O^6-methyl-guanine-DNA methyltransferase methylation and IDH1/2 mutation in small cell lung cancer

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Abstract. Small cell lung cancer (SCLC) is sensitive to first-line chemotherapy and radiotherapy, but frequently recurs. Temozolomide is a chemotherapeutic drug suitable for the treatment of relapsed SCLC, particularly when brain metastases are present. The response of SCLC to temozolomide may be associated with the methylation status of O^6-methyl-guanine-DNA methyltransferase (MGMT). Isocitrate dehydrogenase (IDH) mutation is an independent prognostic factor of good outcome in gliomas and appears to be a significant marker of positive chemosensitivity in secondary glioblastoma. In order to identify the status of MGMT promoter methylation and IDH1/2 mutation of SCLC in China, 33 SCLC specimens from patients that underwent surgery were retrospectively collected in Zhejiang Cancer Hospital (Hangzhou, China) between 2008 and 2014. High-resolution melting analysis and methylation-specific polymerase chain reaction were used to detect MGMT promoter methylation, and polymerase chain reaction amplification and Sanger sequencing were utilized to detect IDH1/2 mutation. Of the 33 examined SCLC specimens, MGMT promoter methylation was detected in 17 patients (51.5%), and no IDH1/2 mutations were detected in the analyzed samples. These findings indicate that the IDH1/2 mutation may not be an ideal marker in SCLC patients treated with temozolomide. Future studies on the predictive and prognostic value of MGMT promoter methylation are urgently required for patients with relapsed SCLC treated with temozolomide in China.

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Abbreviations: SCLC, small cell lung cancer; MGMT, O^6-methyl-guanine-DNA methyltransferase; IDH, isocitrate dehydrogenase; PCR, polymerase chain reaction; HRM, high-resolution melting; MSP, methylation-specific polymerase chain reaction; α-KG, α-ketoglutarate; NADP, nicotinamide adenine dinucleotide phosphate

Key words: small cell lung cancer, O^6-methyl-guanine-DNA methyltransferase, methylation, isocitrate dehydrogenase 1, isocitrate dehydrogenase 2

Introduction

The prognosis of small cell lung cancer (SCLC) remains poor, although modest improvements in survival have been achieved. The proportion of SCLC among all histological types of lung cancer decreased from 17.26% in 1986 to 12.95% in 2002 (1). Although SCLC is sensitive to first-line chemotherapy and radiotherapy, the majority of patients experience relapse within 2 years and succumb to systemic metastasis. The 5-year survival rate of limited stage SCLC is 15% or less and the 2-year survival of extensive stage SCLC is 5.2-19.5% (1,2). Temozolomide, a chemotherapeutic drug, is a suitable option for relapsed SCLC, particularly when brain metastases are present (3,4), and it has been recommended for second-line treatment of SCLC in the National Comprehensive Cancer Network guidelines (5). In a phase II study of the efficacy of temozolomide in the treatment of SCLC, Pietanza *et al* (3) established the methylation status of the O^6-methyl-guanine-DNA methyltransferase (MGMT) promoter in 27 patients, and found that the overall promoter methylation rate in those patients was 48%. A significantly greater proportion of cases with methylated MGMT exhibited a response to temozolomide compared with those with unmethylated MGMT (38 vs. 7%; P=0.08). These results suggest that the response to temozolomide treatment may be associated with the MGMT methylation status in SCLC (3). Temozolomide administered at a dose of 200 mg/m^2/day for 5 days in 28-day cycles is tolerable and active in patients with relapsed SCLC (4). The incidence of mutations in the epidermal growth factor receptor in non-small cell lung cancer is higher in China than in the United States and European countries (6,7). The difference in MGMT methylation rates among China, the United States and European countries remains unknown and, to the best of our knowledge, there are currently no reports on the status of MGMT promoter methylation for SCLC in China.

