Cancer Biol Med 2017. doi: 10.20892/j.issn.2095-3941.2017.0061

ORIGINAL ARTICLE

Promoter methylation of Wnt/β-Catenin signal inhibitor *TMEM88* is associated with unfavorable prognosis of non-small cell lung cancer

Rongna Ma[1*], Nannan Feng[1*], Xiao Yu[1], Hongyan Lin[1], Xiaohong Zhang[1], Oumin Shi[1], Huan Zhang[2], Shuo Zhang[1], Lei Li[1], Min Zheng[1], Ming Gao[2], Herbert Yu[3], Biyun Qian[1]

[1]Hongqiao International Institute of Medicine, Shanghai Tongren Hospital/Faculty of Public Health, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China; [2]Key Laboratory of Cancer Prevention and Therapy, Tianjin Medical University Cancer Institute and Hospital, Tianjin 300060, China; [3]Cancer Epidemiology Program, University of Hawaii Cancer Center, HI 96813, USA

ABSTRACT

Objective: Recent research has indicated that altered promoter methylation of oncogenes and tumor suppressor genes is an important mechanism in lung cancer development and progression. In this study, we investigated the association between promoter methylation of *TMEM88*, a possible inhibitor of the Wnt/β-Catenin signaling, and the survival of patients with non-small cell lung cancer (NSCLC).

Methods: Twelve pairs of tumor and adjacent non-tumor samples were used for microarray analyses of DNA methylation and gene expression. For validation, more than two hundred additional samples were analyzed for methylation using bisulfite pyrosequencing and for gene expression using qRT-PCR. Then the cell function were tested by wound healing, transwell, CCK8 and cell cycle assay.

Results: Our analysis of patient specimens showed that *TMEM88* methylation was higher in NSCLC tumors (82.2% ± 10.3, P < 0.01) compared with the adjacent normal tissues (65.9% ± 7.2). The survival analysis revealed that patients with high *TMEM88* methylation had a shorter overall survival (46 months) compared with patients with low *TMEM88* methylation (>56 months; P=0.021). In addition, we found that demethylation treatment could inhibit tumor cell proliferation, migration, and invasion, which was supportive of an association between methylation and survival.

Conclusions: Based on these consistent observations, we concluded that *TMEM88* may play an important role in NSCLC progression and that promoter methylation of *TMEM88* may serve as a biomarker for NSCLC prognosis and treatment.

KEYWORDS *TMEM88*; lung cancer; methylation; prognosis; Wnt/β-Catenin signaling

Introduction

Lung cancer is one of the most common malignant tumors in the world. According to the data from GLOBOCAN 2012, 1.8 million people are diagnosed with lung cancer every year, which represents 13% of all newly diagnosed cancer patients. Lung cancer is also the number one cause of cancer-related death, accounting for 1.6 million deaths per year, which represents 19% of all cancer deaths[1]. Non-small cell lung cancer (NSCLC) is the most common form of lung cancer and constitutes approximately 85% of all lung cancer cases. Studies have shown that the disruption of normal epigenetic regulation is an important mechanism in the initiation and progression of cancer[2]. Research has also indicated that the Wnt/β-catenin signaling pathway is critical in tumorigenesis[3].

DNA methylation plays a crucial role in the epigenetic regulation of gene activity and function[4-6]. Substantial alterations in DNA methylation and gene expression have been observed in many types of cancer, including breast[7], prostate[8], and lung[9] cancer. It has also been reported that the downregulation of the Wnt/β-Catenin signal inhibitors by hypermethylation is common in NSCLC[10-13]. Recently, Zhang et al.[14] found that *TMEM88* could inhibit canonical Wnt/β-Catenin signaling through interaction with the Wnt pathway factor Dishevelled (DVLS). *TMEM88*, located on chromosome 17p13.1, is a transmembrane protein that plays

*These authors contributed equally to this work.

Correspondence to: Biyun Qian
E-mail: qianbiyun@shsmu.edu.cn

Received May 16, 2017; accepted August 31, 2017.
Available at www.cancerbiomed.org

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an important role in embryonic development and stem cell differentiation[15]. However, it remains unclear whether *TMEM88* hypermethylation in lung cancer has any biological relevance to tumorigenesis and if this has any clinical implications for disease prognosis.

In this study, we investigated the role of *TMEM88* methylation in NSCLC with regard to its association with the clinical and pathological features of patients with NSCLC. Using the demethylation agent DAC, we evaluated the methylation and expression of *TMEM88* in lung cancer cells and its impact on cancer cell migration, invasion, proliferation, and cell cycle progression. We also analyzed the association of *TMEM88* methylation with the overall survival of patients with NSCLC.

