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Methylation of the ATM promoter in glioma cells alters ionizing radiation sensitivity

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Abstract

Glioblastomas are among the malignancies most resistant to radiation therapy. In contrast, cells lacking the ATM protein are highly sensitive to ionizing radiation. The relationship between ATM protein expression and radiosensitivity in 3 glioma cell lines was examined. T98G cells exhibited normal levels of ATM protein, whereas U118 and U87 cells had significantly lower levels of ATM and increased (>2-fold) sensitivity to ionizing radiation compared to T98G cells. The ATM promoter was methylated in U87 cells. Demethylation by azacytidine treatment increased ATM protein levels in the U87 cells and decreased their radiosensitivity. In contrast, the ATM promoter in U118 cells was not methylated. Further, expression of exogenous ATM did not significantly alter the radiosensitivity of U118 cells. ATM expression is therefore heterogeneous in the glioma cells examined. In conclusion, methylation of the ATM promoter may account for the variable radiosensitivity and heterogeneous ATM expression in a fraction of glioma cells.

Keywords: Glioblastoma; Radiation therapy; Ionizing radiation; ATM; Methylation; Radiosensitivity

Ataxia Telangiectasia (*A-T*) is an inherited disease characterized by immunodeficiency, cerebellar ataxia, chromosomal instability, and increased incidence of cancer [1]. The product of the *A-T* gene, the ATM protein kinase, is a key component of the signal transduction pathway activated by DNA damage [2,3]. ATM's kinase activity is activated by genotoxic events which generate DNA strand breaks, and ATM is recruited to sites of DNA damage [4]. Once activated, the ATM protein kinase co-ordinates the DNA damage response through the direct phosphorylation of proteins involved in the activation of cell cycle checkpoints and DNA repair [3]. Cells lacking functional ATM protein show increased sensitivity to ionizing radiation (IR) and other genotoxic events. This increased sensitivity of *A-T* cells to IR is linked to defects in DNA repair, including elevated levels of chromatid aberrations and chromosome end joining [5–7], and defects in the repair of DNA double strand breaks [8].

Loss of functional ATM is associated with both decreased genomic integrity and increased cancer risk. 10% of *A-T* homozygotes develop cancer, mainly of lymphoid origin [1], and there is accumulating evidence that *A-T* heterozygotes have increased susceptibility to breast cancer [9,10]. Inactivating mutations in ATM have also been detected in T cell prolymphocytic leukemia, B cell chronic lymphocytic leukemia, and mantle cell lymphoma [11,12]. These observations imply a key role for ATM in suppressing mutational events which can ultimately lead to cancer.

In addition to mutations, tumor suppressor genes can be inactivated by methylation of cytosine residues within CpG islands which are located in the promoter of many genes [13]. This methylation of the promoter leads to transcriptional repression and subsequent loss of protein expression. For example, the p16 [14], Brca1 [15], and MLH1 [16] genes have all been shown to be inactivated by methylation during tumor progression. Studies have also shown that the

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ATM promoter is methylated in some head and neck carcinomas [17], and in the MLH defective HCT116 cell line [18], implying decreased levels of ATM protein in these tumor cells. Methylation of the ATM promoter will therefore decrease ATM protein levels, which should lead to increased sensitivity of the cells to DNA-damaging agents such as IR [18]. Here, we have explored the methylation status of the ATM promoter in a representative group of human glioblastoma cell lines to determine if the methylation status of the ATM promoter correlates with cellular radiosensitivity.

Methods

Cell culture conditions. U87, T98G, and U118 cells were obtained from the American Type Culture Collection and were maintained in alpha-MEM supplemented with fetal bovine serum (10%). 5-Azacytidine 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. For cell survival experiments, cells were plated at the appropriate dilution, irradiated, and surviving colonies were stained with crystal violet 10 days later [19].

Western blot analysis and immunofluorescent staining. Western blot analysis to detect the ATM protein was carried out as previously described by us [19]. For immunofluorescent detection of ATM, cells were seeded onto LabTek II chamber slides (Nunc, NY). Cells were fixed and stained with ATM antibody 5C2 (Genetex, TX) and Texas Red or GFAP protein (Santa Cruz, CA) and FITC. Slides were mounted with Fluoromount-G (Southern Biotech, AL) and visualized with a Nikon Eclipse TE 2000.