Mutations of isocitrate dehydrogenase (IDH)1 and IDH2 are the most frequent genomic alterations detected in gliomas, affecting 40% of these tumors, and are some of the earliest aberrations occurring in gliomagenesis (8). IDH mutations have been found to be independent prognostic factors of a good outcome for glioma, and IDH mutation appears to be a significant marker of chemosensitivity in secondary glioblastoma (8). A study of secondary glioblastomas indicated that the combined use of the IDH status and MGMT promoter status may be deserving and appropriate for examination as a stratification factor in clinical trials involving the use of temozolomide for the treatment of patients with secondary glioblastoma (9). However, prognostic and predictive values of IDH1/2 mutation for chemosensitivity in SCLC are unknown and further studies are required for clarification. Therefore, it would be valuable to elucidate the status of MGMT methylation and IDH1/2 mutation in SCLC patients treated with temozolomide.

In the present study, MGMT promoter methylation in resected SCLC specimens was detected by the use of polymerase chain reaction (PCR) amplification and Sanger sequencing. The IDH1/IDH2 mutation was identified by methylation-specific PCR (MSP) and high-resolution melting (HRM) analysis.

Table I. Clinical characteristics of the patients with small cell lung cancer.

Characteristics	Cases (n)
Sex	
Male	27
Female	6
Location	
Right lung	16
Left lung	17
Smoking	
Non-smoker	6
Light smoker (≤10 pack-years)	2
Moderate smoker (≤10 pack-years)	5
Heavy smoker smokers (≥20 pack-years)	20
Stage	
IA	9
IB	5
IIA	4
IIB	3
IIIA	11
IIIB	1

Materials and methods

Patient characteristics. A total of 33 SCLC specimens were retrospectively collected in Zhejiang Cancer Hospital (Hangzhou, China) between April 2008 and June 2014. All specimens were obtained from SCLC patients who underwent surgery. A total of 3 patients received pneumonectomy with lymph node dissection, 1 patient received wedge resection with lymph node dissection, and 29 patients received lobectomy with lymph node dissection. All patients were diagnosed with conventional SCLC, the pathological diagnosis of which was based on the standard criteria defined by WHO classification (10). The stage was classified according to seventh edition of the TNM classification for lung cancer as follows: IA, 9 cases; IB, 5 cases; IIA, 4 cases; IIB, 3 cases; IIIA, 11 cases; and IIIB, 1 case. The 33 specimens were obtained from 6 female and 27 male patients (age range, 38 to 77 years; median age, 58 years; body weight range, 44 to 80 kilograms; median body weight, 66 kilograms). There were 6 non-smokers, 2 light smokers (≤10 pack-years), 5 moderate smokers (10-20 pack-years) and 20 heavy smokers (≥20 pack-years). The median pack-years of smoking history was 30 (Table I). The present study was approved by the Ethics Committee of Zhejiang Cancer Hospital. The patient specimens were retrospectively collected and, as a number of patients were deceased, exempt written informed consent was also approved by the Ethics Committee of Zhejiang Cancer Hospital. A total of 21 patients signed the written informed consent prior to surgery to preserve their specimens in Biological Sample Bank of Zhejiang Cancer Hospital to be used for research.