Materials and methods

Patients

Patients with NSCLC were recruited from the Tianjin Medical University Cancer Hospital (TMUCH) between May 2006 and July 2011. The fresh tumor samples and adjacent non-tumor tissues were collected from the patients during the surgical resection of tumors. Twelve pairs of tumor and adjacent non-tumor samples were used for microarray analyses of DNA methylation and gene expression. For validation, an additional 213 tumor samples and 30 matched adjacent non-tumor tissues were analyzed for methylation by using bisulfite pyrosequencing; further, the gene expression of 201 tumor samples and 66 matched adjacent non-tumor tissues was tested by using qRT-PCR; and, from these two batches, samples from 173 patients were analyzed for both the methylation and expression of *TMEM88*. The clinical and pathological information for each patient was extracted from hospital medical records and pathology reports. All patients were followed after surgery until August 2013. The study was approved by the Medical Ethical Review Committees at Tianjin Medical University Cancer Institute and Hospital and Shanghai Jiao Tong University School of Public Health.

RNA extraction and qRT-PCR

Total RNA was extracted from the tissue samples and cultured cancer cells by using a commercial kit. Quantitative RT-PCR assays were performed as previously described[16].

DNA extraction and analysis of DNA methylation

Genomic DNA was extracted from the tissue samples and cancer cells by using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany, Cat no. 51304). The extracted DNA samples were treated with sodium bisulfite by using the EpiTect plus DNA Bisulfite Kit (Qiagen, Cat no. 59124) according to the manufacturer's protocol. Genome-wide DNA methylation profiles were measured in 12 pairs of tissue samples by using the Illumina HumanMethylation450 BeadChip (Illumina, Cat no. WG-314-1003); the procedure for the microarray analysis of DNA methylation has been described elsewhere[14]. Bisulfite pyrosequencing was performed to validate our microarray results from tissue samples. For pyrosequencing, the treated DNA samples were amplified by PCR and fragmented. The processed samples were then precipitated, suspended, and genotyped in the Pyro Mark Q96 system (Qiagen, Hilden, Germany). The sequencing primers were designed to include CpG sites within 0.5 kb of the transcription start site. A methylation level of 15% or higher was considered positive for methylation and any levels equal to or lower than that were considered undistinguishable from the background.

Cell culture

The NSCLC cell lines A549 and H1299 were purchased from Cell Bank of Chinese Academy of Sciences (Cell Bank of CAS, Cat nos. TCHu150 and TCHu160) and maintained in RPMI-1640 medium at 37 °C supplemented with 10% fetal calf serum (Sigma, Cat no. F2442) in a humid atmosphere containing 5% CO_2, as recommended by CAS. For the experiments that evaluated cell behavior, cells in the exponential growth phase were used.

Treatment with 5-aza-2'-deoxycytidine (DAC)

NSCLC cells in culture were treated with 10 μM/L 5-aza-2'-deoxycytidine (DAC) (Sigma-Aldrich, Cat no. A3656) for 72 h. After treatment, the cells were harvested to extract genomic DNA and total RNAs for use in gene expression analysis and DNA methylation. For the experiments involving DAC treatment, DMSO was used as the control treatment.

Assays for cell proliferation, migration, invasion, and cell cycle progression

Cell proliferation was analyzed by using Cell Counting Kit-8 (Dojindo, Cat no. CK04, Japan). After DAC treatment for 0 h, 24 h, and 48 h, the cells were treated with 10% CCK-8 in new media for 2 h at 37 °C and the absorbance at 450 nm was measured. Cell migration was measured by using a wound

healing assay in 6-well plates. After the cultured cells reached full confluence, a 100-μL pipette tip was used to scrape a line in each well. The images of the cultures were taken at 72 h after DAC treatment. The invasion assay was performed by using Transwell chamber inserts (Corning, USA) with Matrigel according to the manufacturer's protocol. Cell cycle progression was analyzed by C6 flow cytometer (BD, Cat no. 653160).

For details, please refer to the protocols described in our previous publication[16].

siRNA transfection

The transfection assays of *TMEM88* siRNA were performed according to the protocols described in our previous publication[17].

