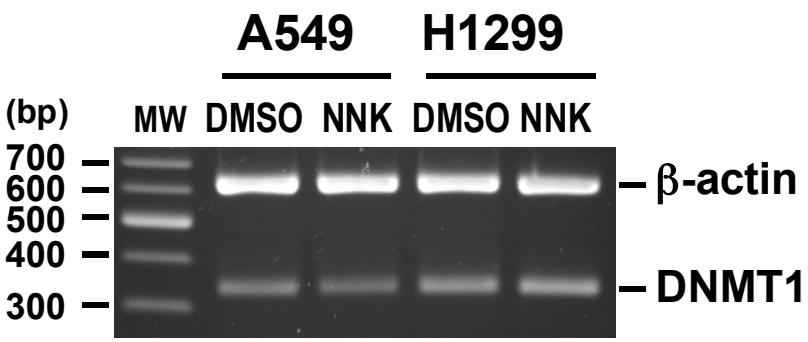
Supplemental Information

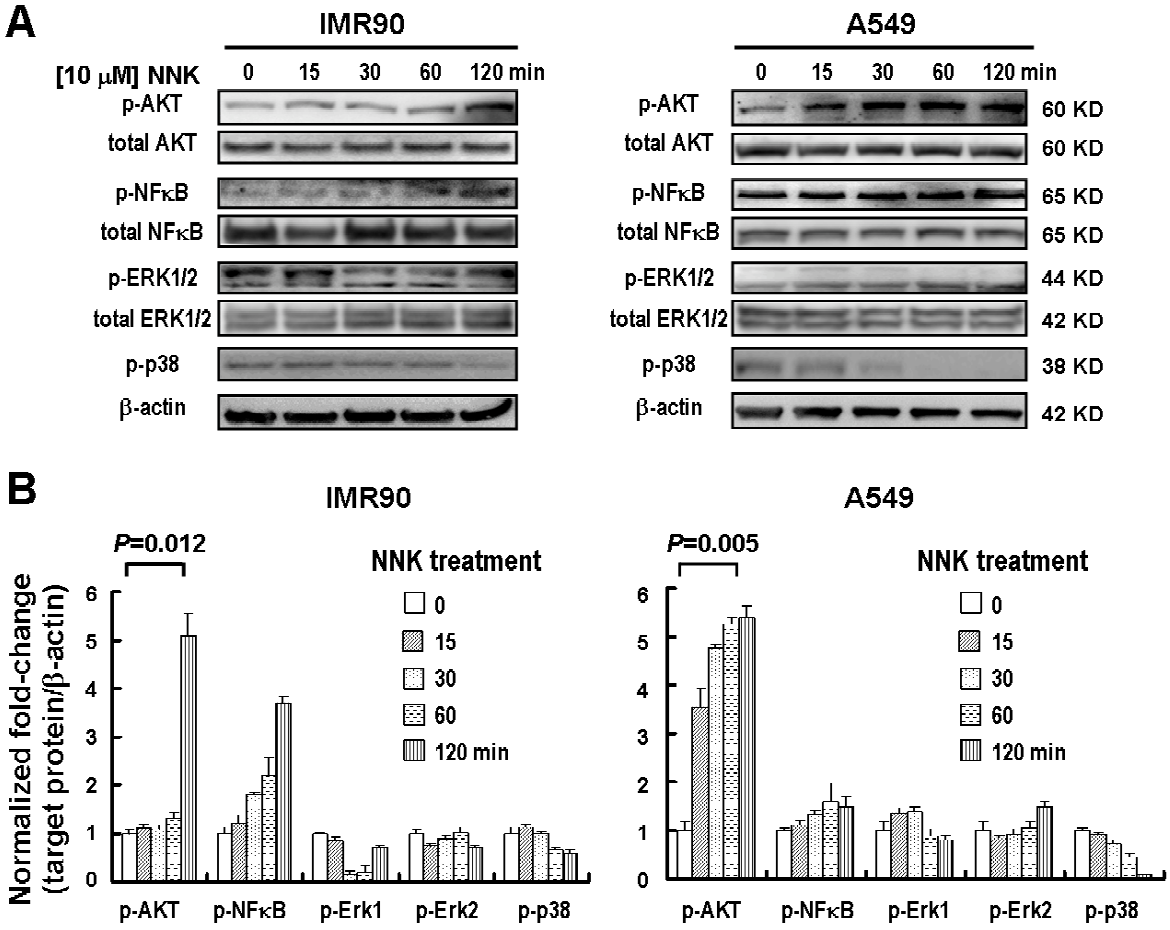
Tobacco-specific Carcinogen Induces DNA Methyltransferases 1 Accumulation through AKT/GSK3β/βTrCP/hnRNP-U in Mice and Lung Cancer patients

