Methylated +322-327 CpG site decreases *hOGG1* mRNA expression in non-small cell lung cancer

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Abstract. hOGG1 plays a role in several disease pathways, including various cancers. Despite such functional importance, how hOGG1 is regulated at the transcriptional level in human non-small cell lung cancer (NSCLC) remains unknown, particularly via DNA methylation changes. We obtained NSCLC tissues and adjacent non-cancerous tissues and examined *hOGG1* mRNA expression levels. NSCLC cells were treated with 5-Aza to test whether DNA methylation can influence the expression of *hOGG1*. The MassARRAY EpiTYPER and luciferase reporter gene assays were used to define the functional region of the *hOGG1* gene (including CpG sites). Finally, ChIP assay was utilized to verify transcription factor binding to the *hOGG1* 5'-UTR region. Our previous studies supported the idea that the methylation of the *hOGG1* gene promoter region occurs frequently in NSCLC. Treatment with 5-Aza, a demethylating agent, led to a significant restoration of *hOGG1* expression in NSCLC cell lines. Quantitative PCR and MassARRAY EpiTYPER assays demonstrated that methylation of the +322-327 CpG site in the 5'-UTR region of *hOGG1* was higher in NSCLC tissues compared with adjacent non-cancerous tissues. Notably, the methylation level of +322-327 site (T/N) was inversely correlated with that of *hOGG1* mRNA level (T/N) in 25 NSCLC tissues. ChIP assay and *in silico* prediction showed an association between the +322-327 CpG site and Sp1, which has been reported to be an activator of transcription. Importantly, luciferase reporter gene and ChIP assays showed that +322-327 CpG site methylation particularly reduced the recruitment of Sp1 to the 5'-UTR sequence in *hOGG1* and reduced transcriptional activity ~50%. In summary, we have demonstrated that *hOGG1* mRNA is downregulated in NSCLC tissues. Moreover, we identified that the methylated +322-327 CpG site in the *hOGG1* 5'-UTR is associated with reduced expression of *hOGG1* by decreasing the recruitment of Sp1 to the 5'-UTR of *hOGG1*.

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Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide (1,2). Non-small cell lung cancer (NSCLC) accounts for ~85% of lung cancer. Despite improvement in cancer treatment, the 5-year survival rate remains less than 10%. The 5-year survival rate is estimated to be 55-80% with an earlier lung cancer diagnosis or surgery at an early stage (3). Preferential understanding of how NSCLC develops and progresses plays an important role in early detection and prevention as well as targeted treatment of NSCLC.

Previous studies have shown that DNA repair pathways commonly exhibit functional overlap to ensure genomic stability; therefore, challenging the perception that distinctive lesions are repaired by different mechanisms in the mammalian genome. This finding is especially true for oxidative damaged DNA, such as oxidized bases (4), which might be caused by oxidant exposure or ionizing radiation, but could also result from normal cellular metabolism. Oxidized bases are mutagenic and cytotoxic, and several previous studies have shown that oxidized bases may contribute to neurodegeneration, aging and cancer (5). Base excision repair (BER) is an important DNA repair pathway that is responsible for the repair of DNA base damage and single strand breaks caused by X-rays, oxygen radicals or alkylating agents (6).

OGG1, a DNA repair glycosylase that localizes to both the nucleus and mitochondria, is the main enzyme responsible for the identification and excision of 8-oxoG lesions, which

produces G:C to T:A transversions (7-9). OGG1 is one of the components of the BER pathway. The human OGG1 (*hOGG1*) gene is found on chromosome 3p26.2, which is one of the most frequent genomic deletion regions that contains some potential tumor suppressor genes in various types of tumors, such as NSCLCs (10). Previous studies suggested that hOGG1 plays a role in several disease pathways, including various cancers (11-13). Despite such functional importance, how *hOGG1* is regulated at the transcriptional level in NSCLC remains largely unclear, particularly via DNA methylation changes.

It is well known that epigenetic regulation, such as DNA methylation, can alter gene expression (14-16). DNA methylation frequently occurs in CpG islands, which are frequently found in the 5'-untranslated regions (5'-UTR) of genes (17). DNA methylation changes at site-specific CpGs may play a crucial role in cancer progression, including hypermethylation of tumor suppressor genes and hypomethylation of oncogenes (14,18). Our previous studies have shown that DNA methylation underlies inactivation of the CpG island methylator phenotype (CIMP) and that TSGs on 3p might be a frequent epigenetic event that confers an increased risk of developing NSCLC (10,19). Moreover, we previously showed that a methylated +58 CpG site in the DCN 5'-UTR was associated with reduced *DCN* mRNA expression in highly metastatic NSCLC cells (20). Hence, we hypothesize that site-specific CpG methylation affects *hOGG1* mRNA expression levels in NSCLC.