Statistical analysis

SPSS software version 16.0 was used for the computation of statistical analyses. Student's *t*-test was performed to compare changes in cell-based experiments. Chi-squared tests were used to analyze *TMEM88* methylation and expression in association with clinical and pathological parameters. The Kaplan-Meier survival curves and log-rank tests were used to evaluate the association between methylation and overall survival. Cox proportional-hazards regression, in which covariates and confounding factors were adjusted, was also used for multivariate survival analysis. The overall survival time was calculated from the date of surgery to the date of death or last live contact. Pearson's correlation coefficient was calculated to assess the correlation between the expression of *TMEM88* and the genes involved in the Wnt signaling pathway by using data from TCGA (The Cancer Genome Atlas). All statistical tests were based on two-tailed analysis.

Results

TMEM88 expression and methylation in NSCLC

In our microarray analyses of DNA methylation and gene expression, we found that *TMEM88* was hypermethylated in 12 NSCLC tumors compared with 12 matched adjacent non-tumor samples and that the methylation was inversely correlated with the expression of *TMEM88* (**Figure. 1A**). High methylation in the *TMEM88* promoter region was confirmed in additional 213 tumor samples compared with 30 adjacent non-tumor tissues by using bisulfite pyrosequencing. A low expression of *TMEM88* was validated in 201 tumor samples in comparison with 66 adjacent non-tumor tissues by using qRT-PCR. The inverse correlation between methylation and expression was also validated in 173 subjects (P=0.001) (**Figure. 1B**), which supported the results of the microarray analyses.

TMEM88 and NSCLC characteristics

The methylation and expression of *TMEM88* associated with the clinical and pathological features of NSCLC are shown in **Table 1**. The analysis indicated that *TMEM88* methylation was associated with tumor size and histology (P=0.002 and P=0.010, respectively). However, neither *TMEM88* methylation nor expression were associated with patient age at diagnosis, gender, BMI, family history of lung cancer, smoking status, and disease stage (**Table 1**).

TMEM88 and NSCLC survival

To evaluate the association between *TMEM88* methylation and overall survival, we performed Kaplan-Meier survival analysis on 213 patients with NSCLC. The analysis showed that patients with high *TMEM88* methylation had a shorter overall survival (median survival of 46 months) than those with low *TMEM88* methylation (median survival >59 months, P=0.021) (**Figure. 1C** and **Table 2**). This survival association with *TMEM88* methylation in NSCLC remained significant after the adjustment for covariates and potential confounding factors (**Table 2**). However, no association was observed between *TMEM88* expression and overall survival in the study (**Table 2**).

TMEM88 expression in NSCLC cell lines after DAC treatment

To assess the functional relevance of *TMEM88* methylation in NSCLC, we analyzed the methylation changes related to *TMEM88* expression after the treatment of the NSCLC cell lines A549 and H1299 with the demethylation agent, DAC. Our experiments showed that DAC treatment reduced *TMEM88* methylation and resulted in elevated *TMEM88* expression in both cell lines (**Figure. 2A**).

DAC-induced *TMEM88* expression and NSCLC cell behavior

To evaluate the effect of *TMEM88* expression on NSCLC cells, we performed a number of *in vitro* experiments. First, we investigated cell migration and invasion after the DAC-