Ruo-Kai Lin,[1] Yi-Shuan Hsieh,[2] Pinpin Lin,[3] Han-Shui Hsu,[4] Chih-Yi Chen,[5] Yen-An Tang[1], Chung-Fan Lee,[6] and Yi-Ching Wang[1,*]

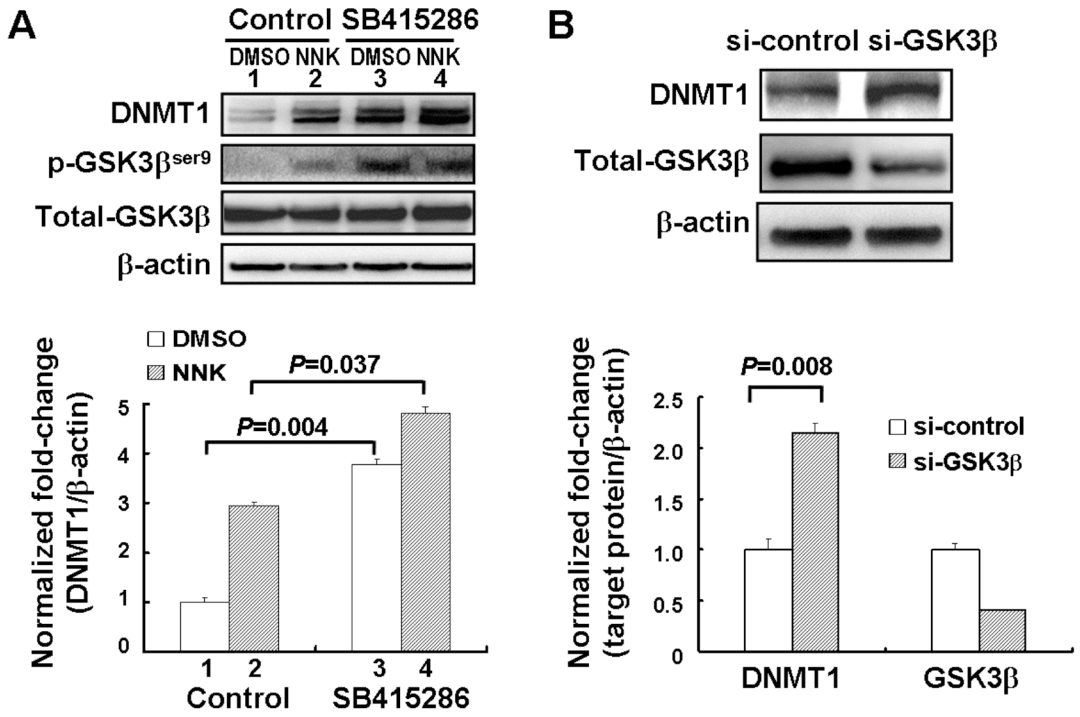
Supplemental figures and legends



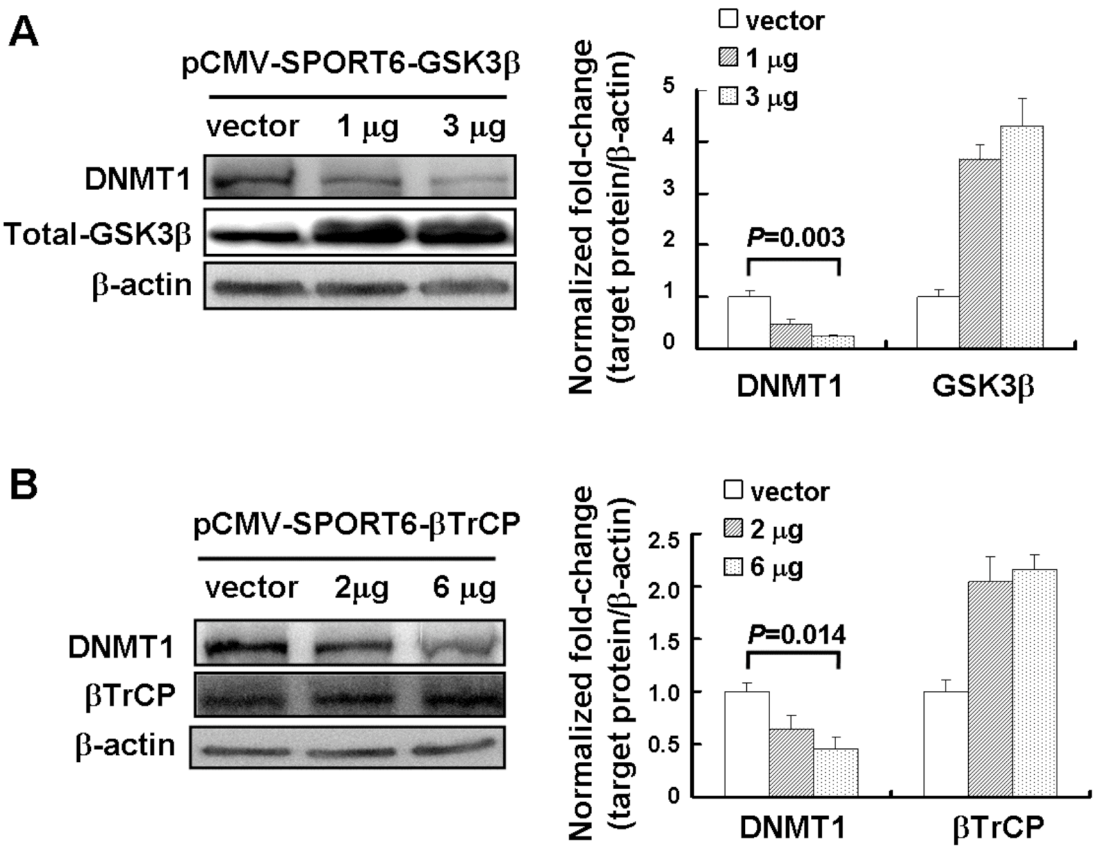
Supplemental Figure 1 NNK-treated A549 and H1299 cells showed no change in the endogenous *DNMTs* mRNA expression levels.
The expression level was measured by RT-PCR The housekeeping gene *β-actin* was used as an internal control.



