Int J Clin Exp Pathol 2016;9(9):9562-9568
www.ijcep.com /ISSN:1936-2625/IJCEP0031666

Original Article

Low APE1/Ref-1 expression significantly correlates with MGMT promoter methylation in patients with high-grade gliomas

Qiushi Wang[1], Hualiang Xiao[1], Qingya Luo[1], Mengxia Li[2], Shirong Wei[1], Xiangfeng Zhu[1], He Xiao[2], Lizhao Chen[3]

Departments of [1]Pathology, [2]Cancer Center, [3]Neurosurgery, Daping Hospital and Research Institute of Surgery, Third Military Medical University, Chongqing, China

Received May 4, 2016; Accepted June 25, 2016; Epub September 1, 2016; Published September 15, 2016

Abstract: Gliomas are aggressive tumors that have poor prognoses. The identification of an accurate prognostic marker and effective therapeutic target is important to improve the treatment for gliomas. Apurinic/apyrimidinic endonuclease/redox effector factor (APE1/Ref-1), a multifunctional enzyme that is part of the BER pathway, exhibits DNA repair activity and has a role in the reductive activation of many transcription factors. The repair of DNA damage by the O6-methylguanine-DNA Methyltransferase (MGMT) protein, which is ubiquitously expressed in normal human tissue, has been identified as the major mechanism of treatment resistance in gliomas. Methylation of CpGs in the MGMT promoter impairs the DNA repair activity and is significantly associated with a better response to chemotherapy, especially TMZ, and longer survival in patients with high-grade glioma. Hence, we aimed to determine whether loss of APE1/Ref-1 expression occurs following MGMT promoter methylationin patients with high-grade gliomas and, if so, to identify the mechanism of this loss. We found that APE1/Ref-1 expression was absent or diminished in primary gliomas compared with normal brain tissue. In summary, the present study showed that loss of APE1/Ref-1 expression is a frequent event with MGMT promoter methylationin patients with high-grade gliomas and is associated with poor prognosis. Promoter methylation may be an underlying mechanism of treatment resistance.

Keywords: APE1/Ref-1, MGMT, glioma, methylation, prognosis

Introduction

Gliomas are the most common type of primary brain tumor in adults, with a standardized incidence of 6/100,000 person/year in Asia in 2012 and a total of 95,732 deaths [1]. Standard treatment-consisting of surgery, radiotherapy, and chemotherapy with the alkylating agent temozolomide (TMZ)-increases median survival to 12-15 months, although the disease typically progresses within 6-9 months, and the 2-year survival rate is less than 25% [2, 3]. The unsatisfactory therapeutic results are partially due to the glioma cells developing resistance against the clinical therapy. The mechanism of this treatment resistance has long been a subject of study. The repaired DNA damage by The O6-methylguanine-DNA methyltransferase (MGMT) protein, which is ubiquitously expressed in normal human tissue, can repair DNA damage, and this DNA repair ability has been identified as the major mechanism of treatment resistance [2, 4, 5]. Methylation of CpGs in the MGMT promoter impairs the DNA repair process and is significantly associated with a better response to chemotherapy, especially TMZ, and longer survival in patients with high-grade glioma [6, 7].

Apurinic/apyrimidinic endonuclease/redox effector factor (APE1/Ref-1), a multifunctional enzyme that is part of the BER pathway, exhibits DNA repair activity and has a role in the reductive activation of many transcription factors [8]. APE1 repairs DNA damage by cutting the DNA backbone at baseless sites (a basic) after the removal of the damaged base [9, 10]. Higher APE1 expression levels are significantly associated with a poor prognosis and reduced treatment response in oral squamous cell carci-

Table 1. Clinical profile of patients with gliomas

Characteristic	No. of Patients (%)
Age (yrs)	
Mean	44.5
Range	10-75
Sex	
Males	60 (62)
Females	37 (38)
WHO classifications	
I-II	28 (29)
III	28 (29)
IV	41 (42)
Total	97

noma patients [11]. Wang et al. [12] reported that 83.3% (20/24) of cisplatin-resistant non-small cell lung cancer patients had high APE1 expression levels, while 8.3% (4/48) of cisplatin-sensitive tumors had high APE1 expression levels ($P<0.01$). Ape1 over-expression correlates with increasing radioresistance in glioma cells [13]. Ape1, which is elevated by oxidative stress, contributes to human glioma cell resistance to clinical alkylators such as TMZ [14].