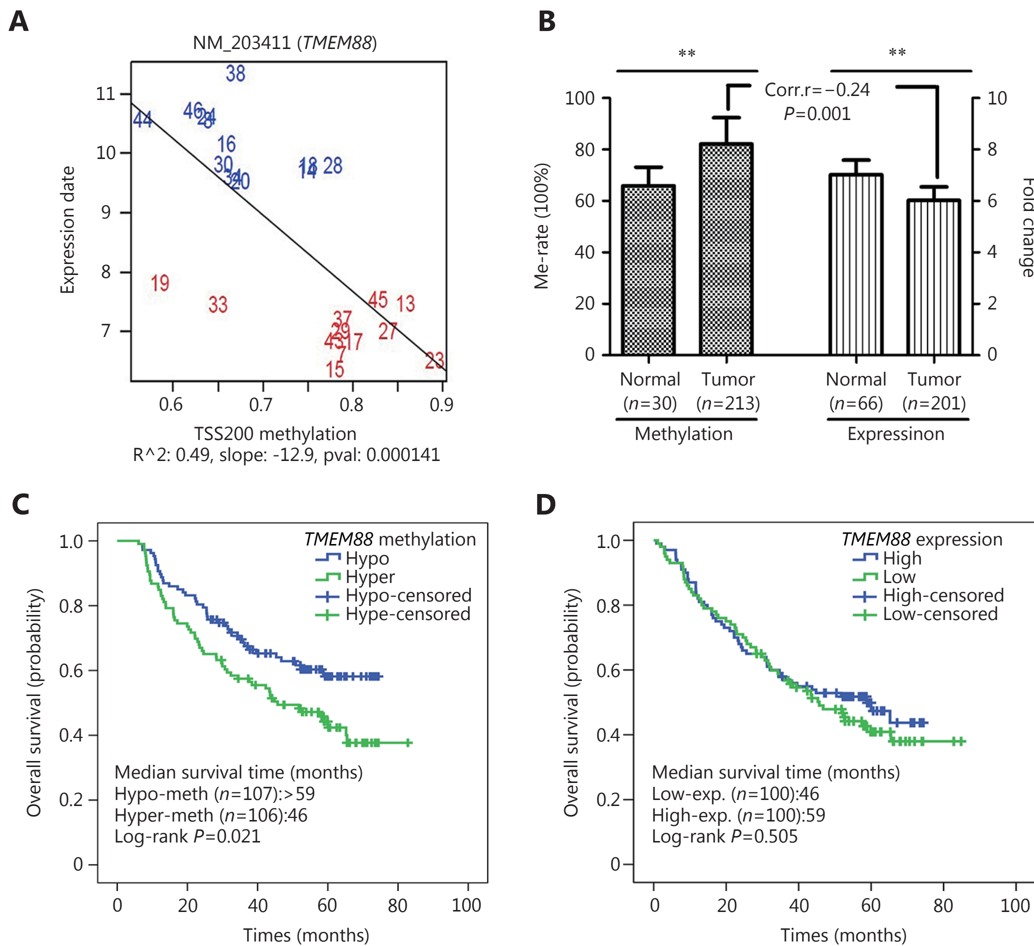


Figure 1 Correlation between *TMEM88* expression and methylation, and the association *TMEM88* expression and methylation with NSCLC overall survival. (A) The correlation between the methylation and expression of *TMEM88*, as shown from the microarray analyses. Red coloring indicates the tumor tissues and blue coloring the adjacent normal tissues. (B) The comparison of *TMEM88* methylation and the expression in tumor and adjacent non-tumor tissues, and the analysis of the correlation between *TMEM88* methylation and expression. (C) Survival curve of *TMEM88* methylation. (D) Survival curve of *TMEM88* expression. The data are presented as the mean±SD, *$P < 0.05$, **$P < 0.01$ from Student's *t*-test in comparison with the control group.

induced upregulation of *TMEM88* expression in H1299 and A549 cells that originally had a low expression and high methylation of *TMEM88*. The experiments showed that the demethylation of *TMEM88* in H1299 and A549 significantly delayed cell migration (**Figure. 2B**). Moreover, the invasion ability of H1299 and A549 cells was also significantly decreased ($P < 0.05$ and $P < 0.01$, respectively) after demethylation treatment (**Figure. 2C**). In order to further validate the effect of *TMEM88* expression on NSCLC cells, we transfected the cells with *TMEM88* siRNA after DAC treatment, which reduced *TMEM88*. The experiments showed that the demethylation of *TMEM88* in H1299 and A549 significantly delayed cell migration (**Figure. 2C**), but that these effects were abolished when the cells were transfected with *TMEM88* siRNA.

We then analyzed cell proliferation and cell cycle progression after DAC treatment. Cell proliferation in DAC-treated H1299 cells was decreased by 60% in comparison with the untreated cells ($P < 0.01$). Proliferation of A549 cells was also decreased, but the change was not statistically significant (**Figure. 2D**). Therefore, the demethylation of *TMEM88* retarded cell cycle progression at the G2/M phase in both cell lines (**Figure. 2E**).

Correlation between *TMEM88* and molecules in the Wnt signaling pathway

TMEM88 was reported to inhibit canonical Wnt/β-Catenin

Table 1 Associations of *TMEM88* methylation and expression with patient clinical and pathological features

