Hypermethylation of *MDFI* promoter with NSCLC is specific for females, non-smokers and people younger than 65

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Abstract. Non-small cell lung carcinoma (NSCLC) is a major subtype of lung cancer. Aberrant DNA methylation has been frequently observed in NSCLC. The aim of the present study was to investigate the role of MyoD family inhibitor (*MDFI*) methylation in NSCLC. Formalin-fixed paraffin-embedded tumor tissues and adjacent non-cancerous tissues were collected from a total of 111 patients with NSCLC. A methylation assay was performed using the quantitative methylation-specific polymerase chain reaction method. The percentage of methylated reference was used to represent the methylation level of the *MDFI* promoter. Data mining of a dataset from The Cancer Genome Atlas (TCGA) demonstrated that *MDFI* promoter methylation levels were significantly increased in 830 tumor tissues compared with 75 non-tumor tissues (P=0.012). However, the results on tissues obtained in the present study indicated that the *MDFI* promoter methylation levels in tumor tissues were not significantly different compared with those in the adjacent non-tumor tissues (P=0.159). Subsequent breakdown analysis identified that higher *MDFI* promoter methylation levels were significantly associated with NSCLC in females (P=0.031), but not in males (P=0.832). Age-based subgroup analysis demonstrated that higher *MDFI* promoter methylation levels were significantly associated with NSCLC in younger patients (≤65 years; P=0.003), but not in older patients (P=0.327). In addition, the association of *MDFI* methylation with NSCLC was significant in non-smokers (P=0.014), but not in smokers (P=0.832). Similar results also have been determined from subgroup analysis of the TCGA datasets. The Gene Expression Omnibus database indicated *MDFI* expression restoration in partial lung cancer cell lines (H1299 and Hotz) following demethylation treatment. However, it was identified that *MDFI* promoter hypermethylation was not significantly associated with prognosis of NSCLC (P>0.05). In conclusion, the present study indicated that the association of higher methylation of the *MDFI* promoter with NSCLC may be specific to females, non-smokers and people aged ≤65.

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Introduction

Lung cancer is the leading cause of cancer-associated mortality globally (1), with >1 million cases reported annually (2). Non-small cell lung cancer (NSCLC) is the dominant histological subtype, accounting for ~85% of lung cancer (3,4). Other types include lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC) and large cell carcinoma (5). Due to the nonspecific symptoms at early stages and poor overall survival rates (6,7), association studies for NSCLC biomarkers have been investigated globally (8,9).

The evolution of lung cancer is a complex process involving the interaction of genetic, epigenetic and environmental factors (10). As a common epigenetic modification, DNA methylation serves an important role in human malignant tumor types, including NSCLC (11). Methylation of the cytosine-phosphate-guanine (CpG) island affects gene silencing (12), and provides a novel insight into lung tumorigenesis and progression. Currently, there are a number of studies on the potential of DNA methylation biomarkers in NSCLC, including *RASSF1* (13), *CDKN2A* (14), *MGMT* (14), *APC* (15), *FHIT* (16), *CDH13* (15) and *DAPK* (13). Furthermore, a large number of tumor-specific methylated genes have been identified using genome-wide CpG island methylation analysis in NSCLC (17). Since aberrant DNA methylation has been indicated as an early stage event during lung carcinogenesis (18), it is characterized as dynamic and reversible (19). DNA methylation biomarkers may be an ideal tool for early diagnosis and prognosis due to their non-invasive, high sensitivity, and high specificity characteristics (20).

MyoD family inhibitor (*MDFI*) is located in chromosome 6p21.1, encoding a transcription factor that negatively regulates

myogenic family proteins (21). *MDFI* has been considered as a candidate tumor suppressor gene (21,22) and the domain protein is involved in transcriptional regulation by affecting the Wnt signaling pathway (23). Additionally, *MDFI* gene silencing induced by promoter hypermethylation was observed in pancreatic cancer (24,25); however, the epigenetic role of *MDFI* methylation in NSCLC pathogenesis remains unclear. The present study aimed to establish the association between the *MDFI* promoter methylation and NSCLC.

Materials and methods

Sample collection and data source. Formalin-fixed, paraffin-embedded (FFPE) tumor tissues and adjacent non-cancerous tissues were collected from 73 male, and 38 female patients with NSCLC at Huzhou First People's Hospital (Huzhou, Zhejiang, China) between August 2010 and October 2013. The patient age range was 33 to 82 years old (mean, 63.59±10.19 years old). All pathological parameters were defined according to the World Health Organization guidelines and Union for International Cancer Control tumor-node-metastasis classifications (26,27). According to the histological type, there were 42 patients with LUSC and 69 patients with LUAD. The adjacent non-cancerous tissues were obtained from ≥5 cm outside the edge of tumors. All specimens were sliced at 4-μm thickness using a Leica RM2245 Semi-Automated Rotary Microtome (Leica Microsystems GmbH, Wetzlar, Germany). Written informed consent form was signed by all of the participants and the present study was approved by the Ethics Committee of Huzhou First People's Hospital.