MSP and HRM analysis. DNA methylation pattern in the CpG island of the *MGMT* gene was determined by nested MSP of bisulphite-converted DNA. DNA bisulfite conversion was conducted using an EZ DNA methylation kit (Zymo Research Corporation, Irvine, CA, USA) following the manufacturer's protocol. DNA was treated with sodium bisulfite, to convert all unmethylated cytosine residues to uracil, whereas methylated cytosine remained unchanged. Bisulfite-converted DNA was stored at -70˚C until use. The primer sequences used for nested MSP, methylated and unmethylated PCR are listed in Table II. For the first round of PCR amplification, 2 μl bisulfite-modified DNA was used in a final volume of 30 μl reaction mixture containing the template DNA (>50 ng), forward and reverse MGMT-outside primers and (0.5 pM), 2X SYBR premix (Bioer Technology Co., Ltd., Hangzhou, China). Amplification was completed in a Mx3000P Thermal Cycler (Stratagene; Agilent Technologies, Inc., Santa Clara, CA, USA) under the following conditions: initial denaturation at 95˚C for 3 min, 35 cycles each comprising denaturation at 94˚C for 30 sec, annealing at 59˚C for 30 sec and extension at 72˚C for 30 sec, followed by a final extension at 72˚C for 5 min. The resulting PCR products of MGMT served as a template for the second MSP. The PCR product from the first step was subjected to the second round of PCR with MGMT forward and reverse primers specific for methylated (MGMT-M) and unmethylated (MGMT-U) sequences of MGMT promoter (Table II). Amplification was completed under the following conditions: Initial denaturation at 95˚C for 3 min, 40 cycles of denaturation each at 95˚C for 30 sec, annealing at 59˚C for 30 sec and extension at 72˚C for 30 sec followed by final extension at 72˚C for 4 min. The PCR products were subjected to HRM analysis using a Light Cycler Nano (Roche Diagnostics GmbH, Mannheim, Germany) according to its high resolution melting software in one cycle of 97˚C for 1 min and a melting range temperature of 70-95˚C, rising by 0.2˚C per second.

Table II. Sequences of primers for MGMT detection.

Primer	Direction	Sequence of primers
MGMT-O	Forward	GYGTTTYGGATATGTTGGGATAGTT
MGMT-O	Reverse	AAACTCCRCACTCTTCCRAAAAC
MGMT-M	Forward	TTTCGACGTTCGTAGGTTTTCGC
MGMT-M	Reverse	GCACTCTTCCGAAAACGAAACG
MGMT-U	Forward	TTTGTGTTTTGATGTTTGTAGGTTTTTGT
MGMT-U	Reverse	AACTCCACACTCTTCCAAAAACAAAACA

MGMT, O^6-methyl-guanine-DNA methyltransferase; O, outside; M, methylated; U, unmethylated.

IDH1 and IDH2 mutation detection. Mutational analyses of IDH1 (R132) and IDH2 (R172) were performed using PCR amplification and Sanger sequencing. The genomic DNA was extracted from paraffin-embedded tumor tissue using an EASYspin FFPE DNA isolation kit (Aidlab Biotechnologies Co., Ltd., Beijing, China) according to the protocol of the manufacture. The primer sequences used for PCR are provided in Table III. PCR amplification was performed using a Mx3000P Thermal Cycler (Stratagene; Agilent Technologies, Inc., Santa Clara, CA, USA) under the following conditions: 95˚C for 2 min, then 40 cycles of 94˚C for 30 sec, 57˚C for 30 sec and 72˚C for 30 sec. The final elongation step was at 72˚C for 5 min. The PCR products were sequenced using an Applied Biosystems 3500 sequencer using IDH1/2 forward primer (Table III).

Statistical analysis. SPSS 15.0 software (SPSS, Inc., Chicago, IL, USA) was used for the Chi-square analysis. $P<0.05$ was considered to indicate a statistically significant difference.

Table III. Sequences of primers for IDH1/2 detection.

Primer	Direction	Sequence of primers
IDH1	Forward	CTCCTGATGAGAAGAGGGTTG
IDH1	Reverse	TGGAAATTTCTGGGCCATG
IDH2	Forward	TGGAACTATCCGGAACATCC
IDH2	Reverse	AGTCTGTGGCCTTGTACTGC

IDH, isocitrate dehydrogenase.

Results and Discussion

MGMT promoter methylation was detected in 17 patients and the MGMT promoter methylation rate in SCLC was 51.5% in the present cohort. No significant difference was observed between patients with MGMT methylation and without MGMT methylation in terms of age, sex and smoking history (Table IV). Furthermore, no IDH1/2 mutation was detected in the 33 examined SCLC specimens (Figs. 1 and 2).