Characteristics	Group	Methylation					Expression				
		n	Low *n* (%)	High *n* (%)	χ^2	*P*	*n*	Low *n* (%)	High *n* (%)	χ^2	*P*
Age (years)	≤ 60	213	56 (54)	47 (46)	1.364	0.243	201	50 (51)	48 (49)	0.046	0.831
	>60		51 (46)	59 (54)				51 (50)	52 (50)		
Stage	I-II	208	74 (53)	65 (47)	0.868	0.351	197	58 (48)	64 (52)	0.943	0.331
	III-IV		32 (46)	37 (54)				41 (55)	34 (45)		
BMI	≤ 24	212	59 (52)	55 (48)	0.162	0.687	199	48 (49)	51 (51)	0.126	0.723
	>24		48 (49)	50 (51)				51 (51)	49 (49)		
Gender	Male	213	58 (47)	66 (53)	1.422	0.233	201	65 (52)	61 (48)	0.242	0.623
	Female		49 (55)	40 (45)				36 (48)	39 (52)		
History of lung diseases	No	213	97 (50)	98 (50)	0.223	0.637	201	94 (51)	91 (49)	0.294	0.588
	Yes		10 (56)	8 (44)				7 (44)	9 (56)		
Family history	No	212	90 (51)	88 (49)	0.004	0.952	200	81 (50)	81 (50)	0.085	0.770
	Yes		17 (50)	17 (50)				20 (53)	18 (47)		
Smoking status	No	213	43 (57)	33 (43)	1.902	0.168	201	25 (42)	34 (58)	2.072	0.150
	Yes		64 (47)	73 (53)				76 (54)	66 (46)		
Histology	LUSC	185	40 (42)	55 (58)	6.683	**0.010**	167	52 (53)	47 (47)	0.776	0.378
	LUAD		55 (61)	35 (39)				31 (46)	37 (54)		
Tumor size (cm)	<3	212	46 (66)	24 (34)	9.713	**0.002**	200	30 (52)	28 (48)	0.097	0.755
	≥ 3		61 (43)	81 (57)				70 (49)	72 (51)		

signaling through interaction with the Wnt pathway factor Dishevelled (*DVLS*)[14] As an inhibitor of the Wnt signaling, *TMEM88* may interact with the molecules in the signaling pathway. To determine whether the expression of *TMEM88* was correlated with the genes involved in Wnt/β-Catenin signaling, we analyzed data from TCGA. We downloaded freely available gene expression data from lung cancer samples and calculated expression correlations between *TMEM88* and several components in the Wnt/β-Catenin signaling pathway, such as Wnt, Frizzled class receptor (*FZD*), Receptor tyrosine kinase like orphan receptor (*ROR*), and Dishevelled segment polarity protein (*DVL*). Modest correlations were found between *TMEM88* expression and some of these genes, including *DVL1* (r=0.21), *FZD4* (r=0.27), *FZD5* (r=0.39), and *ROR1* (r=0.30) (**Table 3**).

Discussion

Our study demonstrated that the *TMEM88* promoter was highly methylated in NSCLC and that this hypermethylation was correlated with a low expression of *TMEM88*. The survival analysis showed that patients with NSCLC and methylated *TMEM88* had poor overall survival compared with those without methylated *TMEM88*. In addition, patients with higher *TMEM88* expression had a longer survival time in more than 36 months survival group (P>0.05). Furthermore, our *in vitro* experiments revealed that *TMEM88* expression was increased after the treatment of NSCLC cells with the demethylation agent DAC; the treatment also inhibited cell proliferation, migration, and invasion, which indicated that our observations in patients were biologically relevant. These findings suggested that promoter methylation of *TMEM88* may serve as a potential biomarker for NSCLC prognosis and treatment.

TMEM88 is a transmembrane protein located on the cell membrane of *Xenopus* embryonic cells[18]. As a newly discovered gene, limited studies have been conducted on *TMEM88*. Although the mechanism is not fully understood, evidence has suggested that *TMEM88* may play an important role in the regulation of human stem cell differentiation and embryonic development and have significant involvement in cell signaling[3]. However, no studies have previously shown that *TMEM88* methylation is implicated in lung cancer prognosis.

Many genes have been reported to undergo aberrant DNA methylation in cancer[19,20], such as the DNA repair gene

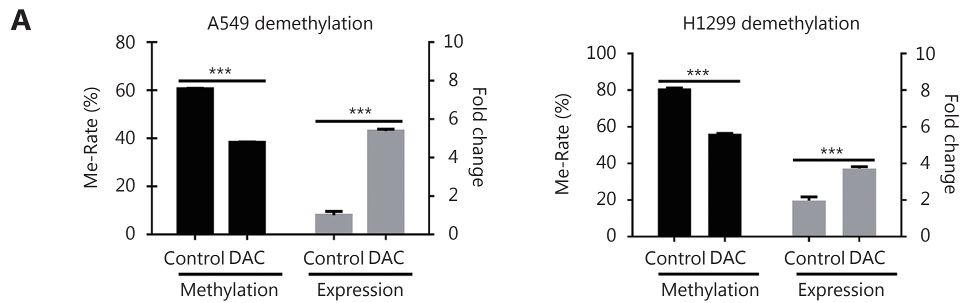
Table 2 Cox regression analysis of overall survival and patient characteristics