Materials and methods

Tissue samples. Seventy-seven paired NSCLC tissues and adjacent non-cancerous tissues were obtained after informed consent from patients in the First Affiliated Hospital of Soochow University between 2009 and 2013. Blood specimens were obtained after informed consent from 30 randomly selected NSCLC and paired non-cancerous lung patients. Blood was isolated by centrifugation at 3,500 rpm for 20 min after blood sampling (10). The Revised International System for Staging Lung Cancer was used to determine histological and pathological diagnostics for NSCLC patients. No chemotherapy or radiotherapy was given to patients with NSCLC before tissue sampling. Tissue samples were stored at -80˚C after being snap-frozen. The present study was approved by the Ethics Committee of the First Affiliated Hospital of Soochow University.

Cell culture and drug treatment. Human lung carcinoma cell lines (A549, H1650, H460, SPC-A-1, 95C, 95D, H226 and SK-MES-1) were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), and human bronchial epithelial (HBE) cells from Shanghai Bogoo Biotechnology, Co., Ltd. (Shanghai, China). Cell lines were seeded and grown in RPMI-1640 medium (HyClone Laboratories, Inc., Logan, UT, USA), with the exception of SK-MES-1, which was seeded in MEM medium (HyClone Laboratories), with 10% heat-inactivated fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA) and L-glutamine and antibiotics (Invitrogen, Carlsbad, CA, USA) in a humidified incubator containing 5% CO_2 at 37˚C. Treatment with 5-aza-2'-deoxycitidine (5-Aza; Sigma-Aldrich, St. Louis, MO, USA) was used to demethylate cells in culture according to the previously described treatment protocol (10).

Quantitative determination of human 8-oxoguanine DNA glycosydase (hOGG1) concentrations and evaluation of DNA damage in serum. The human 8-oxoguanine DNA glycosydase (hOGG1) ELISA kit (cat. no. CSB-E12686h; Wuhan, Hubei, China) was used to quantitatively determine hOGG1 concentrations in serum. Briefly, standard controls and samples (100 μl) were added to each well and covered with a provided adhesive strip; cells were incubated at 37˚C for 2 h. The standard and sample results were recorded in a plate layout that was provided by the manufacturer. Next, each well received 100 μl of biotin-antibody (1x), and the plate was covered with a new adhesive strip. Cells were incubated at 37˚C for 1 h. If the biotin-antibody (1x) appeared cloudy, it was warmed to room temperature and mixed gently until the solution appeared uniform. Next, each well received 100 μl of HRP-avidin (1x), and the plate was covered with a new adhesive strip. Cells were incubated at 37˚C for 1 h. The aspirate was washed five times, and 90 μl of TMB substrate was added to each well. Cells were incubated at 37˚C for 15-30 min. Cell plates were protected from light. A total of 50 μl of stop solution was added to each well, thoroughly mixed and hOGG1 was detected using a microplate reader.

8-OHdG, 8-hydroxyguanine and its 2'-deoxynucleoside equivalent, 8-hydroxy-2'-deoxyguanosine (8-OHdG) are common byproducts of DNA damage. During the repair of damaged DNA *in vivo* by exonucleases, 8-hydroxy-2'-deoxyguanosine (8-OHdG) is excreted (21,22); therefore, we used an OxiSelect Oxidative DNA Damage ELISA kit (8-OHdG Quantitation; Cell Biolabs, Inc., San Diego, CA, USA) to evaluate the level of DNA damage in serum according to the manufacturer's instructions (23-25). The extracted DNA was dissolved in water to reach a concentration of 1-5 mg/ml. The DNA was converted to single-stranded DNA by incubating at 95˚C for 5 min, then promptly chilling on ice. The denatured DNA samples were digested to nucleosides by incubating with 5-20 units of nuclease P1 at 37˚C for 2 h in 20 mM sodium acetate (pH 5.2). DNA samples were treated with 5-10 units of alkaline phosphatase at 37˚C for 1 h in 100 mM Tris (pH 7.5), followed by centrifugation for 5 min at 6,000 x g. Subsequently, the supernatant was used for the 8-OHdG enzyme-linked immunosorbent assay (ELISA).

RNA extraction, cDNA synthesis and quantitative real-time PCR (qRT-PCR). Total RNA of cells and tissues were extracted by adding 1.0 ml RNAiso Plus (Takara Bio, Osaka, Japan) according to the manufacturer's protocol. The RNA concentration was measured using a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA) and synthesis of cDNA was performed using Reverse Transcriptase M-MLV (Takara Bio) with reverse transcriptase. The sequences of qRT-PCR for *hOGG1* and *GAPDH* were as follows: *hOGG1*, forward, 5'-ATCGTACTCTAGCCTCCACTCC-3' and reverse, 5'-GTC AGTGTCCATACTTGATCCGC-3'; *GAPDH*, forward, 5'-TG CACCACCAACTGCTTAGC-3' and reverse, 5'-TGCACC ACCAACTGCTTAGC-3'. qRT-PCR was performed using SYBR Premix ExTaq™ (Takara Bio) according to the manufacturer's instructions on an ABI StepOnePlus Real-Time PCR

system (Applied Biosystems, Foster City, CA, USA). The PCR program was 50˚C for 2 min, 95˚C for 10 min, followed by 45 cycles of 95˚C for 15 sec and 60˚C for 1 min.