In the present study, we aimed to (i) evaluate the correlation between MGMT methylation status and the clinical features of gliomas; (ii) evaluate the relationship between APE1 expression and the clinical features of gliomas; and (iii) explore the possible correlation between MGMT promoter methylation status and APE1 protein expression in TMZ-resistant gliomas.

Materials and methods

Patients and tissues

A total of 97 formalin-fixed, paraffin-embedded (FFPE) tissue samples from consecutive patients were obtained from the Department of Neurosurgery of Daping Hospital in Chongqing, China, between January 2013 and November 2015 (**Table 1**). Patients were further diagnosed by pathologists using the different WHO glioma classifications. No patient had received radiotherapy or chemotherapy before surgery.

The FFPE tissues used for the pathological diagnosis of these cases were used in this study. The patients' clinical and pathological data were obtained from their medical records. This study was approved by the Clinical Ethics Committee of Daping Hospital and Research Institute of Surgery, Third Military Medical University.

DNA isolation

Genomic DNA was extracted and purified from five sequential sections of 10-μm-thick FFPE tumor tissue using the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany). DNA quality was assessed using a NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific Inc., MA, USA).

Bisulfite modification and specific polymerase chain reaction for MGMT promoter methylation

Purified DNA underwent bisulfite modification according to the manufacturer's instructions for the EZ DNA Methylation-Gold Kit (Zymo Research Corporation, Irvine, CA, USA). Positive controls for methylated and unmethylated MGMT promoters were used in each test. A real-time PCR assay, using specific primers for either the methylated or unmethylated MGMT promoter, after bisulfite modification was used to identify MGMT promoter methylation status. The primer sequences were as follows according to published data [15, 16]: methylated primers: forward 5'-TTTCGACGTTCGTAGGTTTTCGC-3' and reverse 5'-GCACTCTTCCGAAAACGAAACG-3' and unmethylated primers: forward 5'-TTTGTGTTTTGATGTTTGTAGGTTTTTGT-3' and reverse 5'-AACTCCACACTCTTCCAAAAACAAAACA-3'.

The real-time PCR reaction mixture consisted of 10 μL 2×SYBR green master mix (Thermo Fisher Scientific Inc., MA, USA), 500 nM primers (methylated or unmethylated), 2 μL bisulfite-modified DNA, and distilled water to obtain a final volume of 20 μL. The reactions underwent a two-step cycling procedure in a LightCycler 480 II system (Roche, Basel, Switzerland) as follows: 95°C for 10 min and 40 cycles of 95°C for 10 s and 60°C for 30 s (the fluorescent signal was measured at this step).

Immunohistochemistry of the APE1/Ref-1 protein

APE1/Ref-1 monoclonal antibodies (Abcam, Cambridge, UK) were tested using conventional immunohistochemistry (IHC). The incubation with the primary antibody was performed on 4-μm-thick, formalin-fixed, paraffin-embedded (FFPE) tissues: the slides were deparaffinized

Table 2. MGMT promoter methylation and APE1 expression status in different grade of gliomas

WHO classification	Num.	MGMT		APE1 expression		
		Negative n (%)	Positive n (%)	Low n (%)	High n (%)	High expression rate n (%)
I-II	28	15 (53.6)	13 (46.4)	12 (42.9)	16 (57.1)	Low grade 16/28 (57.1)
III	28	12 (42.9)	16 (57.1)	17 (60.7)	11 (39.3)	High grade 27/69 (39.1)
IV	41	24 (58.5)	17 (41.5)	25 (61.0)	16 (39.0)	
P		0.437		0.270		0.106

Table 3. MGMT promoter methylation status has significant negative correlation with APE1 expression

		APE1 expression		Total
		Low	High	
MGMT	Negative	19	32	51
	Positive	35	11	46
Total		54	43	97

κ=-0.390, P<0.001.

and pre-treated with 1 mmol/L ethylene diamine tetraacetic acid (EDTA) at pH 9.0 in a high-pressure cooker for 3 min and then treated with 3% hydrogen peroxide for 10 min. The primary APE1/Ref-1 antibody was applied to the slides followed by incubation in a hydrated chamber at 4°C overnight. The next day, the slides were washed, stained using conventional 3, 3'-diaminobenzidine staining, and reviewed under a microscope by two pathologists independently. The slides were scored as strong staining (3+), moderate staining (2+), faint staining (1+), or no staining (0). Cases scored 3+ and 2+ were classified as high expression, while those scored 1+ and 0+ were classified as low expression.