Variable	Univariate		Multivariate	
	RR (95%CI)	*P*	RR (95%CI)	*P*
Age	1.53 (1.06–2.20)	**0.023**	1.51 (0.96–2.37)	0.077
Gender				
Male	1.00	0.672	1.00	0.484
Female	1.08 (0.75–1.56)		0.80 (0.44–1.48)	
Smoking status				
No	1.00	0.241	1.00	0.211
Yes	0.80 (0.56–1.16)		0.67 (0.36–1.26)	
Stage				
I–II	1.00	**< 0.001**	1.00	**< 0.001**
III–IV	4.18 (2.86–6.10)		3.44 (2.18–5.44)	
Family history of cancer				
No	1.00	0.341	1.00	0.519
Yes	1.25 (0.79–1.97)		1.21 (0.68–2.17)	
TMEM88 methylation				
Low	1.00	**0.021**	1.00	**0.018**
High	1.57 (1.07–2.38)		1.74 (1.10–2.74)	
TMEM88 expression				
Low	1.00	0.521	1.00	0.940
High	0.88 (0.60–1.30)		0.98 (0.63–1.53)	

RR: relative risk; 95%CI: 95% confidence interval

O(6)-methylguanine DNA methyltransferase (*MGMT*) in B-cell lymphoma[21], dickkopf 3 (*Dkk3*) gene in gastric cancer[22], secreted frizzled-related protein 1 (*SFRP1*) in breast cancer[23] and colorectal cancer[24], *MGMT* and *MLH1* in colorectal cancer[25], and *WIF-1*[26] and *Axin*[27] in lung cancer. In our study, we discovered that the hypermethylation of *TMEM88* in NSCLC was associated with an unfavorable prognosis. In addition to our findings, Mullapudi et al.[28] performed a genome-wide analysis of the lung cancer methylome and identified the hypermethylation of *TMEM88*. Lee et al.[26] demonstrated that promoter methylation of Wnt inhibitory factor 1 (*Wif1*) was an early and frequent event and the hypermethylation of Wif1 was an indicator of the unfavorable prognosis of non-small cell lung cancer. Hypermethylated Axin was also correlated with the progression of lung cancer[27]. Zhang et al.[23] revealed that the hypermethylation of frizzled-related protein 1 (*SFRP1*) was a common contributory factor in lung carcinogenesis and could serve as a potential biomarker for NSCLC in the Chinese population.