Quantitative methylation analysis of DNA was performed using MassARRAY EpiTYPER assays. Quantitative methylation analysis of DNA was performed using MassARRAY EpiTYPER assays (Sequenom, Inc., San Diego, CA, USA) according to the protocol recommended by the manufacturer (26). This system uses matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry in combination with RNA base-specific cleavage (Mass Cleave). After bisulfite modification, genomic DNA was amplified using MassArray primers. PCR products were introduced to a T7 promoter sequence by the Beijing Bio-Miao Biotechnology, Co., Ltd. (Beijing, China). Next, RNA products were transcribed *in vitro* using T-base-specific cleavage, in which small RNA fragments were obtained. The molecular weight of each fragment was detected by flight mass spectrometry (MALDI-TOF) and methylation levels were analyzed using EpiTyper software. PCR amplification bias was controlled for by using DNA methylation standards (0, 20, 40, 60, 80 and 100%) and data was normalized by correction algorithms based on an R statistical computing environment.

Construction of luciferase reporter plasmids, transient transfection and luciferase assay. To construct a plasmid containing the *hOGG1* promoter, we used pGL3 basic vector (Promega, Madison, WI, USA). Briefly, an 88-bp fragment containing the predicted Sp1 target site (positions +322-327) was chosen for the luciferase assay. The wild-type and mutated fragment was directly synthesized (Genewiz, Suzhou, China) and subcloned into the pGL3 basic vector to generate pGL3-wild-type (WT: tggtccttgtctgggCGgggtctttgggCGtCGaCGaggcctggt tctggg taggCGgggctactaCGgggCGgtgcctgctgtggaa) and pGL3-mutant plasmid (Mut: tggtccttgtctgggCGgggtctttgggCGtCG aCGaggcctggttctgggtaggCGgggctactaCTggAATgtgcctgctgt ggaa). Subsequently, A549 and SPC-A1 cells were plated in a 24-well plate and cotransfected with wild-type plasmid, mutated plasmid, or pRL-TK plasmid using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA). After 48 h, cells were collected, and luciferase activities were measured by the Dual-Luciferase reporter assay kit (Promega). Each experiment was performed in triplicate.

Chromatin immunoprecipitation (ChIP) assay. ChIP assay was carried out as previously described (20). Briefly, immunoprecipitation was performed using 5 μg anti-Sp1 antibody (Cell Signaling Technology, Beverly, MA, USA). Purified ChIP DNA was subjected to PCR, using primers specific for the *hOGG1* promoter region (positions +247 to +398) encompassing the putative Sp1-binding site. Specific ChIP primers used for PCR were as follows: forward, 5'-TAAGGGTCGTG GTCCTTGTC-3' and reverse, 5'-TGGAGGCTAGAGTACGA TGC-3'.

Results

Serum levels of hOGG1 are decreased and 8-OHdG levels are increased in NSCLC samples. *hOGG1* gene encodes a DNA glycosylase that catalyzes the excision and removal of 8-OH-dG adducts (27). A previous report has shown a decrease of hOGG1 in the brain of Alzheimer's patients (28), which caused the accumulation of 8-oxoG in the mitochondrial DNA of neurons and calpain-dependent neuronal loss (12). In addition, an association between the *hOGG1* gene and lung cancer risk has been reported (27,29). Here, we detected the level of hOGG1 and 8-OH-dG using ELISA assays. As illustrated in Fig. 1A, the level of hOGG1 was lower in NSCLC serum than in paired normal serum. Furthermore, we detected 8-OH-dG and found that the level of 8-OH-dG was higher in NSCLC serum than in paired normal serum (Fig. 1B).

