Pathol. Oncol. Res. (2011) 17:587–591
DOI 10.1007/s12253-010-9354-7

RESEARCH

Relationship Between Hypermethylated MGMT Gene and Osteosarcoma Necrosis Rate After Chemotherapy

Qiu Cui · Weihao Jiang · Jun Guo · Cheng Liu · Dingfeng Li · Xiaohong Wang · Yanjun Zeng

Received: 15 August 2010 / Accepted: 28 December 2010 / Published online: 20 March 2011

Abstract To investigate the relativity of MGMT(O-6-methylguanine-DNA methyltransferase) gene methylation from patients with protein expression and osteosarcoma necrosis rate after chemotherapy. Fifty-one oteosarcoma tissues were collected, Methylation of MGMT gene promoter was detected by methylation-specific PCR method, and protein expression of MGMT was examined by immunohistochemistry procedure, the relationship between methylated MGMT gene expression and patients response to chemotherapy was analyzed. The positive ratio of methylation MGMT gene promoter in 51 patients was 23.5% (12 in 51). Negative percentage of protein expression of MGMT was 27.5% (14 in 51). It seemed that methylation of MGMT gene in osteosarcoma tissues had no evident relationship with the patient's age, sexuality, and the size and type of neoplasms, etc. The necrosis rates of methylated MGMT of osteosarcoma (tumor grade from I to IV) were 0 (0/51), 3.9% (2/51), 5.9% (3/51), 13.7% (7/51), respectively. In contrast, the necrosis rates of unmethylated MGMT of osteosarcoma (tumor grade from I to IV) were 45.1% (23/51), 25.5% (13/51), 3.9% (2/51), 2.0% (1/51), respectively. It suggest that methylated and unmethylated MGMT gene of osteosarcoma have significant difference in protein expression. The unmethylated MGMT gene has higher positive protein expression (u=−4.92, P<0.001). Methylation of MGMT gene has higher tumor necrosis rate in osteosarcoma patients. Methylation in MGMT promoter may be important for judging the effect of chemotherapy in Osteosarcoma patients.

Keywords Osteosarcoma · DNA methylation · CpG island · Drug resistance, Neoplasm · O^6-Methylguanine-DNA Methyltransferase

Q. Cui · W. Jiang · J. Guo · C. Liu · D. Li (✉)
Department of Orthopedics, the Affiliated Hospital of Academy of Military Medical Sciences, PLA 307th Hospital,
Beijing 100071, China
e-mail: 307yygk@sina.com

X. Wang · Y. Zeng (✉)
Biomechanics and Medical Information Institute, Beijing University of Technology,
Beijing 100022, China
e-mail: yjzeng@bjut.edu.cn

Introduction

The drug-resistance of carcinoma cells has been one of the reasons of improper effect of chemotherapy to the osteosarcoma. So how to predict and overcome the problem of drug-resistance of carcinoma cells is to be resolved. Lots of reports showed that O6-methylguananine-DNA methyltransferase (MGMT) could repair the injury of DNA caused by alkylating agent, which was the main reason of drug-resistance of carcinoma cells [1, 2]. There are many clinical trials based on the inhibition of MGMT and using alkylating agent reasonably for the treatment of cancer domestic and overseas [3, 4]. But there is no report by using this method for the treatment of osteosarcoma. In the article we detected the methylation of the promoter and the expression level of protein of MGMT in the osteosarcoma and analyzed the relationship between methylation of promoter of the MGMT and the tumor necrosis rate in

order to investigate the significance of methylation of the promoter of MGMT in the chemotherapy.

Material and Method

The Resource of Osteosarcoma Tissue

Fifty-one osteosarcoma tissue were collected in the 307 hospital of PLA by surgical resection and biopsy specimens, and immediately stored in liquid nitrogen after operation. In the 51 patients, 30 were male and 21 are female, the age of patients from 7–64,and median age was 17.9, and the diameter of osteosarcoma was 3.1–25.0 (average 9.2 cm). All the osteosarcoma tissue were primary tumors, and the number of the osteosarcoma tissue of osteoblast progenitor cell type was 38, fibroblast progenitor cell type was 6, chondroblast progenitor type was 4 and telangiectatic was 3. All the 51 patients were treated with chemothreapy for 3–7 cycle before operation, and the medicine for chemothreapy contained cisplatin, adriamycin and ifosfamide.