In our study, we demonstrated that *TMEM88* expression level was increased after the demethylation treatment of NSCLC cells and that the treatment resulted in the inhibition of cell migration, invasion, and proliferation. These results suggested that *TMEM88* may behave as a tumor suppressor in NSCLC and that the hypermethylation of *TMEM88* in NSCLC may be associated with an unfavorable prognosis. Previous studies have suggested that the activation of the Wnt signaling pathway promotes tumorigenesis[29]; conversely, the inhibition of Wnt signaling suppresses this process[3]. Our analysis of online data also indicated that *TMEM88* expression was correlated with some of the molecules involved in the Wnt signaling pathway. *TMEM88* inhibits Wnt/β-Catenin signaling through interaction with the Wnt pathway factor Dishevelled (*DVLS*)[14]. Therefore, the hypermethylation of the *TMEM88* gene may inhibit its function as a negative modulator of Wnt/β-Catenin signaling, which results in the constitutive activation of this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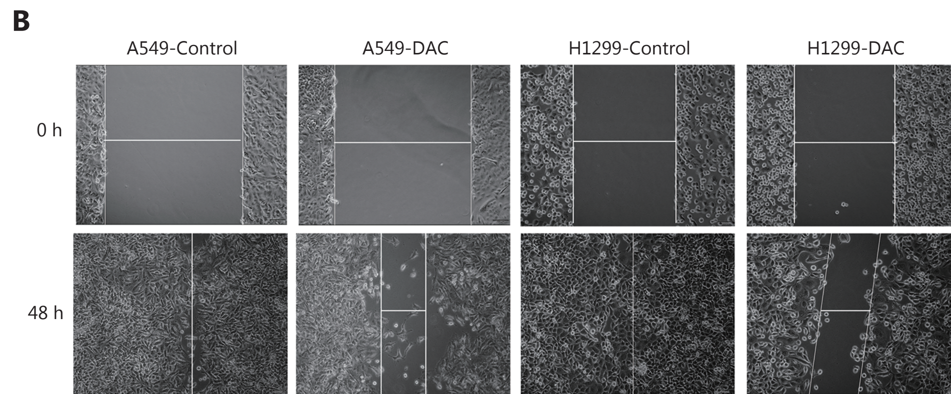
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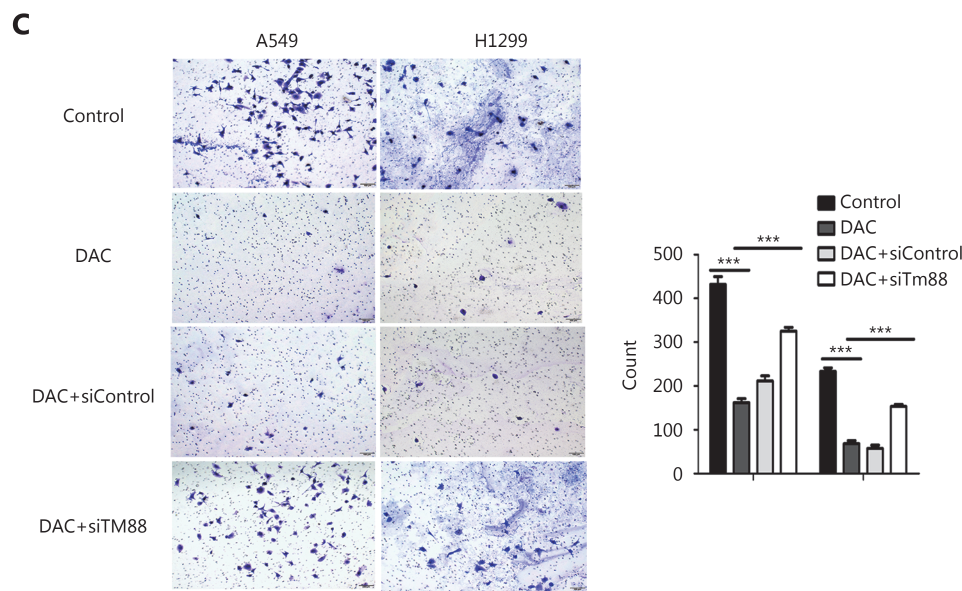
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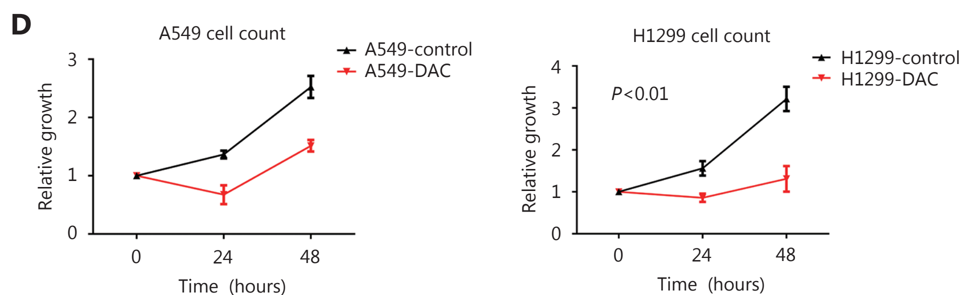
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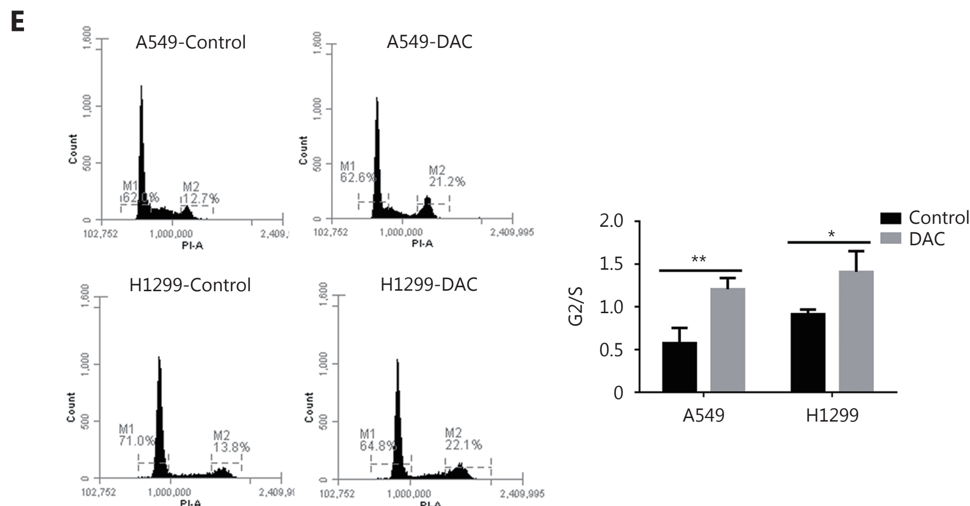