DNA methylation profiles (Illumina Human Methylation 450K, HM450K; Illumina, Inc., San Diego, CA, USA) and clinical characteristics (age, sex and smoking status) generated from 830 NSCLC tumor tissues and 75 non-tumor tissues were obtained from The Cancer Genomics Browser of The University of California Santa Cruz database (https://genome-cancer.ucsc.edu/). The browser contains data generated from the Cancer Genome Atlas (TCGA) project (https://cancergenome.nih.gov/). Therefore, larger samples were used to verify the findings of the study cases. These samples were then used as a control for the study data.

DNA extraction and bisulfite treatment. Genomic DNA from tissues was extracted using the E.Z.N.A® FFPE DNA kit (Omega Bio-tek, Inc., Norcross, GA, USA). DNA concentration measurements and bisulfite treatment were performed as previously described (28).

SYBR® Green-based quantitative methylation-specific polymerase chain reaction (PCR; qMSP). The 20 μl PCR consisted of 20 ng converted DNA, 0.5 μl forward primer (10 μM), 0.5 μl reverse primer (10 μM), 10 μl LightCycler® 480 SYBR-Green I Master mix (Roche Diagnostics, Basel, Switzerland) and 8 μl DNAase/RNAase-free water. All the experiments were performed on the LightCycler 480 system (Roche Diagnostics) utilizing a 384-well plate platform. The PCR program was conducted as follows: 95˚C for 10 min; followed by 45 cycles at 95˚C for 20 sec and 58˚C for 20 sec; and a single step of 72˚C for 30 sec. Following amplification, melting curve analysis was performed for PCR product identification that consisted of one cycle of 95˚C for 15 sec, 58˚C for 1 min and 95˚C for 10 sec (slope 0.11˚C/s, acquisition mode: Continuous). The primer sequences of *MDFI* were as follows: Forward, 5'-AGAGACGGTGAGGATTGT-3' and reverse, 5'-CGACTACTACATTCTTACCTACTT-3', and the product length was 80 bp. The primer sequences of *β-actin* were as follows: Forward, 5'-TGGTGATGGAGGAGGTTT AGTAAGT-3' and reverse, 5'-TGGTGATGGAGGAGGTTT AGTAAGT-3', and the product length was 133 bp. Sperm DNA from a healthy individual was methylated with excess SssI methyltransferase (Thermo Fisher Scientific, Inc., Waltham, MA, USA) to serve as a positive control. Water without DNA served as a negative control in each assay. The percentage of methylated reference (PMR) of the *MDFI* in each sample was calculated using the $2^{-\Delta\Delta Cq}$ method, whereby ΔΔCq was calculated as follows: Sample DNA ($Cq_{\text{target gene}}$-$Cq_{\text{ACTB control}}$)-fully methylated DNA ($Cq_{\text{target gene}}$-$Cq_{\text{ACTB control}}$) (29). All products were confirmed by Sanger sequencing and capillary gel electrophoresis as previously described (30).

Statistical analysis. Statistical analysis was performed using SPSS software 18.0 (SPSS Inc., Chicago, IL, USA). Due to the skewed distribution of methylation data, the non-parametric Wilcoxon signed-rank test and Mann-Whitney-U test was used to compare the methylation levels between tumor tissues and non-tumor tissues in the study cohort, and TCGA dataset. Data were presented as the median ± interquartile range. Fisher exact test or χ^2 test was used to determine the associations between the methylation status and the clinical characteristics (age, sex, smoking history, histological types and clinical stage). Kaplan-Meier curve was implemented to assess the prognostic value of *MDFI* methylation in postoperative patients with NSCLC. P<0.05 was considered to indicate a statistically significant difference.

Results

In order to assess the methylation level of *MDFI* promoter region, a fragment (chr6: 41604618-41604697) amplified with a suitable primer pair was selected. As there was a high number of CG sites in the promoter region, a specific primer pair of qMSP method was difficult to design. The primers used were designed to obtain a single melting curve. Furthermore, this fragment was located in the region rich in histone modifications (H3K4me1, H3K4me3 and H3K27Ac) according to human 2009 (GRCh37/hg19) assembly (http://genome.ucsc.edu/), indicating a potential regulatory mechanism in gene transcription (Fig. 1A). Additionally, the amplified fragment was demonstrated to match the target sequences by Sanger sequencing and capillary gel electrophoresis (Fig. 1B).