DNA extraction and methylation-specific PCR. Genomic DNA was extracted from T98G, U87, and U118 glioma cells using the QiaQuick™ kit (Qiagen, CA). Unmethylated, human male genomic DNA was used as negative control (Promega, WI). To prepare a fully methylated control DNA, human male genomic DNA (10 μg) was methylated in vitro with M. SssI methyltransferase (20 U; New England Biolabs, MA) at 37 °C for 2 h in the presence of 160 μM *S*-adenosylmethionine. DNA from glioma cells as well as from methylated/unmethylated controls was subjected to standard bisulfite modification conditions prior to performing methylation-specific PCR (MSP) [20] using the Chemicon commercial kit. Bisulfite modified DNA (1 μl) was amplified in a 20 μl PCR using TITANIUM Taq™ (Clontech laboratories, Inc., Palo Alto, CA). MSP primers for analysis of the ATM gene promoter were: 5′-GTTTTGGAGTTTGAGT TGAAGGGT-3′ (sense) and 5′-AACTACCTACTCCCACTTCCAA-3′ (antisense) for amplification of the promoter existing in an unmethylated state and 5′-GGAGTTCGAGTCGAAGGGC-3′ (sense) and 5′-CTACC TACTCCCGCTTCCGA-3′ (antisense) for amplification of the promoter existing in a methylated state. The thermal cycler conditions consisted of 5 min at 95 °C and 38 cycles at 95 °C for 30 s, 57.5 °C for 30 s, 72 °C for 30 min, and a final 72 °C for 10 min.The PCR products were subsequently assayed by electrophoresis on 2% agarose gels and were visualized by ethidium bromide staining.

Results

The levels of ATM protein expression were examined in a panel of representative brain tumor cell lines, including U87, T98G, and U118 cells. Western blot analysis (Fig. 1A) demonstrates that T98G cells expressed similar levels of ATM protein to that detected in HeLa cells. By comparison, ATM protein levels were extremely low in the U87 and U118 cell lines. To further characterize this, the cellular location of ATM was examined by immunofluorescent staining with ATM-specific antibodies. In

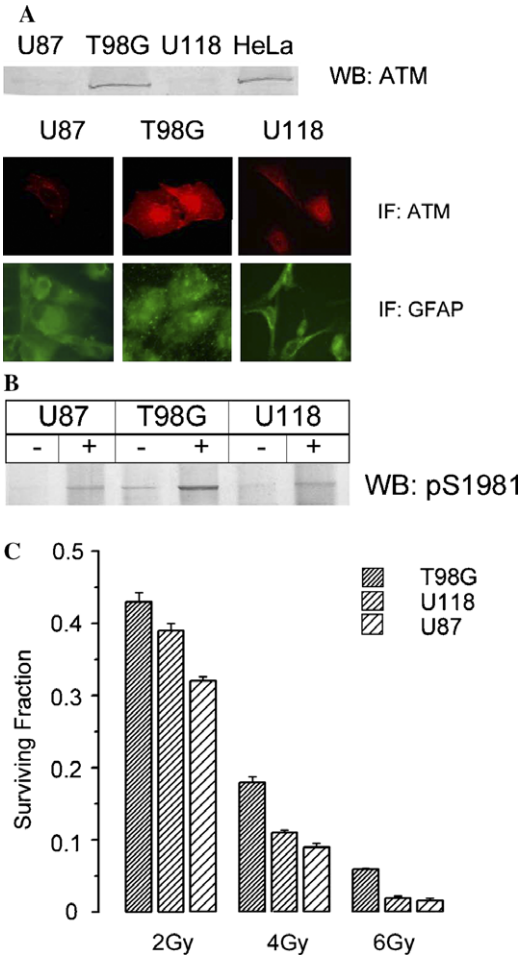

Fig. 1. ATM expression in glioma cell lines U87, T98G, and U118. (A) Cell extracts were examined for the presence of ATM protein by either Western blot (WB) analysis or immunofluorescent (IF) staining. (B) Cells were incubated with (+) or without (−) bleomycin (5 μM) for 30 min. Cell extracts were separated by SDS–PAGE and the levels of autophosphorylation of serine 1981 (pS1981) of ATM determined by Western blot (WB). (C) Cells were irradiated at the indicated dose, and colony formation measured by clonogenic cell survival 10 days later. Results ± SE ($n = 4$).

T98G cells, ATM was localized to the nucleus of the cells, with some ATM detected in the cytoplasm (Fig. 1A, IF). U87 cells showed only a weak ATM signal confined to the cytoplasm of the cells. U118 had intermediate levels of staining, with some cells showing nuclear ATM, although the majority displayed cytoplasmic ATM. Glioma cell lines can lose expression of GFAP, a protein expressed in astrocytes, when maintained in culture [21]. Fig. 1B demonstrates that all 3 cell lines retained significant levels of GFAP expression in culture.

Low levels of ATM expression may occur through several mechanisms, including point mutations within the coding region of ATM which decrease protein stability, or through decreased transcription of the ATM gene. Although most ATM mutations result in protein truncations [22], a subset of point mutations has been identified from *A-T* patients which reduce ATM protein levels and