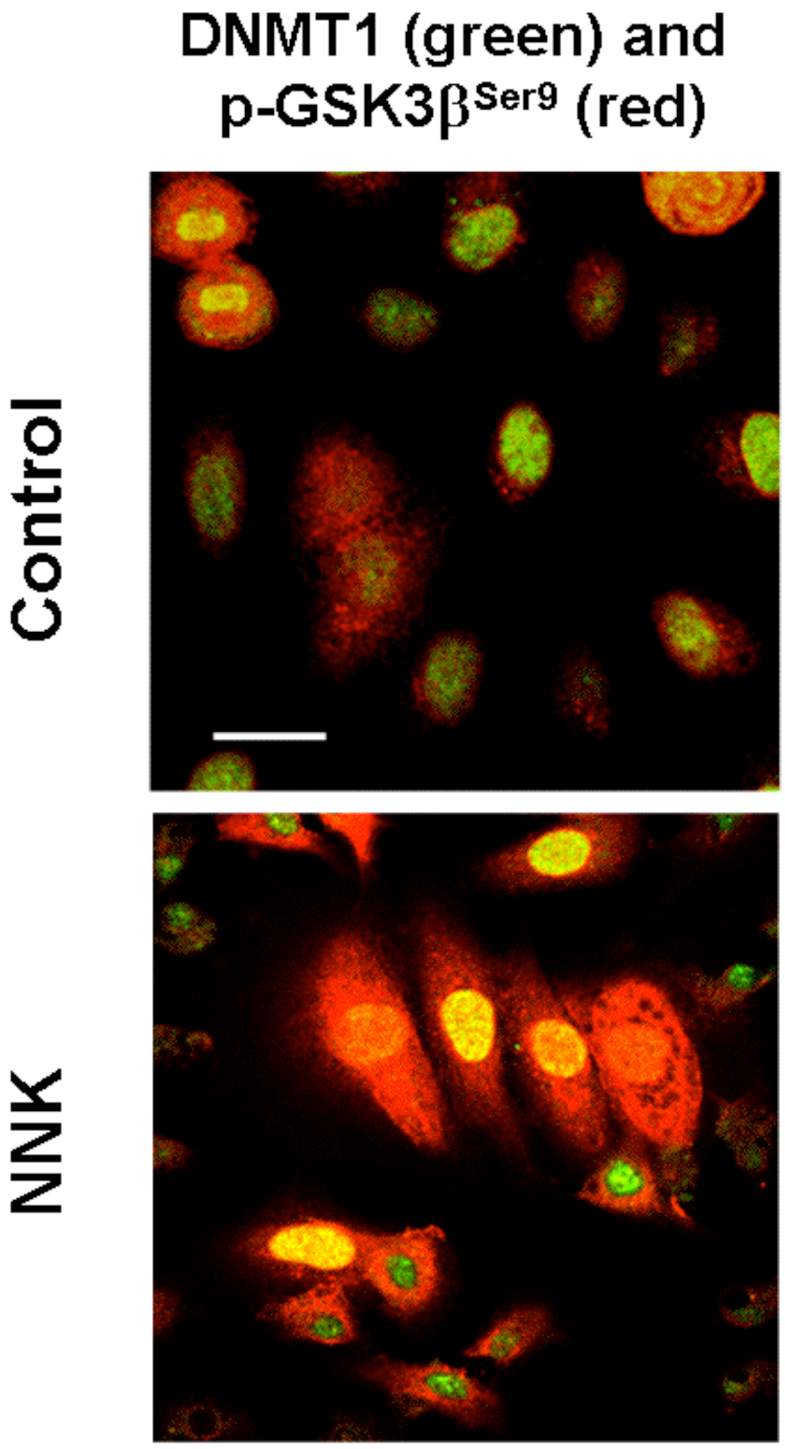
Supplemental Figure 2 The signaling pathways induced by NNK.
(A) IMR90 normal lung cell line and A549 lung cancer cell lines were treated with 10 μM NNK for various times as indicated. Phosphorylation of AKT was analyzed by Western blotting using phospho-AKT at Ser473 antibody. Total AKT antibody was used to confirm and quantify AKT protein. Western blot analysis was performed to detect phosphorylated NFκB or total NFκB by using a phospho-specific NFκB or total NFκB antibodies; phosphorylated ERK1/2 or total ERK1/2 by using a phospho-specific ERK1/2 or a mixture of ERK1 and ERK2 antibodies. Phosphorylation of p38 was analyzed by Western blotting using phospho-p38 antibody. β-actin used an internal control. **(B)** Quantitative figures were determined from at least three experiments. Columns, mean; bars, ±SEM. and *P* values are as indicated.



Supplemental Figure 3 GSK3β involved in DNMT1 protein degradation and the increase of DNMT1 protein level by NNK in IMR90 cells.
(A) IMR90 cells were treated with or without 25 μM GSK3β inhibitor, SB415286 for 24 hr, and then treated with 10 μM NNK for 2 hr. Increased in DNMT1 protein expression after inhibition of GSK3β activity by NNK and SB415286 was observed (lanes 2-4 versus lane 1 in DNMT1 level). Phosphorylated GSK3β^{ser9} (inactive form can be seen after NNK and SB415286 treatment (lanes 2-4 versus lane 1 in GSK3β^{ser9} level). Protein expression levels of DNMT1, phosphorylation of GSK3β^{ser9}, and total GSK3β were analyzed by Western blotting with β-actin as an internal control. Densitometry showing the change of DNMT1 protein level. **(B)** Knockdown of GSK3β in cells treated by siRNA enhanced DNMT1 protein level. Columns, mean; bars, ±SEM (n=3). *P* values are as indicated.