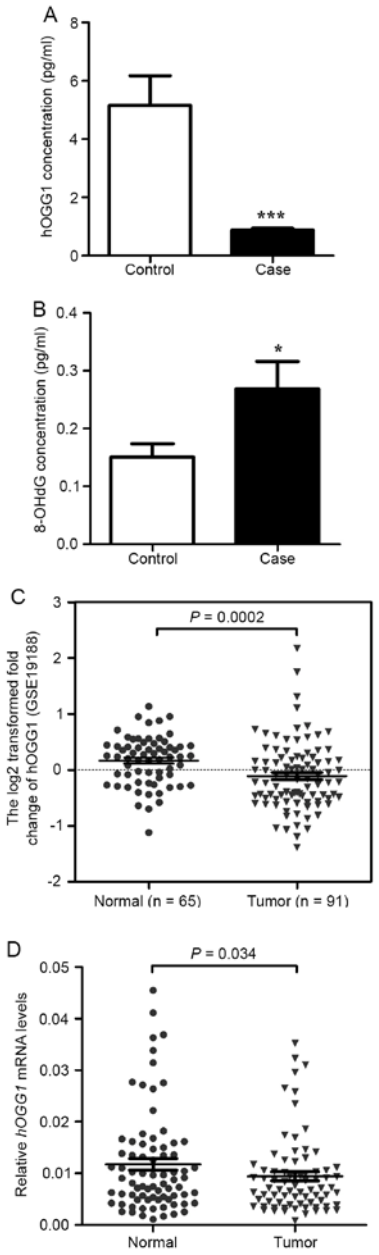

Figure 1. Expression of hOGG1 and 8-OHdG in NSCLC patients. (A) ELISA kit analysis of the level of hOGG1 in 30 NSCLC serum and paired normal serum. (B) The concentration of 8-OHdG in 30 NSCLC serum and paired normal serum. (C) Scatter plots showing relative *hOGG1* mRNA expression levels of NSCLC tumors and adjacent normal lung tissues in a public data set (GSE19188). (D) *hOGG1* mRNA levels expressed in 77 NSCLC tissues and paired non-cancerous lung tissues. *P<0.05; ***P<0.001.

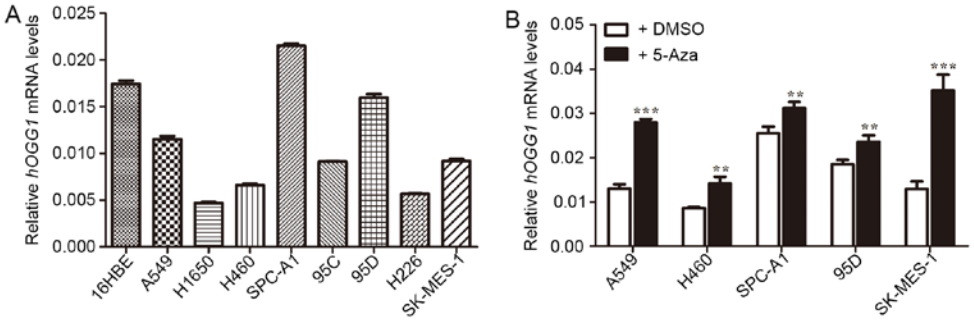

Figure 2. *hOGG1* mRNA expression in NSCLC cell lines and 5-Aza treated NSCLC cells. (A) Real-time PCR analysis of *hOGG1* mRNA levels in HBE, A549, H1650, 95C, 95D, H226 and SK-MES-1 cells. *hOGG1* mRNA levels are expressed as a relative index normalized against *GAPDH*. (B) Expression of mRNA in A549, H460, SPC-A1, 95D and SK-MES-1 cells in the presence or absence of 5-Aza (10 μM). Data summarized in the bar charts are represented as mean ± SD of three independent fields or experiments. **P<0.01; ***P<0.001.

Table I. Demographic and clinical characteristics of NSCLC patients and the association with hOGG1 mRNA expression in tumor tissue specimens.

Characteristics	No. of cases (%)	*hOGG1* expression	P-value
Age (years)			
≤65	36 (46.8)	0.0098±0.0011	0.6832
>65	41 (53.2)	0.0091±0.0013	
Sex			
Male	52 (67.5)	0.0092±0.0011	0.7653
Female	25 (32.5)	0.0098±0.0014	
Histology			
Adenocarcinomas	35 (45.5)	0.0119±0.0016	0.0707
Squamous cell carcinomas	29 (37.7)	0.0073±0.0010	
Others	13 (16.8)	0.0075±0.0011	
Smokers			
Yes	44 (57.1)	0.0098±0.0012	0.6264
No	33 (42.9)	0.0090±0.0016	
Clinical stage			
I	21 (27.3)	0.0077±0.0012	0.2214
II	19 (24.7)	0.0112±0.0017	
III	26 (33.8)	0.0078±0.0012	
IV	11 (14.2)	0.0134±0.0036	
Lymph node			
Yes	36 (46.8)	0.0095±0.0012	0.9264
No	41 (53.2)	0.0093±0.0012	
Distant metastases			
Yes	11 (14.3)	0.0134±0.0036	0.0562
No	66 (85.7)	0.0088±0.0008	

Data are presented as mean ± SE. Unpaired t-test for two groups. Kruskal-wallis test for three or more groups.

hOGG1 mRNA expression is downregulated in NSCLC tissues. *hOGG1* mRNA levels were significantly lower in NSCLC tissues compared with adjacent non-cancerous lung tissues (P=0.034; Fig. 1D). No significant differences were observed in *hOGG1* mRNA levels between NSCLC tissues classified by various clinicopathological characteristics (Table I). Moreover, a public data set (GSE19188) containing 91 NSCLC tissues and 65 normal lung tissues showed that *hOGG1* mRNA expression was downregulated in human NSCLC tissues (P=0.002; Fig. 1C).