Tumor Cell Necrosis Rate Evaluation

Twelve pieces of tissue were exscinded from middle to verge of the 51 osteosarcoma tissues after operation and then stained with HE, chose two pieces of sections of the 12 pieces of tissue to observe the live tumor cells of five visual field random. If there was no live tumor cell in one section, tested the whole piece. Choose the visual field and the average number of the live tumor cells was N. Choose three visual field of the representative sections which was made before operation random, and counted the number of the live tumor cells and the average of the tumor cells was M. The formula to calculate tumor cell necrosis rate was $\mathrm{TCNR} = (1 - \mathrm{N}/\mathrm{M}) \times 100\%$. The grade of TCNR was divided to four degree: grade I TCNR ≤50%, grade II 50% <TCNR ≤90%, grade III 90% <TCNR ≤99%, grade IV = 100% [5].

DNA Extract

The genomic DNA was extracted by Genomic DNA Mini Preparation Kit from the frozen tissue of osteosarcoma tissues, and the purity of DNA detected by agarose gel electrophoresis and UV detector.

Methylation Specific PCR (MSP)

The DNA extracted from genome and serum were modified of sulphited by EZ DNA Methylation-gold™ Kit. Methylated cytosines (C) did not change, but unmethylated cytosines transfroming to uracil (U) after modification, which was turned into thymine (T) by PCR subsequently. PCR was progressed by primers of methylated and unmethylated. The sequence of the primers [6] were synthesized by Aoke biotec. co.lit. See Table 1 for the sequence and annealing temperature of the primers.

The DNA polymerase was Hotstart Taq DNA polymerase (TAKARA), the condition of the PCR was 95 5 min; 95 45 s, annealing 45 s for each primer, 72 60 s, for 35 cycle;72 extend for 5 min. Normal DNA of PBL as Positive control of unmethylated group, and the DNA treated with methylase as positive control of methylated group, and the distilled water was a negative control of non template. The product of PCR was separated by 3% agarose, which was stained by EB, and observed by UV hydrogel imaging system.

Immunohistochemical Staining for Expression of MGMT

Osteosarcoma tissues were fixed by formalin and embedded in paraffin, then stained with the Immunohistochemical staining Kit invented by Institute of blood transfusion of field of Academy of military medical science. The MGMT

Table 1 Methylation-specific PCR primers, annealing temperature and the length of amplified products

Gene	Sequence of primer	Length of products (bp)	Tm
MF	TTTCGACGTTCGTAGGTTTTCGC	81	55
MR	GCACTCTTCCGAAAACGAAACG		
UF	TTTGTGTTTTGATGTTTGTAGGTTTTTGT	93	63
UR	AACTCCACATCTTCCAAAAACAAAACA		

MR forward primer of methylaed group; Reverse primer of methylated group; *UF* forward primer of unmethylaed group, *UR* reverse primer of unmethylated group

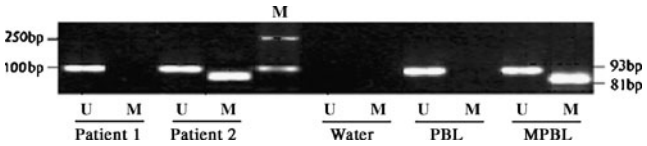

Fig. 1 Methylation-specific PCR (MSP) analysis of MGMT hypermethylation in osteosarcoma patients

positive cell was stained to brown. In a section if the rate of positive cell was <5%, it was regarded as negative, 5–10% was weak positive, 10–30% was positive and ≥30% was strong positive.

Statistical Analysis

The data was analyzed with SPSS13.0, and the relationship of the methylation of MGMT and clinical parameters (age, gender, size of osteosarcama, pathological type) was tested by $\chi 2$-test and rank sum test. $P<0.05$ was statistical significance.

Result

The Rate of Methylation in the CpG Island in the Promoter of MGMT in Osteosarcoma Tissue

As the result of MSP in the Fig. 1, if the electrophoretic band was appeared only in the unmethylated group(U),the MGMT gene was not methylated, and if the electrophoretic band was appeared in both methylated group(M) and unmethylated group(U), the MGMT gene was methylated. The rate of methylation of the promoter gene was 23.5% (12/51) in the 51 osteosarcoma tissues. Statistical analysis showed that the relationship between methylation and the age of the patients(rank sum test),gender, pathological type, size of osteosarcoma tissue ($\chi 2$ -test) was not statistical significance ($P>0.5$) (Fig. 2; Table 2).

The Relationship Between the Methylation of CpG Island in the Promoter of MGMT Gene and the Expression of MGMT Protein

The result of Immunohistochemical staining was shown in the Fig. 2, the rate of osteosarcoma that expressed protein of MGMT was 72.5% (35/51). In 12 patients of methylated MGMT, nine patients negative expressed MGMT, two patients low expressed MGMT and one patient was positive express MGMT. In 39 cases of unmethylated MGMT patients, five cases were MGMT negative expression, and the rest were MGMT positive expression. The results showed that the expression of MGMT was different in the methylated and unmethylated MGMT groups, the expression protein of MGMT was increased obviously ($u=-4.92$, $P<0.001$, Wilcoxon rank sum test).