Statistics

The correlation analyses of MGMT with APE1, MGMT with gliomas, and APE1 with gliomas were analyzed using Pearson's χ^2 test. P<0.05 was considered statistically significant. All analyses were performed using SPSS software (version 18.0; SPSS Inc., IL, USA).

Results

MGMT promoter methylation status was not significantly correlated with the different WHO glioma classifications

Ninety-seven glioma cases were screened for MGMT promoter methylation (**Table 2**). Among these cases, 28 were classified as WHO I-II grade, 28 as WHO III grade, and 41 as WHO IV grade. The positive rates of MGMT promoter methylation (MGMT+) in gliomas with a WHO grade of I-II, III, and IV were 13/28 (46.4%), 16/28 (57.1%), and 17/41 (41.5%), respectively. MGMT+ was more frequent in grade III gliomas than in the others, but this trend was not statistically significant (P=0.437).

High APE1 expression was not significantly correlated with the different WHO glioma grades

Immunohistochemistry analysis showed that APE1 was expressed in most cases but at different intensities. Cases with strong staining (3+) and moderate staining (2+) were classified as high APE1 expression, while those with faint staining (1+) and no staining (0) were classified as low expression. The results showed that APE1 was highly expressed in 16/28 (57.1%), 11/28 (39.3%), and 16/41 (39.0%) of grade I-II, III, and IV gliomas, respectively (**Table 2**). High APE1 expression was more frequently detected in low-grade gliomas (WHO I and II) than in high-grade gliomas (WHO III and IV), but this trend was not statistically significant (57.1% vs. 39.1%, P=0.106) (**Table 2**).

MGMT promoter methylation was significantly negatively correlated with APE1 expression in high-grade gliomas.

MGMT promoter methylation was significantly correlated with low APE1 expression in this study (**Table 3**, P<0.001; **Figure 1**). When considering the different WHO grades, we found that MGMT promoter methylation was only significantly negatively correlated with APE1 expression in patients with high-grade WHO gliomas (WHO III and IV) (**Table 4**), as 13/16 WHO III and 15/17 WHO IV cases with MGMT promoter methylation showed low APE1 expression.

Multivariate logistic regression analysis was used to eliminate interference from age, gender, and WHO grade. The results indicated that