hOGG1 mRNA expression is downregulated in NSCLC cell lines and associated with DNA methylation. As shown in Fig. 2A, *hOGG1* mRNA levels were significantly lower in A549, H1650, H460, 95C, 95D, H226 and SK-MES-1 cells compared with control HBE cells, except for SPC-A-1. Our previous study supported the idea that DNA methylation could be epigenetically responsible for inactivation of tumor suppressor genes in NSCLC, and the methylation of the *hOGG1* gene promoter region occurs frequently in NSCLC (10,19). Therefore, to determine whether methylation of *hOGG1* gene promoter is an alternative mechanism underlying inactivation of *hOGG1* mRNA expression, we detected the mRNA expression after using demethylating agent 5-Aza on NSCLC cell lines. As illustrated in Fig. 2B, after 5-Aza treatment, *hOGG1* mRNA expression was increased in NSCLC cell lines (A549, H460, SPC-A1, 95D and SK-MES-1); therefore, we suggest that *hOGG1* expression is silenced by DNA methylation.

Methylation levels of the +322-327 CpG site is higher in NSCLC than adjacent non-cancerous lung tissues and inversely correlated with hOGG1 mRNA expression. It is known that CpG islands are located -200 to -1,000 bp from the transcription start site of a gene. Based on this knowledge, we used Methyl Primer Express® software to identify potential CpG sites in the *hOGG1* promoter and observed a GC-rich region (Fig. 3A). Furthermore, by using the MassARRAY EpiTYPER application, methylation levels of CpG sites in the *hOGG1* gene were observed in 10 paired NSCLC tissues and adjacent non-cancerous lung tissues (Tables II-IV). In the present study, we detected three separate regions (position -1000 - -643, -463 - +34 and +35 - +412), including 68 CpG sites, in the *hOGG1* promoter. Several CpG sites were detected between positions -1000 - -643 and -463 - +34, and differences in methylation levels were detect (P<0.05). However, we found

Table II. CpG methylation of -1000 - -643 of the *hOGG1* promoter in NSCLC and paired normal tissues.

		Tumor		Paried-normal		
No.	CpG site	n	∑X/n±s	n	∑X/n±s	P-value
1	CpG_1	10	0.417±0.175	10	0.261±0.061	0.016
2	CpG_3	10	1	10	0.983±0.018	0.010
3	CpG_4.5	10	0.933±0.018	10	0.929±0.014	0.594
4	CpG_6	10	0.900±0.024	10	0.889±0.026	0.339
5	CpG_7.8	10	0.951±0.015	10	0.960±0.009	0.129
6	CpG_10	10	0.835±0.064	10	0.828±0.082	0.835
7	CpG_11.12	10	0.938±0.013	10	0.937±0.012	0.863
8	CpG_13	10	1	10	1	

Table III. CpG methylation of -463 - +34 of the *hOGG1* promoter in NSCLC and paired normal tissues.

		Tumor		Paried-normal		
No.	CpG site	n	∑X/n±s	n	∑X/n±s	P-value
1	CpG_1	10	0.328±0.066	10	0.376±0.086	0.202
2	CpG_2.3.4	9	0.121±0.095	9	0.086±0.070	0.403
3	CpG_5	9	0.03±0.026	9	0.017±0.013	0.233
4	CpG_6	9	0.048±0.023	9	0.038±0.023	0.398
5	CpG_9	9	0.015±0.016	9	0.014±0.212	0.952
6	CpG_10	9	0.003±0.007	9	0.022±0.020	0.029
7	CpG_11	9	0	9	0.001±0.003	0.396
8	CpG_12	9	0.333±0.074	9	0.38±0.086	0.258
9	CpG_13	9	0.005±0.007	9	0.024±0.015	0.004
10	CpG_14.15	9	0.015±0.009	9	0.03±0.02	0.114
11	CpG_16	9	0.005±0.007	9	0.024±0.015	0.004
12	CpG_17	9	0.011±0.008	9	0.02±0.014	0.147
13	CpG_19.20.21	9	0.063±0.016	9	0.051±0.019	0.169
14	CpG_22	9	0.031±0.031	9	0.022±0.023	0.511
15	CpG_23.24	9	0.015±0.013	9	0.012±0.004	0.556
16	CpG_25	9	0.071±0.095	9	0.061±0.026	0.764
17	CpG_26	9	0.045±0.062	9	0.026±0.041	0.483
18	CpG_27	9	0.007±0.008	9	0.021±0.023	0.139
19	CpG_28.29.30	9	0.263±0.091	9	0.232±0.097	0.504
20	CpG_31	9	0.017±0.021	9	0.002±0.004	0.575
21	CpG_32.33	9	0.055±0.037	9	0.057±0.017	0.844
22	CpG_34	9	0.038±0.045	9	0.025±0.007	0.405
23	CpG_35	9	0.036±0.028	9	0.051±0.022	0.251
24	CpG_36	9	0	9	0.005±0.007	0.047

that these regions have no transcriptional binding sites or have lower frequency of methylation. Consequently, we expanded the sample size to 25-paired tissues to detect potential CpG sites within the third area (position +35 - +412). We observed significantly higher methylation of CpG site-3 in NSCLC patients compared with the control group (Fig. 3B; Table V). Notably, the methylation level of +322 - 327 site (T/N) was inversely correlated with *hOGG1* mRNA level (T/N) in 25 paired tissues (P=0.0104; Fig. 4C).