The Relationship of Methylation of MGMT and Tumor Cell Necrosis Rate in Osteosarcoma Tissues

The number of patients(12 cases),whose MGMT were methylated, that the tumor cell necrosis rate in grade I was 0(0%), in grade II was 2(16.7%), in grade III was 3 (25%), in grade IV was 7(58.3%) after chemotherapy; and in the 39 cases of unmethylated MGMT patients treated with chemotherapy, the cases that tumor necrosis rate in grade I was 23(59%), in grade II was 13(33,3%),in grade III was 2 (5,1%),in grade IV was 1(2.6%). The comprehensive evaluation of tumor cell necrosis through the data of pathological tissues and the statistical analysis showed that the effect of chemotherapy is better in the MGMT methylated group than the unmethylated group. ($P<0.05$, Table 3)

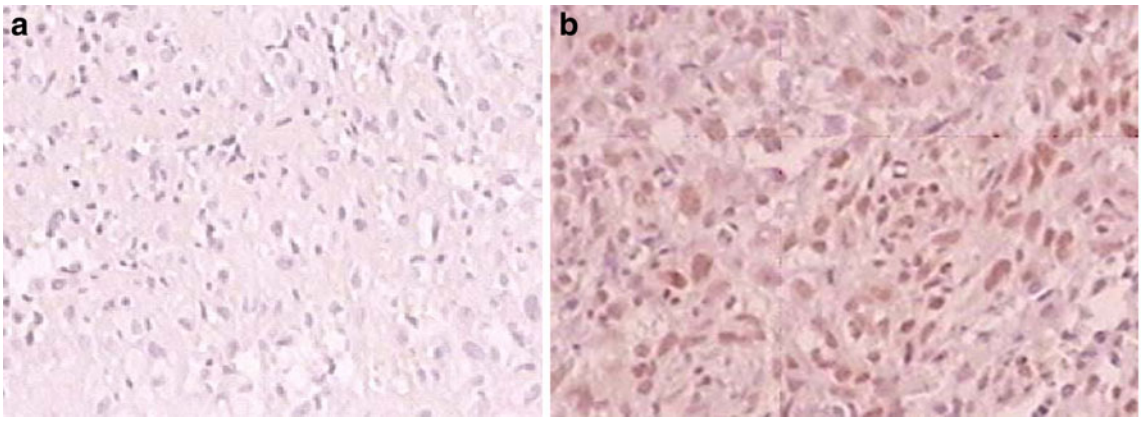

Fig. 2 Expression of MGMT protein in osteosarcoma tissue (DAB×100)

Table 2 Relevance of MGMT gene methylation and clinical pathological parameters in 51 cases of patients with oseosarcoma

Item	Cases	Rate of methylated MGMT(%)
Gender		
Male	30	7(23.3)
Female	21	5(23.8)
Age(year)		
≤10	6	1(16.7)
11–20	19	5(26.3)
21–30	17	4(23.5)
≥31	9	2(22.2)
Pathological type		
Telangiectatic	3	1(33.3)
Osteoblast type	38	9(23.7)
Cartilage progenitor type	4	1(25)
Fibroblast progenitor type	6	1(16.7)
Size of osteosarcoma		
≥10	18	4(22.2)
<10	33	8(24.2)

Discussion

These years, the survival rate and the successful limbs-saving operation rate of osteosarcoma patients has been increased significantly, thereinto, chemotherapy plays an important role in the general therapy of osteosarcoma. Numerous studies found that the high-expression of DNA repaired protein MGMT was negatively correlated with alkylating agent tumor necrosis rate. Through monitor the level of MGMT to estimate the sensitivity of this tumor to generalized alkylating agent and combined application of MGMT activity inhibitor and chemotheraputic drugs, the sensitivity of CDDP to tumor chemotherapy could be improved obviously [7].

As DNA repairing protein, MGMT could remove the mutagenic toxicity and cell toxicity adduct alkylation at O^6 site of guanine in DNA, and recovered from damaged guanine, so that cells could confront the damage of alkylation group, which was the main reason the tumor cell tolerance the alkylating regent. However, the alkylaing MGMT protein was inactive and then degradated through the ubiquitin path. The ability of cell overcoming the damage of O^6-AG was closely related to the re-inducing ability of MGMT. Many studies indicated that the expression of MGMT in tumor was the main reason caused the drug tolerance of alkylating regent [8, 9], but the relative study report of its function in osteosarcoma was less. These years, the study of MGMT silencing got great advances: on one side, the silencing of MGMT gene led to the accumulation of related gene transformation for tumorigenesis. On the other hand the over methylated MGMT gene related to the sensitivity of the tumor cell to the stimulation of alkylating agent [10, 11]. So the detection of the methylation of MGMT gene was valuable for the early prediction of tuomorigenesis and the sensitivity of tumor cell to the stimulation of alkylating agent.