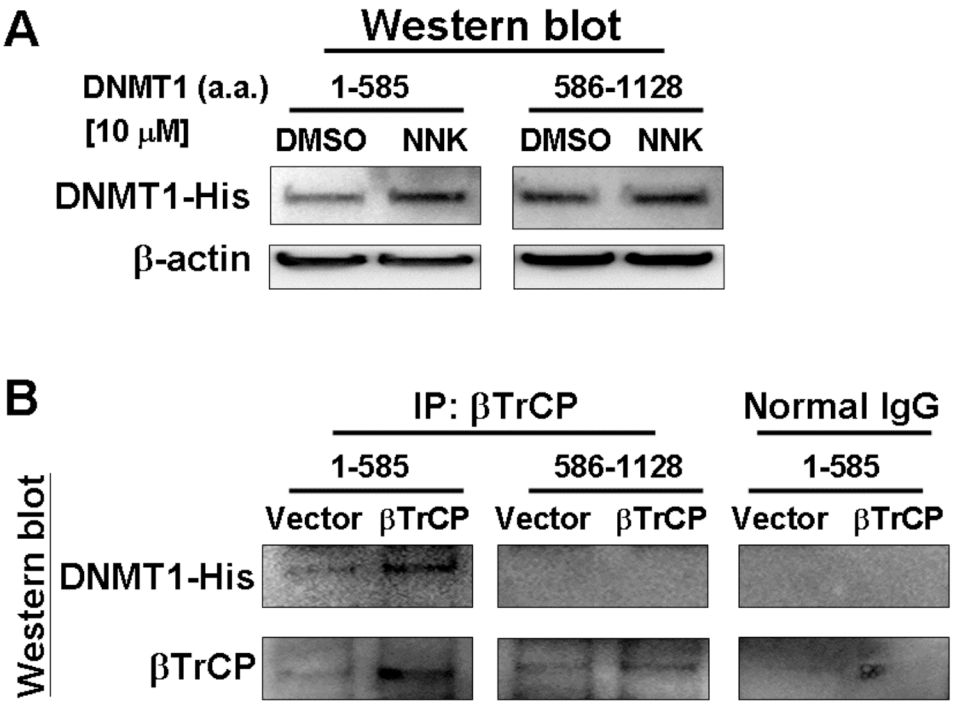


Supplemental Figure 4 Exogenous GSK3β and βTrCP induced DNMT1 protein degradation.
(A) GSK3β and **(B)** βTrCP promoted DNMT1 protein degradation in a dose-dependent manner as seen using both Western blot and densitometry assays. A549 cells were transfected with pCMV-SPORT6-GSK3β or pCMV-SPORT6-βTrCP at indicated doses for 24 h. Columns, mean; bars, ±SEM (n=3). *P* values are as indicated.