Methylation of the +322-327 CpG site inhibits hOGG1 mRNA expression by inhibiting Sp1 binding to the hOGG1 promoter region. Because methylation of individual CpG dinucleotides may contribute to cancer development (20,30), we postulated

Table IV. CpG methylation of +35 - +412 of the *hOGG1* promoter in NSCLC and paired normal tissues.

		Tumor		Paried-normal		
No.	CpG site	n	∑X/n±s	n	∑X/n±s	P-value
1	CpG_2	9	0.088±0.114	9	0.101±0.137	0.840
2	CpG_3.4.5	9	0.031±0.056	9	0.048±0.068	0.554
3	CpG_6.7	9	0.356±0.201	9	0.291±0.221	0.520
4	CpG_8	9	0.035±0.040	9	0.021±0.035	0.431
5	CpG_9.10	9	0.035±0.044	9	0.023±0.035	0.529
6	CpG_11	9	0.195±0.196	9	0.17±0.166	0.769
7	CpG_12	9	0.071±0.055	9	0.063±0.046	0.750
8	CpG_14	9	0.022±0.017	9	0.021±0.018	0.898
9	CpG_15	9	0.012±0.016	9	0.017±0.019	0.526
10	CpG_16.17	9	0.101±0.075	9	0.073±0.062	0.405
11	CpG_18.19	9	0.058±0.032	9	0.054±0.034	0.780
12	CpG_20	9	0.025±0.026	9	0.032±0.025	0.599
13	CpG_21.22	9	0.133±0.073	9	0.078±0.065	0.117
14	CpG_23	9	0.051±0.039	9	0.035±0.031	0.370
15	CpG_24.25	9	0.034±0.030	9	0.025±0.019	0.471

Table V. CpG methylation of +35 - +412 of the *hOGG1* promoter in NSCLC and paired normal tissues.

		Tumor		Paried-normal		
No.	CpG site	n	∑X/n±s	n	∑X/n±s	P-value
1	CpG_2	25	0.057±0.050	25	0.055±0.092	0.945
2	CpG_3.4.5	25	0.010±0.033	25	0.024±0.045	0.210
3	CpG_6.7	24	0.158±0.079	24	0.092±0.066	0.001
4	CpG_8	25	0.044±0.028	25	0.039±0.030	0.535
5	CpG_9.10	25	0.082±0.091	25	0.034±0.042	0.226
6	CpG_11	25	0.111±0.145	25	0.105±0.120	0.874
7	CpG_12	25	0.082±0.043	25	0.074±0.035	0.475
8	CpG_14	25	0.033±0.024	25	0.035±0.035	0.815
9	CpG_15	24	0.011±0.015	24	0.006±0.013	0.276
10	CpG_16.17	24	0.140±0.058	24	0.135±0.095	0.827
11	CpG_18.19	24	0.092±0.032	24	0.092±0.043	0.955
12	CpG_20	25	0.061±0.052	25	0.054±0.044	0.625
13	CpG_21.22	25	0.321±0.209	25	0.306±0.195	0.802
14	CpG_23	25	0.080±0.064	25	0.048±0.046	0.073
15	CpG_24.25	25	0.026±0.017	25	0.035±0.032	0.245

that site-specific CpG methylation could alter the expression of *hOGG1* in NSCLC. We found one putative functional CpG site at position +322-327 in the proximal promoter region of *hOGG1* that was located within a transcription factor Sp1-binding sequence (5'-CGGGGCGG-3') using TRANSFAC, TFSEARCH and Methyl Primer Express® software (Fig. 4A). We utilized ChIP analysis to examine whether Sp1 binds to the *hOGG1* proximal promoter region at the +322-327 CpG site, and found that Sp1 was recruited to the +322-327 CpG site in A549 cells (Fig. 4B). Collectively, the results demonstrated that Sp1 may be target *hOGG1* 5'-promoter containing the +322-327 CpG site and thereby upregulate *hOGG1* expression in NSCLC cells. Subsequently, we synthesized segments of the hOGG1 promoter that contained a wild-type and a mutant Sp1 binding site for use in luciferase reporter constructs; these constructs were transfected into A549 and SPC-A1 cells. The mutant Sp1 binding site construct displayed a significant decrease in luciferase activity compared with the wild-type construct (Fig. 4C). In summary, our results suggest that the methylation of the +322-327 site in the *hOGG1* promoter represses Sp1 binding and regulates the expression of *hOGG1* in NSCLC cells.