PMR was used to represent the methylation level of the *MDFI* promoter. The results indicated that there was no significant difference in *MDFI* promoter methylation between tumor tissues and adjacent non-tumor tissues [median (quartile range): 94.47% (71.11-152.20%) vs. 81.21% (65.12-138.60%); P=0.159; Fig. 2A]. Considering the limited sample size, the TCGA dataset was utilized for further investigation. There were seven CG probes (cg01520588, cg08625380, cg14086013, cg06484572, cg02914379, cg17094014 and cg13612207) located in TSS200 and TSS1500 regions of *MDFI*. Mean β-value represented the

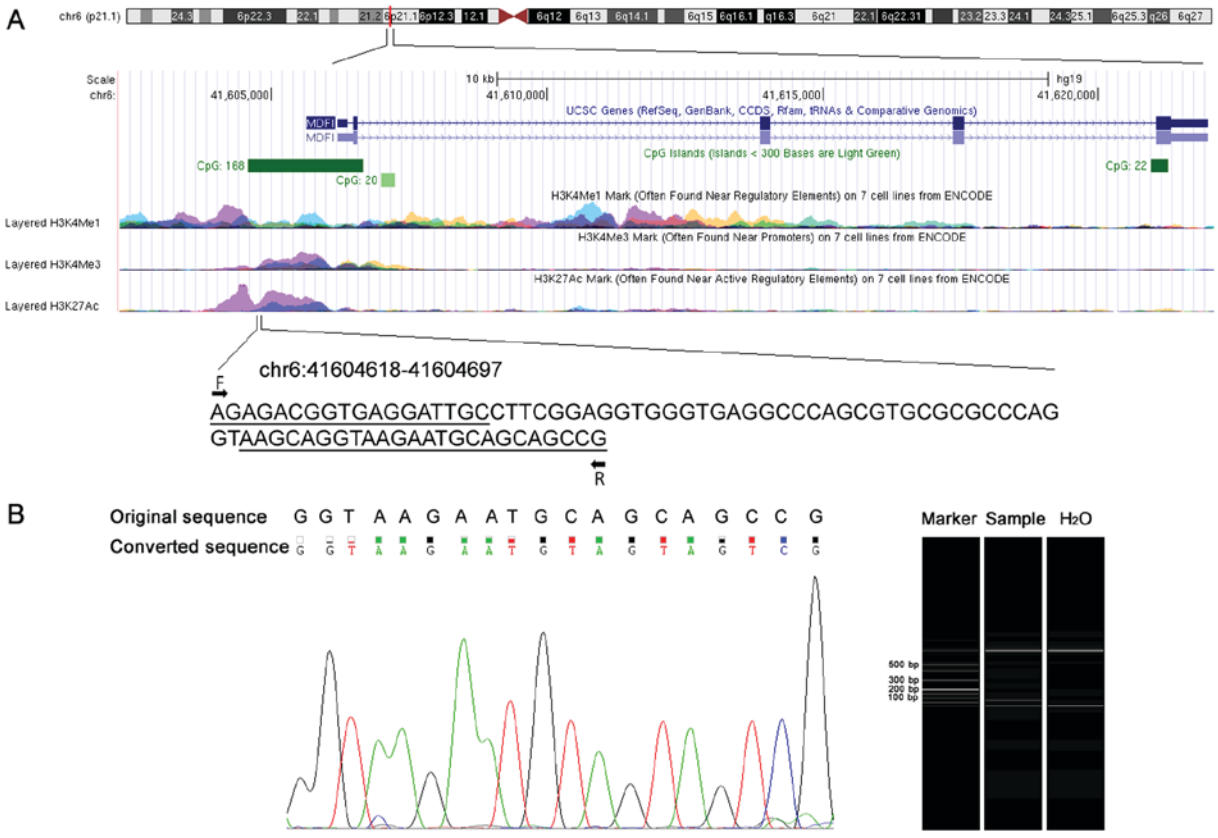

Figure 1. A schematic of the amplified fragment (chr6: 41604618-41604697) at the *MDFI* promoter CpG island. (A) The genomic positions and function annotations of representative sequences were obtained from the Cancer Genomics Browser of the UCSC according to human 2009 (GRCh37/hg19) assembly. qMSP primers are underlined. (B) The top row of the sequence represents the original sequence of the gene and the second row illustrates the converted sequences. The image on the right was the capillary electrophoresis of the representative qMSP product. F, forward primer; R, reverse primer; UCSC, University of California Santa Cruz; CpG, cytosine-phosphate-guanine; MDFI, MyoD family inhibitor; qMSP, quantitative methylation-specific polymerase chain reaction.