Temozolomide is an alkylating agent that is widely used in the chemotherapy of cancer (11,12). The enzyme MGMT, via its dealkylating effect, repairs potentially tumorigenic DNA mismatches caused by environmentally induced alkylation, but also acts against the effects of alkylating molecules that are administered in the chemotherapy of different types of cancer (13). MGMT is the primary mediator of the alkylator resistance of glioma cells. Epigenetic silencing of the MGMT gene in glioma cells by promoter methylation compromises the DNA repair mechanism and thus increases chemosensitivity to alkylating drugs. Therefore, MGMT promoter methylation is a strong prognostic biomarker in pediatric and adult patients with glioblastoma that are treated with temozolomide (14). There have been few investigations on MGMT in SCLC, despite the use of temozolomide in relapsed SCLC. The response of SCLC to temozolomide may be associated with MGMT methylation (3). The rate of MGMT promoter methylation was 51.5% in the present study, which was close to that reported by Pietanza *et al* (48%) (3) and Miglio *et al* (35.2%) (15). In the study conducted by Miglio *et al* (15), results from methylation analysis were obtained in 54 samples (54/56) and failed in two bronchial washes. The MGMT promoter was methylated in 35.2% of the cases without any significant difference between histological and cytological samples (37.9 vs. 32%) (15). All specimens in the current study were collected from surgery, and they may better reflect the respective pathological characteristics. However, it is very difficult to obtain tissues from surgery because only ~5% of SCLC patients with a TNM stage of T1-2N0M0 are suitable for surgery. In MGMT promoter methylation detection, cytological samples may be a substitution for histological samples. These results indicate that the rate of MGMT promoter methylation in patients with SCLC from China may be higher than that in those from the USA and European countries. This difference may be attributable to different ethnicities, specimen sources and limited sample quantity. Elucidation of the predictive prognostic value of MGMT promoter methylation in patients with relapsed SCLC treated with temozolomide in China is required.

IDH occurs in three isoforms: IDH1, located in the cytoplasm; IDH2, located in the mitochondria; and IDH3, which functions as part of the tricarboxylic acid cycle. IDH catalyzes the conversion of isocitrate to α-ketoglutarate (α-KG). Mutations in the active site of IDH1 at position R132 and an analogous mutation in the IDH2 gene at position R172 have been identified (16). IDH1 catalyzes the oxidative decarboxylation of isocitrate to α-KG and in parallel converts nicotinamide adenine dinucleotide phosphate ($NADP^+$) to

Table IV. Clinical features of 33 patients with small cell lung cancer with and without O^6-methyl-guanine-DNA methyltransferase methylation.

Clinical features	Methylation	Without methylation	P-value
Patients	17	16	-
Median age (years)	55	60	0.58
Sex (female/male)	4/13	3/13	0.935
Smoking history (median pack-years)	23	40	0.29