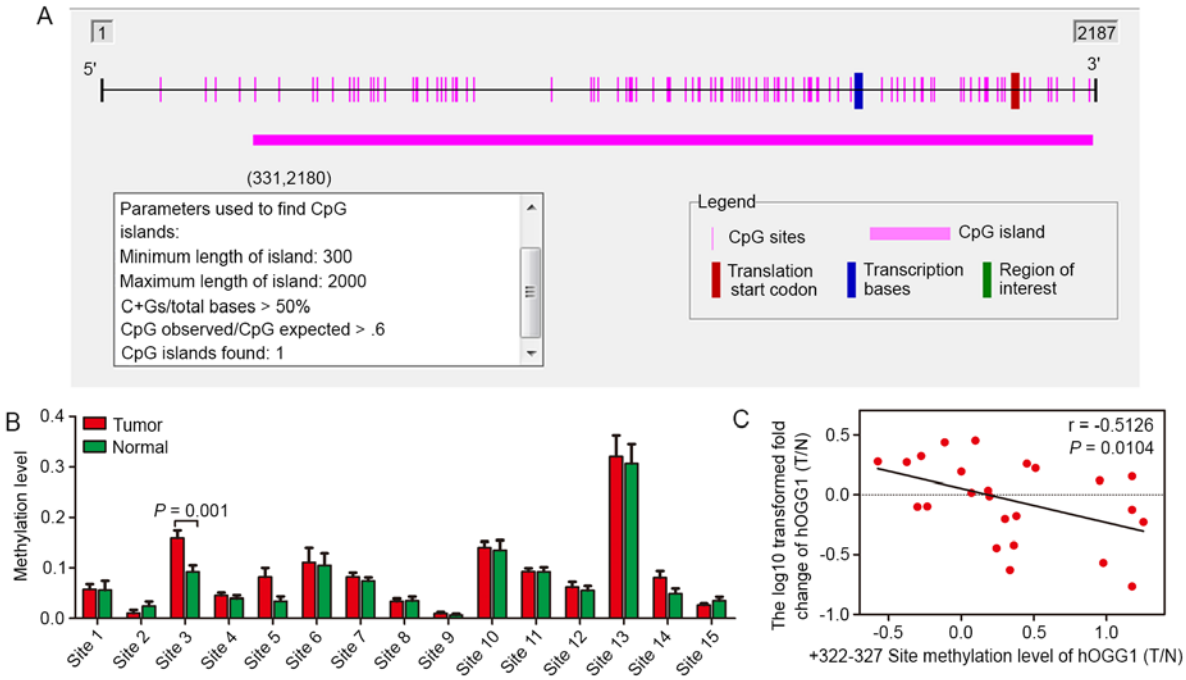

Figure 3. Schematic presentation of CpG site distribution in *hOGG1* proximal promoter. (A) A map of CpG site distribution in *hOGG1* proximal promoter and 5'-UTR regions with a total length of 2,187 bp. There are many CpG sites, each of which is represented by a red vertical line. (B) Methylation levels of CpG sites in the *hOGG1* gene (position +35 - +412 region) were observed in 25 paired NSCLC tissues and adjacent non-cancerous lung tissues. (C) The methylation level of +322-327 CpG site is inversely correlated with *hOGG1* mRNA expression in NSCLC tissues.