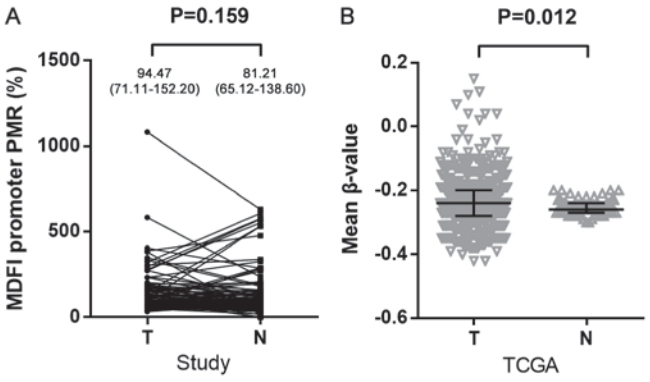

Figure 2. Comparisons of MDFI promoter methylation levels between T and N. (A) A total of 111 patients with NSCLC in the current study were analyzed. PMR represents the methylation level. The line between tumor and non-tumor groups represents the changes in the methylation level for one patient. The P-value was calculated using the Wilcoxon matched-pairs signed rank test. (B) A total of 830 NSCLC tumor tissues and 75 non-tumor tissues in TCGA database were analyzed. Mean β-value represents the methylation level. The P-value was calculated using the Mann-Whitney U test. Data are presented as the median ± interquartile range. MDFI, MyoD family inhibitor; T, tumor tissues; N, non-tumor tissues; NSCLC, non-small cell lung cancer; TCGA, The Cancer Genome Atlas; PMR, percentage of methylated reference.

promoter methylation level. Notably, a significantly higher mean methylation level was determined in 830 NSCLC tumor tissues compared with 75 non-tumor tissues [median (quartile range): -0.242 (-0.276-0.200) vs. -0.255 (-0.269-0.239); P=0.012; Fig. 2B]. Subsequently, sex, age and smoking history-based comparisons of *MDFI* promoter methylation levels between tumor tissues, and paired adjacent tissues were performed. Significantly increased methylation levels were observed in tumor tissues compared with paired adjacent tissues in females [PMR: 90.66% (74.45-153.60%) vs. 75.88% (48.17-117.53%); P=0.031; Fig. 3A]; however, no significant difference was identified in males (P=0.832). Furthermore, a significant difference was determined in younger patients [≤65 years; PMR: 92.98% (70.14-125.37%) vs. 73.74% (60.79-101.68%); P=0.003; Fig. 3A], but not in older patients (>65; P=0.327). Additionally, non-smokers exhibited a significant increase in methylation levels in tumor tissues compared with non-tumor tissues [PMR: 94.10% (72.85-153.20%) vs. 75.67% (48.17-115.20%); P=0.014; Fig. 3A], but this association was not observed in smokers (P=0.832). Following this, the same analysis process was performed in a TCGA cohort (Fig. 3B). The sample size of tumor tissues/non-tumor tissues was 481/46 in males, 335/28 in females, 341/32 in those ≤65 years old, 448/42 in those >65 years old, 578/57 in non-smokers and 219/12 in current smokers. The data demonstrated consistent results with the study cohort whereby significant differences in the methylation levels between tumor and non-tumor tissues were only observed in females (P=0.029), and non-smokers and ex-smokers (P=0.022). No significant difference was determined in age-based subgroup analysis (all P>0.05).