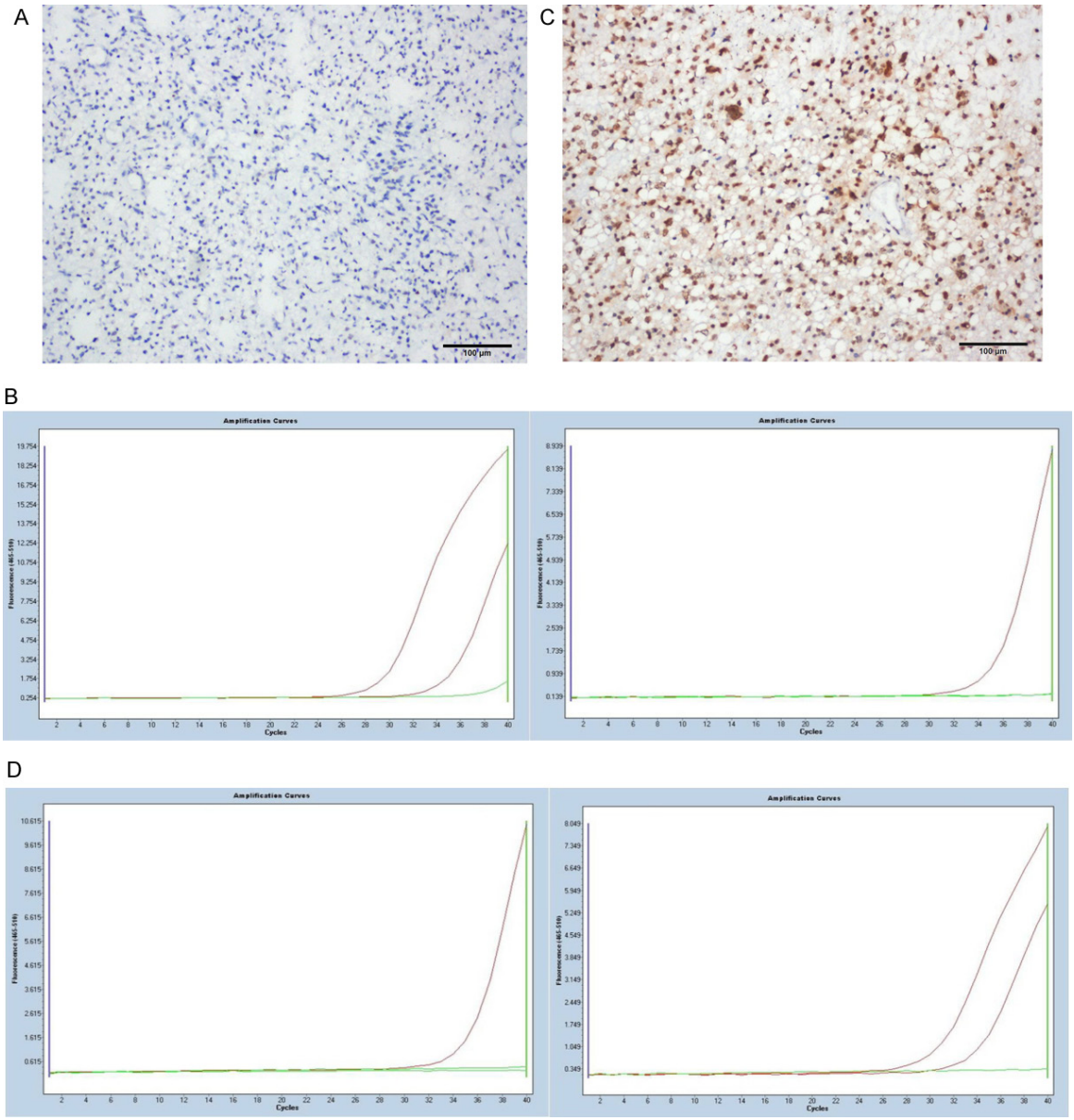

Figure 1. Measurement of APE1 expression and MGMT promoter methylation in two high-grade gliomas. A. Low APE1 expression in GBM patient No. 2383; B. Methylated MGMT promoter in GBM patient No. 2383 (Left, positive result tested using specific methylation primers; Right, negative result tested using specific unmethylation primers). C. High APE1 expression in GBM patient No. 2379; D. Unmethylated MGMT promoter in GBM patient No. 2379 (Left, negative result tested using specific methylation primers; Right, positive result tested using specific unmethylation primers).

Table 4. MGMT promoter methylation shows significant negative correlation with APE1 expression in high grade gliomas

		APE1 expression					
		I-II		III		IV	
		Low	High	Low	High	Low	High
MGMT	Negative	5	10	4	8	10	14
	Positive	7	6	13	3	15	2
	κ	-0.202		-0.455		-0.470	
	P	0.274		0.01		0.003	

MGMT promoter methylation status still significantly correlated with APE1 expression (**Table 5**). The rate of high expression of APE1 in cases with MGMT promoter methylation was significantly lower compared with cases without MGMT methylation (OR=0.153 95% CI 0.058-0.399). Similarly, cases with high APE1 expression had a significantly

Table 5. Multivariate logistic regression analysis showed that low APE1 expression is significantly correlated with MGMT promoter methylation

Factors	B	S.E.	Wald	P	OR	OR 95% C.I.
Gender (M# vs F*)	-0.243	0.507	0.229	0.632	0.785	0.290-2.119
Age	0.021	0.016	1.838	0.175	1.022	0.990-1.054
WHO classification			4.582	0.101		
III vs I-II	-0.902	0.630	2.049	0.152	0.406	0.118-1.395
IV vs I-II	-1.391	0.653	4.539	0.033	0.249	0.069-0.895
MGMT (M† vs Un-M¶)	-1.880	0.491	14.661	0.000	0.153	0.058-0.399

#: Male; *: Female; †: Methylation; ¶: Un-Methylation.

lower probability of MGMT promoter methylation than cases with low APE1 expression (OR=0.156 95% CI 0.060-0.404).