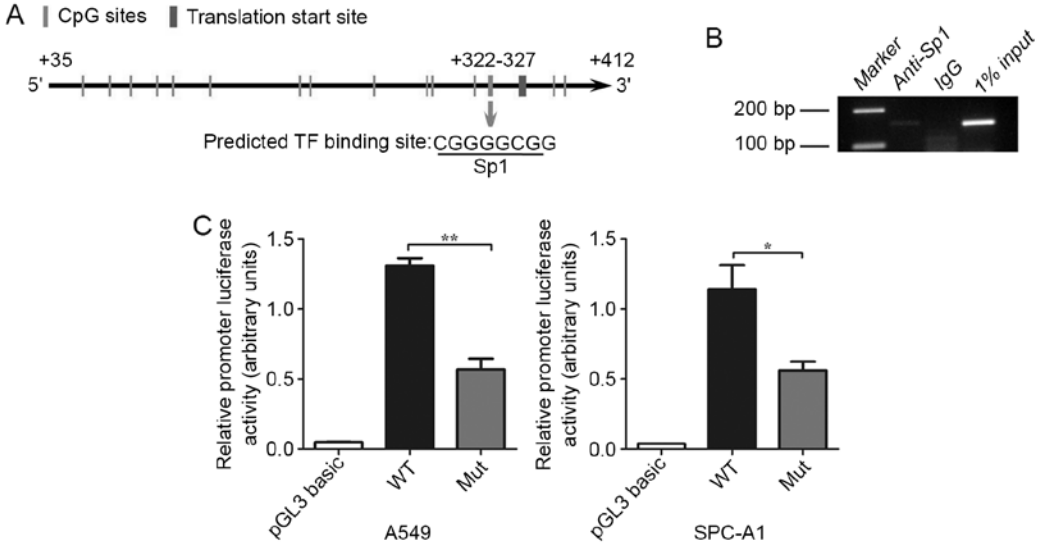

Figure 4. Methylation of +322-327 CpG site inhibits *hOGG1* transcriptional expression by interfering with the recruitment of Sp1 to *hOGG1* proximal promoter. (A) A sketch of CpG sites distributed in *hOGG1* proximal promoter region (+35 to +412). Of 15 CpG dinucleotides, the +322-327 CpG site was predicted to be within a putative transcription factor Sp1-binding sequence (5'-CGGGGCGG-3'). (B) ChIP analysis for the interaction of Sp1 with *hOGG1* promoter region. A549 cells were subjected to ChIP assay using anti-Sp1 antibodies. Presence of *hOGG1* promoter sequence containing +322-327 (+247 to +398) was confirmed by PCR (1% Input) before immunoprecipitation. IgG antibody was used as negative control. PCR products were separated on 2.0% agarose gels. ChIP assay shows an enrichment of *hOGG1* promoter or HPRT1 DNA, which was obtained in the immunoprecipitation using anti-Sp1 antibody. (C) The pGL3 luciferase constructs containing the wild-type or mutated +322-327 CpG site were transiently transfected into A549 and SPC-A1 cells, *Renilla* pRL-TK construct was co-transfected as a normalizing control. Data are shown as mean ± SD of three independent experiments. *P<0.05; **P<0.01.

Discussion

Lung cancer is a leading cause of death throughout the world. The morbidity and mortality of lung cancer has significantly increased in the past decade in China (2). Studies have shown that NSCLC may result from the accumulation of multiple genetic and/or epigenetic aberrations. DNA methylation could be responsible for the inactivation of the tumor suppressor genes found in NSCLC.

Endogenous and exogenous sources cause oxidatively induced DNA damage in living organisms by a variety of mechanisms. The resulting DNA lesions are mutagenic and,

unless repaired, lead to a variety of mutations and consequently to genetic instability, which is a hallmark of cancer. The BER is known to preserve genome integrity by removing damaged bases. It is the main pathway for the repair of oxidized modifications in both nuclear and mitochondrial DNA. Compelling evidence has shown that hOGG1 plays an important role in tumorigenesis (11-13). Lower hOGG1 activity has been reported in patients with NSCLC and downregulation of hOGG1 mRNA and protein levels are compromised in their ability to remove 8-oxoG from their DNA (31,32). Despite such functional importance, it remains largely unknown how hOGG1 is regulated at the transcriptional level in human NSCLC, particularly through epigenetic mechanisms, such as DNA methylation. Genetic polymorphisms in individuals have recently been implicated to account for some of the observed differences in lung cancer susceptibility. The Ser326Cys hOGG1 polymorphism may be the most frequently reported; it is associated with increased risk for lung cancers, but its function is still controversial (33). Our previous studies showed that DNA methylation could underlie epigenetic inactivation of the CpG island methylator phenotype (CIMP) involving TSGs on 3p, suggesting that this is a frequent epigenetic event that may confer an increased risk of NSCLC (19,20). A previous study reported that the CpG methylation of an adjacent cytosine could moderately decrease the oxoGua excision rate, whereas methylation opposite oxoGua could lower the rate of product release (34).

We showed that +322-327 CpG methylation in the *hOGG1* 5'-UTR decreased *hOGG1* mRNA expression in NSCLC tissues and cells using MassARRAY Epi-TYPER applications. Our findings revealed that +322-327 CpG methylation may reduce the recruitment of the transcriptional activator Sp1 to the *hOGG1* 5'-UTR. Sp1 is a well-characterized sequence-specific transcriptional factor that regulates a large number of housekeeping and tissue-specific genes by binding to GC-rich DNA sequences in the promoter region of many human genes (35,36).

Our findings support the idea that site-specific CpG methylation may play an important role in cancer progression (14,18,20,31). To date, the mechanistic roles of individual CpG site methylation are rarely reported in cancer (20,31). This encourages us to investigate how the +322-327 CpG site epigenetically affects the regulation of *hOGG1* expression. In the present study, we identified that the +322-327 CpG in the *hOGG1* proximal promoter is within a putative transcription factor Sp1-binding sequence. Furthermore, cell-based and biochemical analyses revealed that +322-327 CpG methylation can inhibit *hOGG1* transcriptional expression by interfering with the recruitment of Sp1 to the *hOGG1* promoter. However, we cannot exclude the possibility of the roles of other functional CpG sites in the *hOGG1* promoter region.

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