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# Relationship between methylation status of ERCC1 promoter and radiosensitivity in glioma cell lines

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**Abstract**

Ionizing radiation represents one of the most important therapies for glioma, a lethal primary brain tumor, while radiotherapy remains a challenge for radiation oncologist because of radioresistance. Radiosensitivity of gliomas determines radiotherapy efficacy. Evidence demonstrated that methylation of CpG Island in the promoter region may result in gene silencing. This study was designed to determine the relationship between methylation status of ERCC1 promoter region and radiosensitivity in glioma cell lines. We investigated the expression levels of ERCC1 transcripts and protein in GBM cell lines. Colony forming experiments was used to measure surviving fraction at 2 Gy (SF2) in four human glioma cell lines, MGR1, MGR2, SF767 and T98G. Methylation status in the promoter region of ERCC1 in these glioma cell lines was determined by using bisulphate sequencing and MSP analysis. Radiosensitivity was examined to be heterogeneous in these glioma cell lines. There was a statistical difference in the radiosensitivity between glioma cell lines with and without methylation of ERCC1 gene promoter CpG islands. Furthermore, we promoted ERCC1 expression by 5-azacytidine treatment which resulted in the reduction of radiation-induced cell killing in radiosensitive cell lines. Our data indicate that methylation status of ERCC1 is associated with radiosensitivity in glioma cell lines. It could be used as a new biomarker for predicting the radiosensitivity of human gliomas.


*Keywords:* Glioma; Methylation; Epigenetics; Radiosensitivity

## 1. Introduction

Glioblastoma multiforme (GBM) is the most lethal primary brain tumor (Legler et al., 1999). Radiotherapy is ideally suited for the treatment of intracranial lesions, because it is not limited by the blood– brain barrier and is able to conform to highly irregular target volumes. But glioblastomas has poor prognosis because of resistance to radiation and other treatments. The intrinsic radiosensitivity of gliomas is a pivotal factor that determines radiotherapy effect. According to tumor radiosensitivity, developing an individualized radiotherapy regimen for every glioma patient is very important.

Ionizing radiation exerts anti-tumor activities through induction of DNA damages. Previous studies have shown that DNA repair capacity is correlated with the radiosensitivity of human gliomas (Connell et al., 2004). The nucleotide excision repair (NER) pathway is a complex network of many proteins assembled in DNA repair system and there are about 16

*Abbreviations:* ERCC1, Excision repair cross complementing protein 1; ERCC2, Excision repair cross complementing protein 2; XPF, Xeroderma pigmentosum complementation group F; NER, Nucleotide excision repair; SF2, Surviving fraction at 2 Gy; BBB, blood–brain barrier; MSP, Methylation-specific PCR.

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essential proteins involved in DNA-damage recognition and excision. ERCC1 is primarily involved in the recognition and excision of DNA damage product and further repair steps are followed by some genes including helicase ERCC2 that is a component of transcription factor TFIIH. It has shown that high ERCC1 expression in tumor tissue has been related to CDDP resistance in many types of cancer (Furuta et al., 2002; Yu et al., 2000). ERCC1-XPF makes a substantial contribution to protecting mammals from radiation-induced DNA damage via an end-joining mechanism in vitro and in vivo (Ahmad et al., 2008). Interaction of ERCC1 and EGFR might correlate with radiation-induced DNA damage in p53 mutant human colon cancer (Hiro et al., 2008).

DNA methylation regulates gene expression epigenetically, and it often leads to transcriptional repression and subsequent loss of protein expression. Some tumor genes can be inactivated by methylation of cytosine residues within CpG islands. CpG islands are commonly located within or in close proximity to the transcriptional start sites of many genes, and CpG methylation generally results in the transcriptional repression of nearby genes (Bird, 1992). Previous studies have also shown that methylation of the ATM promoter in glioma cells alters ionizing radiation sensitivity (Roy et al., 2006).

In this study, we designed to elucidate the relationship of the ERCC1 promoter in a panel of human glioma cell lines to determine if the methylation status of the ERCC1 promoter correlates with cellular radiosensitivity.