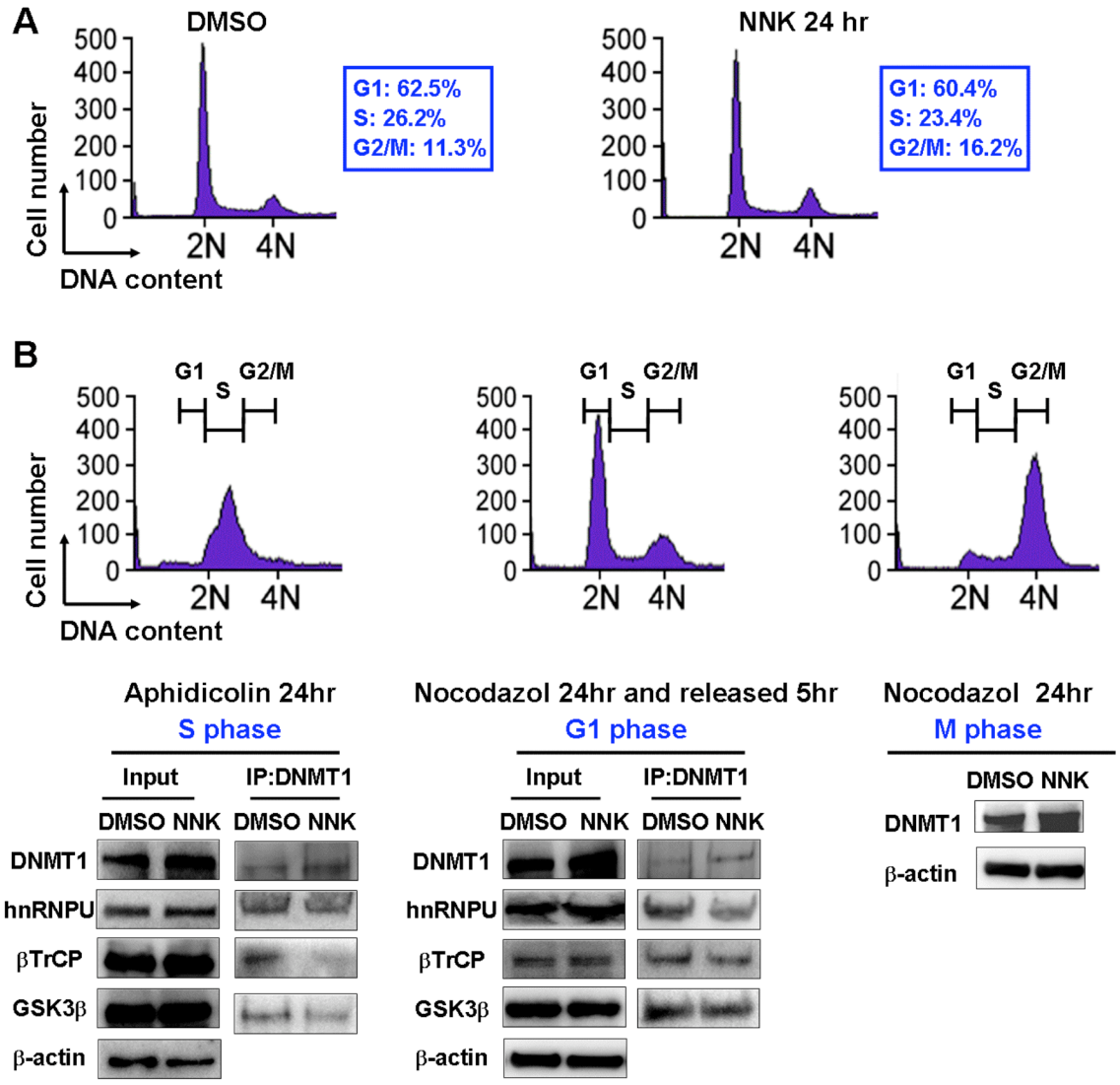


Supplemental Figure 5 An increase of DNMT1 and p-GSK3β^{Ser9} expression and their co-localization (yellow color) were observed in cells treated with NNK.

Immunofluorescence staining of DNMT1 (green color) and p-GSK3β^{Ser9} (red color) was performed and visualized by confocal microscopy. Scale bar: 40 _m.



Supplemental Figure 6 The N-terminal domain of DNMT1 could be induced by NNK treatment which was important for βTrCP interaction.
(A) Analysis of two cDNA deletion constructs of His-tag DNMT1 expression vector, which contained either amino acids 1-585 or 585-1128 of DNMT1, are shown. The N-terminal domain of 1-585 amino acids could be induced by NNK treatment. **(B)** Immunoprecipitation assay was performed in A549 cells transfected with control vector or pCMV-SPORT6-βTrCP with various regions for 24 hr. Cell lysates were immunoprecipitated with anti-βTrCP antibody and Western blotted with anti-His-DNMT1 antibody. Normal-IgG is a negative control. Data demonstrated that N-terminal domain of 1-585 amino acids of DNMT1 was important for βTrCP interaction.



Supplemental Figure 7 NNK-induced DNMT1 accumulation by AKT/GSK3β/βTrCP/hnRNP-U signaling pathway was not due to the impact of NNK treatment on the cell cycle.

(A) Flow cytometry showed that 10μM of NNK treatment for 24 hours did not change cell cycle distribution in A549 cells (upper panel). **(B)** Flow cytometry was performed in A549 cells treated with aphidocolin, nocodazole, and 5 hour post-release from nocodazole to confirm the induction of S, G2/M, and G1 phases, respectively. Western or Immunoprecipitation assay (lower panel) of cell treated with 10 μM NNK at specific cell cycle phases to show the induction of DNMT1 protein level and the interaction between DNMT1 and hnRNPU, βTrCP, and GSK3β. Data demonstrated that NNK-induced DNMT1 accumulation could be seen in all phases of cell cycle.

Supplemental Tables

Supplemental Table 1 Multivariate Analysis of factors associated with poor survival [A].