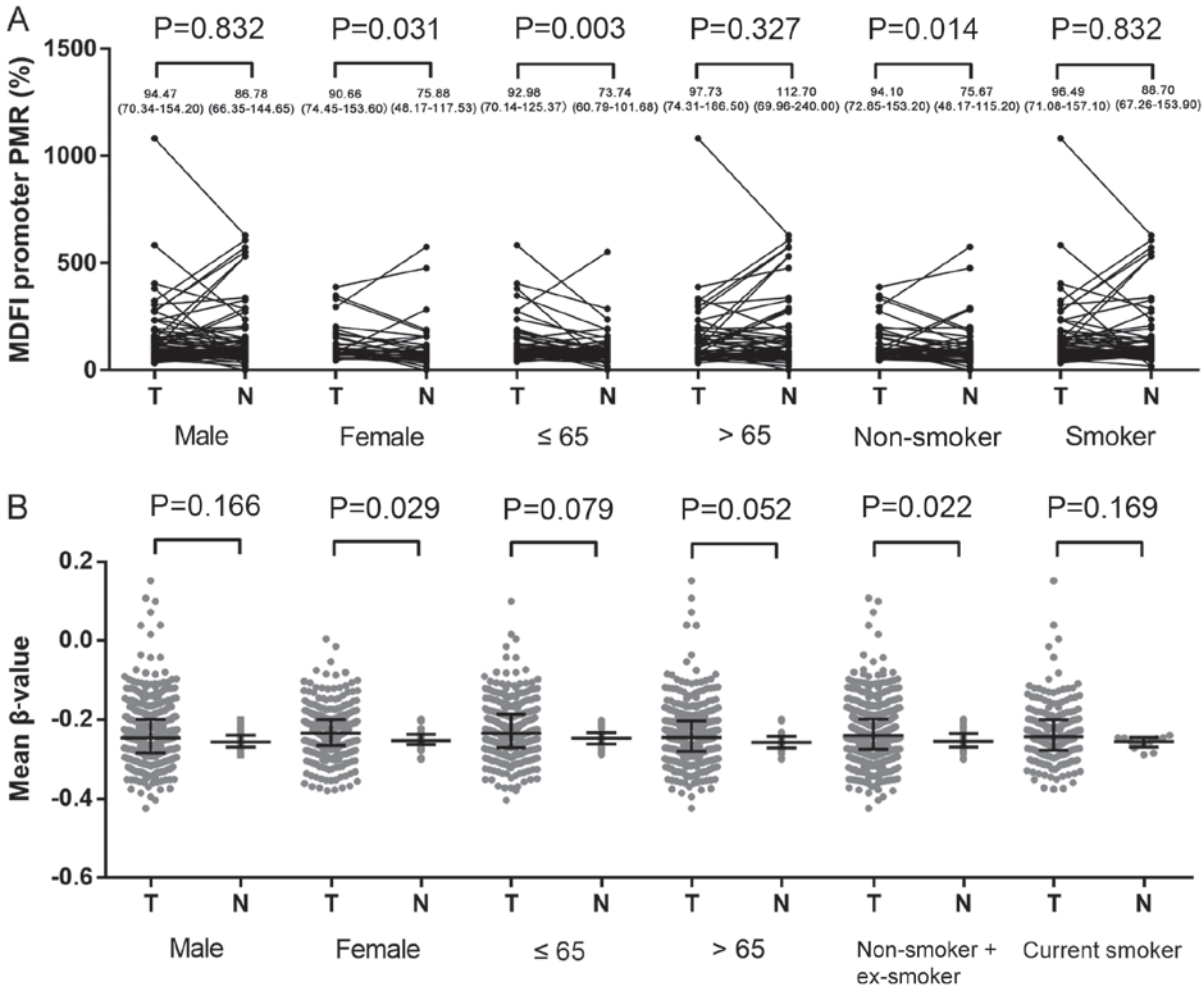

Figure 3. Subgroup-based comparisons of MDFI promoter methylation levels between T and N. (A) A total of 111 patients with NSCLC from the study cohort were used in the subgroup analysis of sex, age and smoking behavior. The line between tumor and non-tumor groups represents the changes in the methylation level from one patient. PMR represents the methylation level. The P-value was calculated using the Wilcoxon matched-pairs signed rank test. (B) A total of 830 NSCLC tumor tissues and 75 non-tumor tissues from The Cancer Genome Atlas cohort were used in the subgroup analysis of sex, age and smoking status. Mean β-value represents the methylation level. The P-value was calculated using the Mann-Whitney U test. Data are presented as the median ± interquartile range. MDFI, MyoD family inhibitor; NSCLC, non-small cell lung cancer; PMR, percentage of methylated reference; T, tumor tissues; N, non-tumor tissues.

The sample was defined as 'hypermethylation' if the PMR value was higher in tumor tissue compared with adjacent non-tumor tissues; otherwise, it was termed 'hypomethylation'. As in Table I depicts, the hypermethylation percentage, used to determine the probability of a hypermethylation event, among 111 patients with NSCLC was 58.56% (65/111). The association analysis with clinical variables indicated that *MDFI* was more significantly frequently hypermethylated in the tumors of female patients compared with male patients (73.68% vs. 50.68%; P=0.020). *MDFI* was also significantly more frequently hypermethylated in younger patients compared with older patients (67.74% vs. 46.94%; P=0.027); and in patients without a history of smoking compared with current smokers (70.00% vs. 49.18%; P=0.027).