## 2. Materials and methods

### 2.1. Cell lines and cell culture, 5-aza-2-deoxycytidine treatment

The cell lines used were MGR1, MGR2, T98G and SF767. All cell lines were maintained in RPMI 1640 (Gibco, USA) supplemented with 10% fetal bovine serum (Hyclone, USA), and cultured at 37 °C in a humidified 5% $CO_2$ atmosphere. Cells were harvested with 0.25% trysin–0.02% EDTA at 37 °C and were washed once in supplemented medium. 5-Azacytidine (Sigma, USA) was added to the culture medium on day one, and the cells maintained in culture for 5 days until 60–70% confluent with frequent changes of media (Roy et al., 2006).

### 2.2. Total RNA isolation and reverse transcription-PCR

*RNA extraction and reverse transcription-PCR*. Total RNAs from cells were extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions. The RNA was pretreated with DNase and used for cDNA synthesis with random hexamers. ERCC1 was PCR amplified from cDNA samples of four glioma cell lines. The nucleotide sequences of the forward and reverse primers for each gene are listed in Table 1. β-Actin was amplified as an internal control. The appropriate size of PCR products was confirmed by agarose gel electrophoresis.

Table 1
PCR primer sequences.

| Target genes | Primers | Size (bp) |
|---|---|---|
| ERCC1 | 5′-CCCTGGGAATTTGGCGACGTAA-3′ (sense)<br>5′-CTCCAGGTACCGCCCAGCTTCC-3′ (antisense) | 273 |
| ERCC1 bisulfite genomic sequencing | 5′-GGATGTTITATTAAAAAAGGTGTT-3′ (sense)<br>5′-CTTCCCCTCCTCTCAACTT-3′ (antisense) | 469 |
| ERCC1 MSP methylated | 5′-CGCGTTATCGCGGTTAAGT-3′ (sense)<br>5′-ACCTTCCCCTCCTCTCAACTT-3′ (antisense) | 223 |
| ERCC1 MSP unmethylated | 5′-TGGGTTGTGTGTTATTGTGGITA-3′ (sense)<br>5′-ACCTTCCCCTCCTCTCAACTT-3′ (antisense) | 230 |
| β-Actin | 5′-ACACTGTGCCCATCTACGAGG-3′ (sense)<br>5′-AGGGGCCGGACTCGTCATACT-3′ (antisense) | 621 |

### 2.3. Western blotting

Total protein was extracted from four glioma cell lines, and protein level was determined by ultraviolet spectrophotometer (Beckman Company). The proteins were separated by 12% SDS-PAGE gel electrophoresis at 100 V for 100 min. The gel was transferred onto PVDF membrane (Roche). The membranes were blocked with PBST containing 5% skim milk overnight at room temperature. The membranes were probed with ERCC1 (Ab-6, Bioworld) mouse monoclonal antibody and β-actin in PBST containing 5% BSA. The blots were then washed with PBST and incubated with horseradish peroxidase-conjugated secondary antibody. The specific complexes were detected using Phototope-HRP Western Blot Detection System (Cell Signaling Technology).

### 2.4. Irradiation

Deep-seated therapeutic X ray machine, which yield 101.38 cGy/min at 210 kV and 12 mA, was used as a source of irradiation. The cell suspension was irradiated with 2 Gy.

### 2.5. Clonogenic survival assay

The procedures were done similarly to previously described methods (Liu et al., 2006). Briefly, exponentially growing cells were trypsinized into single cell suspension. Cell viability was assessed by trypan blue dye exclusion. Viable Cells ($10^2$) were plated in 60 mm Petri dishes, and exposed to 2 Gy of irradiation. Following exposure, cells were incubated at 37 °C, 5% $CO_2$ for colony formation. After 10–14 days of growth, the colonies were fixed with methanol, stained with 1% crystal violet. Only colonies having a minimum of 50 viable cells were counted. Colony plating efficiency was calculated to be the number of