Variable	Risk Ratio	95%CI	*P*
Age	0.965949	(0.629407-1.425018)	0.8660
Tumor type	1.000318	(0.709554-1.391339)	0.9985
Tumor stage	1.085139	(0.767648-1.541339)	0.6432
Smoking and DNMT1(+)	1.524797	(1.099739-2.143316)	0.0114

[A.] Results were analyzed by the Cox proportional hazards regression modal.

Supplemental Table 2 The mRNA expression of DNMT1 was not associated with the smoking status of patients [A]

Characteristics		DNMT1 mRNA	
	Total	Non-overexpression	Overexpression
	N	N (%)	N (%)
Overall	85	48 (51.1)	37 (48.9)
Smoking habit			
Smoker	44	25 (58.6)	19 (43.2) $^{0.947}$
Non-smoker [B]	41	23 (56.1)	18 (43.9)

A. Results were analyzed by the Pearson Chi-Square test. Degree of freedom = 1. *P* value with significance is shown as superscript.

B. The non-smoker group includes ex-smoker and never smoker.

Supplemental Table 3. Overexpression of DNMTs proteins and clinical parameters in NSCLC tumors.[A]

Characteristics		Total [B]	DNMT1	
		n	High expression n (%)	Normal expression n (%)
Overall		124	60 (48.4)	64 (51.6)
Clinicopathological parameters				
Age	< 65	35	19 (54.3)	16 (45.7)
	≥ 65	89	41 (46.1)	48 (53.9)
Tumor type				
	SQ	48	24 (50.0)	24 (50.0)
	AD	67	33 (49.3)	34 (50.7)
Tumor stage				
	I + II	60	28 (46.7)	32 (53.3)
	III + IV	64	32 (50.0)	32 (50.0)

[A.] Results were analyzed by the Pearson Chi-Square test. Degree of freedom = 1. *P* value with significance is shown as superscript.

[B.] Total number of samples (n) in some categories is less than the overall number analyzed because clinical data was not available for some samples.

Supplemental Table 4 The antibodies and their reaction conditions used in the present study

Target	K.D.	Raised In	Application	Dilution	Source	Catalog No.
Anti-DNMT1	190	Chicken	Western blot	1:10000	Asia Hepato Gene Co.	A22659
			Immunoprecipitation	1:100		
			Immunohistochemistry	1:500		
			Immunofluorescence			
		Mouse	Immunohistochemistry	1:30	Imgenex	IMG261A
Anti-_-actin	42	Mouse	Western blot	1:5000	Novus Biologicals	NB 600-501
Anti-LAMIN A/C	62/69	Mouse	Western blot	1:2000	Santa Cruz	sc-7292
Anti-GAPDH	38	Mouse	Western blot	1:2000	Santa Cruz	Sc-32233
Anti-AKT	60	Rabbit	Western blot	1:1000	Cell Signaling	9272
			Immunoprecipitation	1:100		
Anti-phospho-AKT^{Ser473}	60	Rabbit	Western blot	1:500	Cell Signaling	9271
			Immunohistochemistry	1:30		3787
Anti-Erk1/2	42,44	Rabbit	Western blot	1:1000	Upstate	06-182
Anti-phospho-Erk1/2	42/44	Mouse	Western blot	1:1000	Upstate	05-481
Anti-phospho-p38^{Thr180}	38	Rabbit	Western blot	1:1000	Chemicon	AB3828
Anti-NF_B	65	Rabbit	Western blot	1:1000	Upstate	06-418
Anti-phophor-NF_B^{Ser536}	65	Rabbit	Western blot	1:1000	Cell Signaling	3031
Anti-GSK3β	46	Rabbit	Western blot	1:5000	Cell Signaling	9315
			Immunoprecipitation	1:125		
Anti-phospho-GSK3β^{Ser9}	46	Rabbit	Western blot	1:1000	Nuvus	NB100 - 81948
			Immunohistochemistry	1:100		
			Immunofluorescence	1:100		
Anti-βTrCP	70	Mouse	Western blot	1:500	Zymed	37-3400
		Goat	Immunohistochemistry	1:300	Santa Cruz	sc-8862
			Immunofluorescence			
			Immunoprecipitation	1:25		
Anti-p53	53	Mouse	Western blot	1:1000	DAKO	DO-7

Anti-His	-- [A]	Rabbit	Western blotting	1:500	Cell Signaling	2365
			Immunoprecipitation	1:50		
Anti-phospho-serine	-- [A]	Mouse	Western blotting	1:500	Sigma	P3430
			Immunoprecipitation	1:50		
Anti-hnRNP-U	130	Mouse	Western blotting	1:2000	Santa Cruz	sc-32315
			Immunofluorescence	1:50		
			Immunoprecipitation			
Anti-hnRNP-U	130	Rabbit	Immunofluorescence	1:50	Santa Cruz	sc-25374
			Immunohistochemistry			
Anti-ubiquitin	-- [A]	Mouse	Western blotting	1:1000	Santa Cruz	sc-8017
			Immunoprecipitation	1:50		

[A]. -- Molecular weight is variable.

