P-values were calculated by the Chi-square test; [b]P<0.05. IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer; Me, methylated status; Un, unmethylated status; miR, microRNA.

cancer, lung cancer and non-cancerous lung disease tissues but not in normal tissue adjacent to breast cancer (Table II) emphasizes the role of *MIR34* methylation in human diseases including cancers. Recently, Piletič and Kunej have reviewed that epigenetic regulation of 63 *MIR* genes including *MIR34s* was strongly correlated with 21 human diseases including 11 types of cancers (43).

Interestingly, *MIR34a* methylation was significantly correlated with tumor type IDC that consists of about 85% of all breast cancer types (Table III). Aberrant methylation of *MIR34a* has been found to have significant relation with the tumor grade from triple negative breast cancers or type II ovarian cancer (17,44). However, we did not find any association between *MIR34a* methylation and breast tumor grade or lymph metastasis (Table III), even if this latter has been frequently reported in various cancer types such as colon, gastric and esophageal carcinomas (22,31,45). Similarly, there were no association of *MIR34a* methylation with clinicopathological features of lung cancer as shown in our study (Table III), as also shown in a previous work from Wang and

Table IV. Methylation status of the *MIR34* genes assessed by the calculation of the κ coefficient.

Methylation status	Breast cancer methylation frequency (%)				Lung tissue methylation frequency (%)			
	Tumor (n=79)	κ (P-value)	Adjacent (n=79)	κ (P-value)	Tumor (n=95)	κ (P-value)	Pulmonary diseases (n=72)	κ (P-value)
miRa+/miRb/c+	29 (36.71)		17 (21.52)		35 (36.84)		26 (36.11)	
miRa+/miRb/c-	10 (12.66)	0.29	7 (8.86)	0.098	11 (11.58)	0.37	8 (11.11)	0.47
miRa-/miRb/c+	18 (22.78)	(0.0039[a])	32 (40.51)	(0.1432)	19 (20.00)	(0.0001[a])	11 (15.28)	(<0.0001[a])
miRa-/miRb/c-	22 (27.85)		23 (29.11)		30 (31.58)		27 (37.50)	

The methylation status in breast and lung cancers was indicated as (+) for methylated and (-) for unmethylated. *miRa*, *MIR34a; miRb/c*, *MIR34b/c*. [a]Significant (P<0.05).

colleagues (23). These observations suggested that the aberrant *MIR34a* methylation is preferentially associated with the development of different types of cancer. This could be supported by some other findings such as the strong association of *MIR34a* methylation with *p53* mutation in Li-Fraumeni syndrome, a highly penetrant cancer predisposition syndrome while on the contrary, no association with *p53* mutation was found in ovarian cancer (44,46).

On the contrary to *MIR34a*, *MIR34b/c* methylation did not differ from breast cancer to adjacent tissues as well as from lung cancer to non-cancerous tissues although it occurred in all investigated tissues in this study. Furthermore, no significant difference in *MIR34b/c* methylation was found associated with clinicopathological features of neither breast nor lung cancer. There were number of previous reports showing that *MIR34b/c* methylation has a strong association with histologic type, pathologic stage and distance metastasis of breast and lung cancer (17,21,40,47). However, it is worth noting that these conclusions have not always been consistent, since other studies did not detect any correlation of *MIR34b/c* methylation with tumor metastasis nor with clinicopathological features in lung, gastric and colon cancers (16,31,32). The inconsistence of our result with previous studies may be explained by the difference concerning the tumor stage of analyzed samples. In our study, breast samples at grade 3 represented 12% [while it was 32% in the study by Zeng *et al* (17)], and lung cancer samples at stage IV represented 52% [while no sample of this stage was analyzed in the study by Kim *et al* (40)]. In addition, there are limitations inherent to our study design that should be noted. The statistical analysis was limited to small samples. Moreover, the clinicopathological characteristics concerning hormone phenotypes ER/PR/HER2 presented some missing data. In our future studies, increasing number of fully characterized samples will be analysed to determine the correlation between *MIR34* promoter methylation and subtypes of cancers.

To summarize, this study has chosen the non quantitative MSP method for the preliminary analysis of *MIR34* methylation, a method that has been widely used in numerous studies (48) given its simplicity, high sensitivity and low cost. We have shown that the methylation frequently and concomitantly occurred at the promoters of *MIR34* gene family in breast, lung cancer and pulmonary diseases. The encouraging results now prompt us to quantitatively investigate the correlation between *MIR34* promoter methylation and the silencing of their expression, as well as with the expression level of the mRNAs targeted by *MIR34s*. In long term, this would allow optimizing detection techniques that are suitable for moderately equipped laboratories in developing countries, using *MIR* methylation markers in clinical applications for diagnosis of human disease including cancers.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available on reasonable request addressed to the corresponding author.

Authors' contributions

VTTL conceived, planned the experiments and wrote the manuscript. VLT contributed to the statistical analysis and manuscript writing. PATD carried out the statistical analysis. HVS and NLT contributed to sample preparation. NTT and NTP carried out the experiments.

Ethics approval and consent to participate

Informed consent for using tissue materials for scientific purposes and publication was obtained from patients in written form and the study was approved by the guidelines of the VNU University of Science ethical committee in Vietnam (no. 9/2016/108/HDTN, VNU University of Science, Hanoi, Vietnam).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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