Figure 2 Demethylation of *TMEM88* by DAC and cell migration, invasion, proliferation, and cell cycle. (A) The levels of *TMEM88* methylation and expression after demethylation. (B) The demethylation of *TMEM88* inhibited cell migration in A549/H1299 cells. (C) The invasion ability of A549/H1299 cells after the demethylation and downregulation of *TMEM88*. (D) The demethylation of *TMEM88* inhibited cell proliferation in A549/H1299 cells. (E) Flow cytometric analysis showed a significant increase in the G2/M cell population after the demethylation of *TMEM88*. The data were presented as the mean±SD, * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ from Student's *t*-test in comparison with the control groups.

Table 3 Pearson correlation coefficients between the expression of *TMEM88* and the genes involved in the Wnt signal pathway in TCGA.

Gene symbol	Description	P	Pearson χ^2
DVL1	Dishevelled segment polarity protein 1	< 0.001	0.206
DVL2	Dishevelled segment polarity protein 2	< 0.050	0.102
DVL3	Dishevelled segment polarity protein 3	< 0.050	–0.107
FZD10	Frizzled class receptor 10	< 0.050	–0.101
FZD2	Frizzled class receptor 2	< 0.010	0.139
FZD4	Frizzled class receptor 4	< 0.001	0.266
FZD5	Frizzled class receptor 5	< 0.001	0.390
FZD7	Frizzled class receptor 7	< 0.050	–0.124
FZD8	Frizzled class receptor 8	< 0.010	0.138
ROR1	Receptor tyrosine kinase like orphan receptor 1	< 0.001	0.301
WNT5A	Wnt family member 5A	< 0.010	–0.155

signal pathway in NSCLC[30]. This may explain why the hypermethylation of *TMEM88* in NSCLC is associated with an unfavorable prognosis. There is evidence to demonstrate that promoter hypermethylation inactivates various Wnt pathway inhibitors, such as *WIF-1*[26,31], *sFRP-1*[12], *sFRP-5*[32], and *Dkk-3*[33], which are also associated with poor prognosis. Therefore, we concluded that the Wnt/β-Catenin signaling pathway was important in NSCLC biology and prognosis and *TMEM88* act as an inhibitor of this signal pathway. Further, the methylation of *TMEM88* may serve as a potential biomarker for Wnt/β-Catenin signaling when the pathway is targeted for therapy.

Recently, Zhang et al.[14] suggested that the presence of the *TMEM88* protein in the cytosol of NSCLC cells could promote cell invasion and metastasis through the activation of the p38-GSK3ß-Snail signal pathway. However, our study suggested that *TMEM88* had high methylation and low expression in tumors compared with normal tissues and that demethylation treatment could inhibit the invasion and migration of NSCLC cells (**Figure. 1** and **2**). Jang et al.[34] demonstrated that mir-708 could promote the invasion of NSCLC cells through the suppression of the expression of *TMEM88*, which suggested that *TMEM88* may be a tumor suppressor. Furthermore, the expression of *TMEM88* in 58 tumor samples and matched adjacent non-tumor tissues in the TCGA database was 25.27±12.58 and 171.88±97.04, respectively ($P < 0.0001$), which supported the behavior of *TMEM88* as a tumor suppressor. Studies have also suggested that *TMEM88* may have different forms of molecules, which are located in different cellular compartments and have different functions and activities[14,15]. Thus, more studies are needed to clarify the role of *TMEM88* in lung cancer.

In summary, we observed that the epigenetic silencing of *TMEM88* by promoter methylation was associated with a poor prognosis of NSCLC and that *TMEM88* could inhibit cell proliferation, migration, and invasion. These findings suggested that *TMEM88* may function as a tumor suppressor and serve as a potential biomarker for NSCLC prognosis and treatment.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (Grant No.81573231) and the Medical Professionals Crossing Project of Shanghai Jiao Tong University (Grant No. YG2015ZD01).

Conflict of interest statement

No potential conflicts of interest were disclosed.

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Cite this article as: Ma R, Feng N, Yu X, Lin H, Zhang X, Shi O, et al. Promoter methylation of Wnt/β-Catenin signal inhibitor *TMEM88* is associated with unfavorable prognosis of non-small cell lung cancer. Cancer Biol Med. 2017; 14: 377-86. doi: 10.20892/j.issn.2095-3941.2017.0061