Supplemental Table 5 List of primers and siRNA sequences and their reaction conditions used in the present study

Gene [A]	primer	5'→ 3' sequences	PCR size	Tm	Cycle
DNMT1-RT-PCR	Forward Reverse	ACCGCTTCTACTTCCTCGAGGCCTA GTTGCAGTCCTCTGTGAACACTGTGG	334	60°C	30
_-*actin*-RT-PCR	Forward Reverse	ACACTGTGCCCATCTACGAGG AGGGGCCGGACTCGTCATACT	618	60°C	30
p16-Bis-sequencing	Forward Reverse	GTTTTTTTGTTTGGAAAGATAT CRCCCRCACCTCCTCTACCC	278	54°C	35
p16-methyl-MSP-O	Forward Reverse	GTTTTTTTGTTTGGAAAGATAT CTACCCATCATCATAACCTAAATC	232	54°C	35
p16-methyl-MSP-U	Forward Reverse	TTATTAGAGGGTGGGGTGGATTGT CAACCCCAAACCACAACCATAA	151	65°C	35
p16-methyl-MSP-M	Forward Reverse	TTATTAGAGGGTGGGGCGGATCGC GACCCCCGAACCGCGACCTAA	150	65°C	35
*RAR*_-methyl-MSP-O	Forward Reverse	AAGTGAGTTGTTTAGAGGTAGGAGGG CCTATAATTAATCCAAATAATCATTTACC	280	54°C	35
*RAR*_-methyl-MSP-U	Forward Reverse	TTGAGAATGTGAGTGATTTGA AACCAATCCAACCAAAACAA	146	54°C	35
*RAR*_-methyl-MSP-M	Forward Reverse	TCGAGAACGCGAGCGATTCG GACCAATCCAACCGAAACGA	146	54°C	35
FHIT-methyl-MSP-O	Forward Reverse	TAGTGGGTATATTTTTAG ATCCCACCCTAAAACCTC	249	50°C	40
FHIT-methyl-MSP-U	Forward	GAAGGTAGGGGTGGGGAGGTAAGTT	116	68°C	35

	Reverse	CATAAACAACACCAACCCCACTA			
FHIT-methyl-MSP-M	Forward Reverse	GAAGGTAGGGGCGGGGAGGTAAGTT CGTAAACGACGCCAACCCACTA	116	68^{o}C	35
p16-promoter-ChIP	Forward Reverse	TTGAAGCTGGTCTTTGGAT ATCGAAATCACCTGTACGACT	400	65^{o}C	35
*RAR*_-promoter-ChIP	Forward Reverse	GGCAACCAACATGGCTATTT TATAGTGGTCAAGAGCATGGGT	401	65^{o}C	35
FHIT-promoter-ChIP	Forward Reverse	ATGCTCTGCGCGTATTGGC GGTGATGACCTGACGCG	322	61^{o}C	35
GAPDH-promoter-ChIP	Forward Reverse	AATGAAAGGCACACTGTCTCTCTC GTTTCTGCACGGAAGGTCAC	218	65^{o}C	35
RNAi of *AKT*	Forward Reverse	AUUCUUGAGGAGGAAGUAGCGUGGC GCCACGCUACUUCCUCCUCAAGAAU			
RNAi of GSK3β	Forward Reverse	AUUACUUGACAGUUCUUGAGUGGUG CACCACUCAAGAACUGUCAAGUAAU			

[A] RT-PCR: reverse-transcriptase polymerase chain reaction; MSP: methylation-specific PCR; O: outer primer; U: unmethylation - specific primer; M: methylation-specific primer; ChIP: chromatin immunoprecipitation; RNAi: interference RNA.