The next focus was on the prognostic value of aberrant *MDFI* promoter methylation status on predicting the outcomes of postoperative patients with NSCLC. Mortality occurred in 11/111 patients with NSCLC; however, the Kaplan-Meier survival curve indicated that there was no significant association between *MDFI* hypermethylation and the overall survival of patients with NSCLC (P=0.344; Fig. 4A). No significant association was determined in the subgroup analysis of sex, age and smoking behavior, even in females (P=0.979; Fig. 4B), the younger population (P=0.709; Fig. 4C) and non-smokers (P=0.837; Fig. 4D).

In order to investigate the potential epigenetic role in regulating gene expression, the gene expression changes of four lung cancer cell lines (A549, H1299, Hotz and U1752) was further analyzed with different regimens of 5-aza-2'-deoxycytidine (5-AZA) treatment from the Gene Expression Omnibus (GEO) database. As demonstrated in Fig. 5, the expression of *MDFI* was increased significantly in the H1299 cell line with the increasing doses (0.3 and 3.0 μM) of 5-AZA for 48 h (GSE29077; P=0.027). Following 5-AZA treatment in the Hotz cell line, the expression of *MDFI* was also increased, compared with the cell line without treatment (GSE14315; P=0.006).

Discussion

The aim of the present study was to determine whether DNA methylation of *MDFI* promoter was associated with

Table I. Association between MyoD family inhibitor promoter methylation and clinical characteristics in patients with non-small cell lung cancer.

Variables	No.	Hypermethylation, n (%)	Hypomethylation, n (%)	P-value
Total	111	65 (58.56)	46 (40.54)	
Sex				0.020[a]
Male	73	37 (50.68)	36 (49.32)	
Female	38	28 (73.68)	10 (26.32)	
Age, years				0.027[a]
≤65	62	42 (67.74)	20 (32.26)	
>65	49	23 (46.94)	26 (53.06)	
Smoking history				0.027[a]
Non-smoker	50	35 (70.00)	15 (30.00)	
Smoker	61	30 (49.18)	31 (50.82)	
Histological type				0.068
LUSC	42	20 (47.62)	22 (52.38)	
LUAD	69	45 (65.22)	24 (34.78)	
Clinical stage				0.824
I+II	88	52 (59.09)	36 (40.91)	
III+IV	23	13 (56.52)	10 (43.48)	

[a]P<0.05. LUSC, lung squamous cell carcinoma; LUAD, lung adenocarcinoma. P-values were calculated using the χ^2 test.

NSCLC risk. The results demonstrated that the association between *MDFI* promoter hypermethylation and NSCLC was specific to younger patients with younger age, females, and non-smokers.

Age is a crucial factor in carcinogenesis (31). Numerous studies have reported that aging is associated with highly reproducible changes in DNA methylation at specific sites in the genome (32,33). Age-associated hypermethylation is enriched close to CpG islands, whereas hypomethylation occurs outside of CpG islands (34,35). DNA methylation may be one of the important mechanisms by which aging predisposes to numerous age-associated diseases, including cancer (36). In the present study, *MDFI* promoter hypermethylation occurrence was determined in the younger population, which provided as a potential age-specific biomarker of NSCLC.

In female patients with NSCLC, *MDFI* hypermethylation occurred more frequently in the tumor groups compared with the non-tumor groups, in the study and TCGA cohorts, which indicated *MDFI* hypermethylation may be an effective marker for female patients with NSCLC. Previous studies have reported that sexual hormones influence neoplastic diseases by altering the DNA methylation levels (37,38). Estrogen induces decreased thymic autoimmune regulator (*AIRE*) expression by increasing the number of methylation sites within the *AIRE* promoter (39). *MDFI* has been considered as a potential estrogen-associated gene in lung tumorigenesis (40); however, further investigation is required to verify the hypothesis of the present study.

Cigarette smoking, the top risk factor, is attributed to >80% cases of lung cancer cases (41). A previous study identified a group of aberrantly-methylated smoking-associated genes in patients with NSCLC (42). Notably, breakdown analysis by smoking history indicated that a significant association with *MDFI* hypermethylation existed in non-smoking patients, but not in smoking patients. Furthermore, this association existed in ex-smokers, but not in the current smokers. Although it was unclear why *MDFI* promoter methylation was predominantly associated with less tobacco exposure, it was considered that the methylation was caused by carcinogens other than those contained in tobacco smoke. A previous study indicated that the infections caused by human papilloma virus was an influencing factor of lung cancer in female non-smokers (43); therefore, further analysis of carcinogenesis and the progression of NSCLC in non-smokers should be performed in the future.