Discussion

MGMT reverses the alkylation function at the O^6 position of guanine to compensate for the effect of alkylating agents [17]. Methylation of the CpG island has been reported to be the essential mechanism for loss of MGMT function [18]. Esteller et al. reported that 50% of anaplastic astrocytoma (WHO III) and 41% of GBM (WHO IV) cases showed MGMT promoter methylation [19]. The incidence of MGMT promoter methylation in patients with GBM was 45% in two clinical trials [20]. Fiano et al. found that 75% of grade II gliomas, 37.5% of grade III gliomas, and 62.5% of grade IV gliomas contained methylated MGMT promoters [15]. Patients with gliomas containing a methylated MGMT promoter could benefit from TMZ therapy [20]. MGMT promoter methylation is the most important mechanism for resistance to TMZ, but it is not the only one. The nucleotide excision repair system has also been reported to be involved in TMZ resistance in gliomas. Chromatin-associated gene poly (ADP-ribose) pomerase-1 (PARP), which functions in nucleotide excision repair, showed increased expression in human tumor cells treated with TMZ [21]. In addition, a PAPR inhibitor enhanced TMZ sensitivity both in vitro [22] and in vivo [23].

Human apurinic/apyrimidinic Endonuclease/redox factor-1 (APE1/Ref-1), an essential enzyme in the BER pathway, has an important function in the excision repair of DNA damage caused by oxidation and alkylation [24]. High expression levels and altered subcellular localization of APE1 have been correlated with cellular resistance to chemotherapeutic agents in several tumor cell lines [25, 26]. Lucanthone, an inhibitor of the APE1 enzyme, reduces APE1 repair activity in cellular extracts and enhances cell apoptosis after treatment with the laboratory alkylating agent MMS and the clinical agent TMZ [27].

In the present study, we screened 97 gliomas cases with different grades for MGMT promoter methylation and APE1 expression status. As shown in **Tables 3** and **4**, MGMT promoter methylation status was significantly negatively correlated with APE1 expression, especially in high-grade (WHO III and IV) gliomas. After excluding interference from age, gender, and WHO classification, the multivariate logistic regression analysis still showed a significantly negative correlation between APE1 expression and MGMT promoter methylation in high-grade gliomas. However, the molecular mechanism of this relation remains elusive and needs further studies. Qiu et al. found that MGMT expression in glioma stem-like cells (GSCs) decreased through the down-regulation of nuclear factor-κB (NF-κB) expression following treatment with the NF-κB inhibitor MG-132. This result indicated that MG-132 may reduce MG-MT expression by inhibiting NF-κB expression, resulting in a synergistic effect on MGMT-positive GSCs [28].

Another study showed that the redox chaperone activity of APE1/Ref-1 is critical for NF-κB-mediated gene expression in human cells and is mediated through its physical association with target transcription factors [29].

In conclusion, we found that MGMT promoter methylation status was significantly negatively correlated with APE1 protein expression in high-grade (WHO III and IV) gliomas. This finding provides new insights, as this correlation may help explain resistance to TMZ treatment in gliomas. The NF-κB nuclear factor might be a critical factor that participates in this regulation process. However, this hypothesis needs further investigation and more evidences.

Acknowledgements

This study was supported by Natural Science Foundation Project of CQ CSTC (cstc2012jjA-10037).

Disclosure of conflict of interest

None.

Address correspondence to: Lizhao Chen, Department of Neurosurgery, Daping Hospital and Research Institute of Surgery, Third Military Medical University, Chongqing 400042, China. E-mail: chenlizhao1971@163.com

References

[1] GLOBOCAN 2012: Estimated Cancer Incidence, Mortality and Prevalence Worldwide in 2012. IARC Press. Lyon, France. Available from: http://globocan.iarc.fr.