So far the methylation of MGMT gene was detected in many tumor tissues, for example lung cancer, brain glioma, esophageal carcinoma, colon cancer, cervical cancer etc [12], and the rate of methylation was different, commonly from 10–60%, but the methylation of MGMT gene in normal tissues of healthy people or cancer patients could not detected. In our study, the rate of methylation of MGMT gene was 23.5% in osteisarcoma tissues, but not detected in normal tissues, which was consistent with reports before [13]. We found that the methylation of MGMT gene was not related to the age of patients, gender, size of osteosarcoma tissues and pathological types. The study showed that the methylation of MGMT gene was cause low protein expression level of MGMT, which suggested that methylation in the promoter of MGMT might be a primary reason for the sliencing of MGMT. The effect of chemotherapy in the groups, whose MGMT gene was methylated was much better than the unmethylated group, which suggested that the methylation of MGMT

Table 3 The relativityof methylated MGMT gene in osteosarcoma tissues with the tumor necrosis rate (%)

MGMT gene	Tumor cell necrosis rate				Sum
	Grade I	II	III	IV	
Methylated	0	2	3	7	12
Unmethylated	23	13	2	1	39
Sum	23	15	5	8	51

gene was not only valuable to the early prediction of tumorgenesis, but also to be a predictor for the effect of chemotherapy using alkylating agent.

The abnormal methylation of MGMT gene in osteosarcoma tissue was closely related to the expression level of MGMT, which suggested that the methylation was important for the silencing of MGMT and might related to tumogenesis of osteosarcoma. The condition of methylated MGMT in tumor cells cound influence the tumor cell necrosis rate, which might be valuable for the therapeutic effect and prognosis estimation.

References

1. Sabharwal A, Middleton MR (2006) Exploiting the role of O6-methylguanine DNA Methyltransferase (MGMT) in cancer therapy[J]. Curr Opin Pharmacol 6(4):355–363
2. Kaina B, Christmann M, Naumann S, Roos WP (2007) MGMT: key node in the battle against genotoxicity, carcinogenicity and apoptosis induced by alkylating agents[J]. DNA Repair (Amst) 6(8):1079–99
3. Jacinto FV, Esteller M (2007) MGMT hypermethylation: a prognostic foe, a predictive friend[J]. DNA Rep 10(1):10–16
4. Helleday T, Petermann E, Lundin C et al (2008) DNA repair pathways as targets for cancer therapy[J]. Nat Rev 8(3):193–204
5. Rosen G, Caparros B, Huvos AG et al (1982) Preoperative chemotherapy for osteogenic sarcoma: selection of Postoperativeadjuvant chemotherapy based on the response of the primary tumor to preoperative chemotherapy [J]. Cancer 49(6):1221–1230
6. Lee S, Kim WH, Jung HY et al (2002) Aberrant CpG island methylation of multiple genes in intrahepatic cholangiocarcinoma [J]. Am J Pathol 161(3):1015–1022
7. Ho JW (2006) Potential and cytotoxicity of cis-Platinum complex with anti-tumor activity in combination therapy[J]. Recent Patents on Anti-Cancer Drug Discovery 1:129–134
8. Sabharwal A, Middleton MR (2006) Exploiting the role of O^6-methylguanine DNA methyltransferase (MGMT) in cancer therapy[J]. Curr Opin Pharmascol 6(4):355–363
9. Verbeek B, Southgate TD, Gilham DE, Margison GP (2008) O6-Methylguanine-DNA methyltransferase inactivation and chemotherapy[J]. Br Med Bull 85:17–33
10. Voelter V, Diserens AC, Moulin A et al (2008) Infrequent promoter methylation of the MGMT gene in liver metastases from uveal melanoma[J]. Int J Cancer 123(5):1215–8
11. Gal-Yan EN, Saito Y, Egger G et al (2008) Cancer epigenetics: modifications, screening, and therapy[J]. Annu Rev Med 59:267–280
12. Weisenberger DJ, Siegmund KD, Campan M et al (2006) CpG island methylator phenotype underlies sporadic microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer. Nat Genet 38:787–793
13. Kawaguchi K, Oda Y, Saito T et al (2006) DNA hypermethylation status of multiple genes in soft tissue sarcomas[J]. Mod Pathol 19 (1):106–114