Similar to other cancer types, NSCLC is influenced by regional hypermethylation of promoters of common cancer-associated genes (10). Significant differences in *MDFI* methylation have been observed between pancreatic tumor tissues and normal controls (24,25); furthermore, *MDFI* methylation has been considered as a promising diagnostic marker in pancreatic cancer (24). Furthermore, compared with non-tumor tissues, the *MDFI* promoter was most frequently hypermethylated in colorectal cancer tissues (44), indicating a tumor suppressor effect of *MDFI* in human cancer types. In the present study, an increased trend in *MDFI* methylation level was determined in patients with NSCLC, although the result was not statistically significant. Using the TCGA database with an increased sample size demonstrated an elevated methylation level in NSCLC tumor tissues. Considering the divergence caused by sample size, different ethnicities and different detection methods, a more comprehensive study is required.

Aberrant methylation of promoter regions is generally associated with gene transcriptional dysfunction through

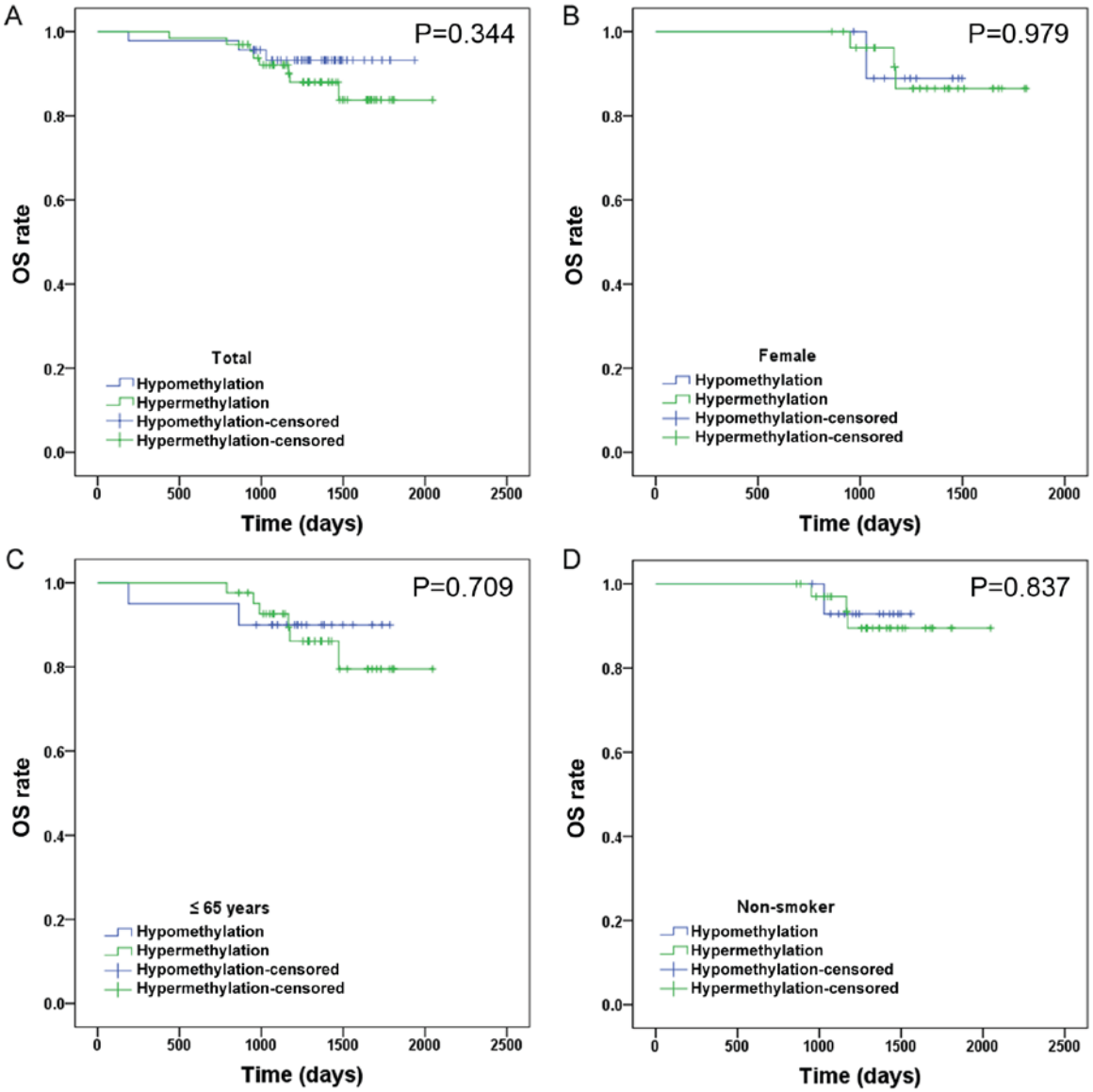

Figure 4. Prognostic analysis of *MDFI* methylation using Kaplan-Meier survival curves (A) in the total cohort, and subgroups of (B) females, (C) patients aged ≤65 and (D) non-smokers. MDFI, MyoD family inhibitor; OS, overall survival.