[2] Wick W, Weller M, van den Bent M, Sanson M, Weiler M, von Deimling A, Plass C, Hegi M, Platten M, Reifenberger G. MGMT testing-the challenges forbiomarker-based glioma treatment. Nat Rev Neurol 2014; 10: 372-85.

[3] Wen PY, Kesari S. Malignant gliomas in adults. N Engl J Med 2008; 359: 492-507.

[4] Grossman SA, Ye X, Piantadosi S, Desideri S, Nabors LB, Rosenfeld M, Fisher J. NABTT CNS Consortium. Survival of patients with newly diagnosed glioblastoma treated with radiation and temozolomide in research studies in the UnitedStates. Clin Cancer Res 2010; 16: 2443-2449.

[5] Weller M, Stupp R, Hegi ME, van den Bent M, Tonn JC, Sanson M, Wick W, Reifenberger G. Personalized care in neuro-oncologycoming of age: why we need MGMT and 1p/19q testingfor malignant glioma patients in clinical practice. Neuro Oncol 2012; 14 Suppl: 100-108.

[6] Fukushima T, Takeshima H, Kataoka H. Antiglioma therapy with temozolomide and status of the DNA-repair gene MGMT. Anticancer Res 2009; 29: 4845-54.

[7] Christians A, Hartmann C, Benner A, Meyer J, von Deimling A, Weller M, Wick W, Weiler M. Prognostic value of threedifferent methods of MGMT promoter methylation analysis in aprospective trial on newly diagnosed glioblastoma. PLoS One 2012; 7: e33449.

[8] Thakur S, Sarkar B, Cholia RP, Gautam N, Dhiman M, Mantha AK. APE1/Ref-1 as an emerging therapeutic target for various human diseases: phytochemical modulation of its functions. Exp Mol Med 2014; 46: e106.

[9] Fishel ML, Kelley MR. The DNA base excision repair protein Ape1/Ref-1 as a therapeutic and chemopreventive target. Mol Aspects Med 2007; 28: 375-395.

[10] Kelley MR, Jiang Y, Guo C, Reed A, Meng H, Vasko MR. Role of the DNA base excision repair protein, APE1 in cisplatin, oxaliplatin, or carboplatin induced sensory neuropathy. PLoS One 2014; 9: e106485.

[11] Hsia KT, Liu CJ, Mar K, Lin LH, Lin CS, Cheng MF, Lee HS, Chiu SY. Impact of apurinic/apyrimidinic endonuclease 1/redox factor-1 on treatment response and survival in oral squamous cell carcinoma. Head Neck 2016; 38: 550-9.

[12] Wang D, Xiang DB, Yang XQ, Chen LS, Li MX, Zhong ZY, Zhang YS. APE1 overexpression is associated with cisplatin resistance in non-small cell lung cancer and targeted inhibition of APE1 enhances the activity of cisplatin in A549 cells. Lung Cancer 2009; 66: 298-304.

[13] Naidu MD, Mason JM, Pica RV, Fung H, Peña LA. Radiation resistance in glioma cells determined by DNA damage repair activity of Ape1/Ref-1. J Radiat Res 2010; 51: 393-404.

[14] Silber JR, Bobola MS, Blank A, Schoeler KD, Haroldson PD, Huynh MB, Kolstoe DD. The apurinic/apyrimidinic endonuclease activity of Ape1/Ref-1 contributes to human glioma cell resistance to alkylating agents and is elevated by oxidative stress. Clin Cancer Res 2002; 8: 3008-18.

[15] Fiano V, Trevisan M, Trevisan E, Senetta R, Castiglione A, Sacerdote C, Gillio-Tos A, De Marco L, Grasso C, Magistrello M, Tondat F, Rudà R, Cassoni P, Soffietti R, Merletti F. MGMT promoter methylation in plasma of glioma patients receiving temozolomide. J Neurooncol 2014; 117: 347-57.

[16] Yu WW, Kong Y, Dai J, Chi ZH, Si L, Cui CL, Sheng XN, Li SM, Mao LL, Zu M, Li Q, Guo J. MGMT gene promoter methylation in malignant melanoma patients. Chinese Clinical Oncology 2011; 16: 498-501.