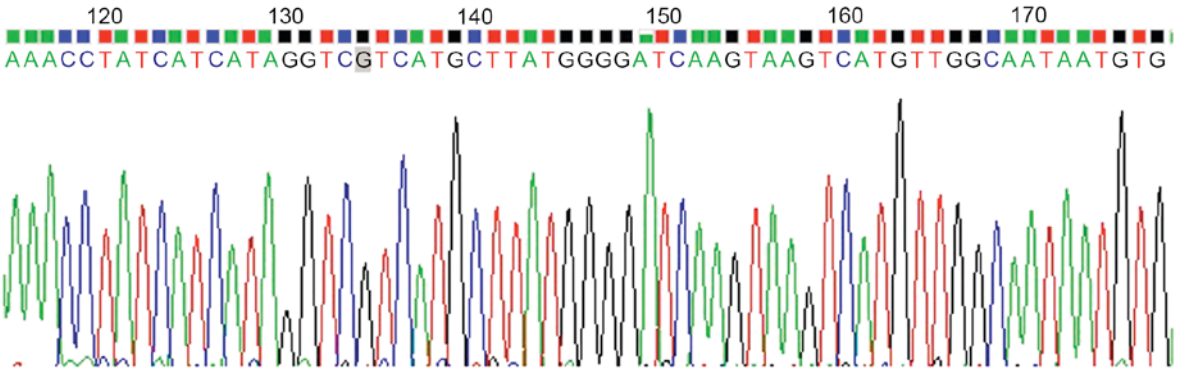

Figure 1. Normal (unmutated) sequence of isocitrate dehydrogenase 1 (R132). Mutation was expected to be in isocitrate dehydrogenase 1 (R132): ATAGGTCGTCATGCTT. No isocitrate dehydrogenase 1 mutation was detected in the 33 examined small cell lung cancer specimens.

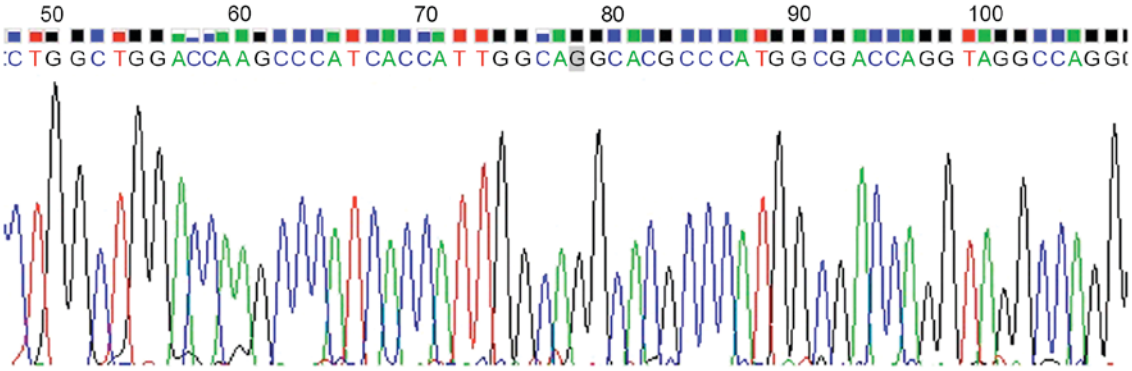

Figure 2. Normal (unmutated) sequence of isocitrate dehydrogenase 2 (R172). Mutation was expected to be in isocitrate dehydrogenase 2 (R172): TGGCAGGCACGCCC. No isocitrate dehydrogenase 2 mutation was detected in the 33 examined small cell lung cancer specimens.

NADPH. The mutant IDH1 acquires novel enzyme activity, converting α-KG to D-2-hydroxyglutarate, an action that is competitive and inhibitory to that of α-KG. As a result, the activity of α-KG-dependent enzyme is reduced (17). IDH2 is a mitochondrial NADP-dependent isocitrate dehydrogenase identified to be a tumor suppressor in different types of tumors (18). The prognosis of Grade II and III glioma was improved in patients with an IDH mutation, compared with those without the mutation (19,20). The IDH mutation was associated with a higher response rate to temozolomide therapy (21). The IDH1 mutation caused cell cycle arrest at the G1 stage and a reduction of proliferation and invasion ability, while raising sensitivity to chemotherapy (22). No IDH1/2 mutations in SCLC were detected in the current study, which may be due to the limited sample quantity, as all samples were acquired from surgical specimens and only ~5% of SCLC patients with a TNM stage of T1-2N0M0 were suitable for surgery. The IDH1/2 mutation may not be an ideal marker in SCLC patients treated with temozolomide.

In conclusion, findings from the present study suggest that the rate of MGMT promoter methylation in patients with SCLC from China may be higher than those from the USA and European countries. Future studies on the predictive and prognostic value of MGMT promoter methylation are urgently required to aid in the treatment for relapsed patients with SCLC that have been treated with temozolomide.

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