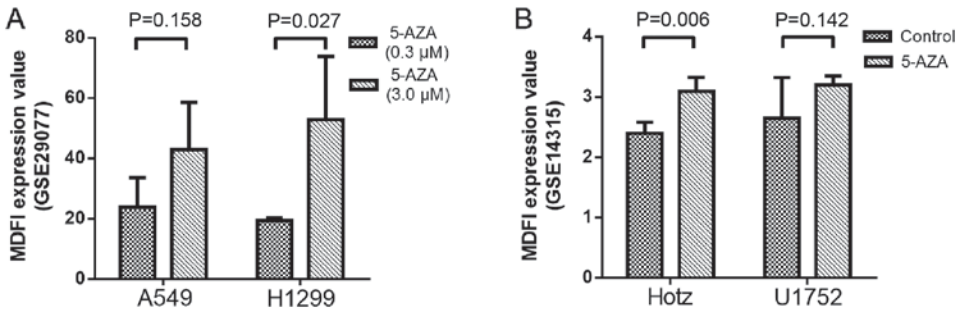

Figure 5. Changes in *MDFI* mRNA expression following different regimens of demethylation treatments. (A) Two lung cell lines (A549 and H1299) were treated with doses (0.3 and 3.0 μM) of 5-AZA for 48 h. (B) Two lung cancer cell lines (Hotz and U1752) were analyzed with and without the treatment with 5-AZA. All P-values were calculated using the moderated Student's t-test. The mRNA expression data were obtained from GEO database (accession nos. GSE29077 and GSE14315). 5-AZA, 5-aza-2'deoxycitidine; MDFI, MyoD family inhibitor.

different underlying mechanisms, including the direct inhibition of transcription factor binding, and the recruitment of methyl-binding domain proteins (MBD1, MBD2 and MeCP2) and their associated complexes (45). Currently, there are a limited number of studies on the underlying epigenetic mechanism of the *MDFI* gene in NSCLC. The *MDFI* gene

is commonly downregulated in invasive hepatic cellular cancer cells and is a repressor of myogenic helix-loop-helix class transportation factors (46). Furthermore, Pan *et al* (46) determined an increasing Wnt reporter gene activity in the canonical Wnt signaling pathway by knocking down endogenous *MDFI* expression, which indicated the *MDFI* gene as a tumor suppressor gene (21). As poor prognosis of NSCLC has been reported to be associated with aberrant methylation through Wnt signaling, including WNT inhibitory factor 1 (47) and secreted frizzled related protein 3 (48), a similar role of *MDFI* promoter methylation may participate in Wnt signaling pathway regulation for different types of cancer with aggressive phenotypes. The evidence for *MDFI* promoter methylation as a regulatory mechanism of gene expression in NSCLC is notable and should be further explored. Additionally, the GEO analysis in the present study demonstrated gene expression changes in partial lung cancer cells following demethylation, indicating that other epigenetic mechanisms, including histone modifications and non-coding RNA, may exert this interaction in NSCLC. Therefore, this complex network of gene activity establishment and maintenance requires further research to be understood.

In conclusion, hypermethylation of *MDFI* promoter may contribute to the risk of NSCLC in females, non-smokers and the younger population. The evidence for *MDFI* promoter methylation as a regulatory mechanism of gene expression is notable and should be further explored.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

SD and HM contributed to the conception, design and final approval of the submitted version. XC, HH and BL contributed to the interpretation of data and completion of figures and tables. XY, CZ and JZ contributed to performing the experiments and analyzing the data. GZ analyzed the data, revised and approved the manuscript. XC and HH contributed to writing the paper. All the authors have read and approved the final manuscript.

Ethics approval and consent to participate

A written informed consent form was signed by all participants prior to their inclusion within the study. The study was approved by the Ethics Committee of Huzhou First People's Hospital.

Consent for publication

All patients have provided written informed consent for the publication of any associated data and accompanying images.

Competing interest

The authors declare that they have no competing interests.

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