[17] Verbeek B, Southgate TD, Gilham DE and Margison GP. O6Methylguanine-DNA methyltransferase inactivation and chemotherapy. Br Med Bull 2008; 85: 17-33.

[18] Paz MF, Yaya-Tur R, Rojas-Marcos I, Reynes G, Pollan M, Aguirre-Cruz L, García-Lopez JL, Piquer J, Safont MJ, Balaña C, Sanchez-Cespedes M, García-Villanueva M, Arribas L and Esteller M. CpG island hypermethylation of the DNA repair enzyme methyltransferase predicts response to temozolomide in primary gliomas. Clin Cancer Res 2004; 10: 4933-4938.

[19] Esteller M, Hamilton SR, Burger PC, Baylin SB and Herman JG. Inactivation of the DNA repair gene O6-methylguanineDNA methyltransferase by promoter hypermethylation is a common event in primary human neoplasia. Cancer Res 1999; 59: 793-797.

[20] Hegi ME, Diserens AC, Gorlia T, Hamou MF, de Tribolet N, Weller M, Kros JM, Hainfellner JA, Mason W, Mariani L, Bromberg JE, Hau P, Mirimanoff RO, Cairncross JG, Janzer RC and

Stupp R. MGMTgene silencing and benefit from temozolomide in glioblastoma. N Engl J Med 2005; 352: 997-1003.

[21] Tentori L, Portarena I, Torino F, Scerrati M, Navarra P and Graziani G. Poly (ADP-ribose) polymerase inhibitor increases growth inhibition and reduces G(2)/M cell accumulation induced by temozolomide in malignant glioma cells. Glia 2002; 40: 44-54.

[22] Curtin NJ, Wang LZ, Yiakouvaki A, Kyle S, Arris CA, CananKoch S, Webber SE, Durkacz BW, Calvert HA, Hostomsky Z and Newell DR. Novel poly(ADP-ribose) polymerase-1 inhibitor, AG14361, restores sensitivity to temozolomide in mismatch repair-deficient cells. Clin Cancer Res 2004; 10: 881-889.

[23] Clarke MJ, Mulligan EA, Grogan PT, Mladek AC, Carlson BL, Schroeder MA, Curtin NJ, Lou Z, Decker PA, Wu W, Plummer ER and Sarkaria JN. Effective sensitization of temozolomide by ABT-888 is lost with development of temozolomide resistance in glioblastoma xenograft lines. Mol Cancer Ther 2009; 8: 407-414.

[24] Abbotts R, Madhusudan S. Human AP endonuclease 1 (APE1): frommechanistic insights to druggable target in cancer. Cancer Treat Rev 2010; 36: 425-435.

[25] Bobola MS, Blank A, Berger MS, Stevens BA, Silber JR. Apurinic/apyrimidinic endonuclease activity is elevated in human adultgliomas. Clin Cancer Res 2001; 7: 3510-3518.

[26] Kelley MR, Cheng L, Foster R, Tritt R, Jiang J, Broshears J, Koch M. Elevatedand altered expression of the multifunctional DNA base excision repair andredox enzyme Ape1/ref-1 in prostate cancer. Clin Cancer Res 2001; 7: 824-830.

[27] Luo M, KELLEY MR. Inhibition of the Human Apurinic/Apyrimidinic Endonuclease (Ape1) Repair Activity and Sensitization of Breast Cancer Cells to DNA Alkylating Agents with Lucanthone. Anticancer Res 2004; 24: 2127-2134.

[28] Qiu ZK, Shen D, Chen YS, Yang QY, Guo CC, Feng BH, Chen ZP. Enhanced MGMT expression contributes to temozolomide resistance in glioma stem-like cells. Chin J Cancer 2014; 33: 115-122.

[29] Ando K, Hirao S, YasuakiKabe, Ogura Y, Sato I, Yamaguchi Y, Wada T and Handa H. A new APE1/Ref-1-dependent pathway leading toreduction of NF-κB and AP-1, and activation oftheir DNA-binding activity. Nucleic Acids Res 2008; 36: 4327-4336.