Analysis of Cellular Factors assisting AGT Regulation

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**Abstract**

O\textsuperscript{6}Methyguanine-DNA methyl transferase is a very critical enzyme in a cell. It protects cells from carcinogens and protects the tumour cells from chemotherapeutic agents. It is considered a unique DNA-repair enzyme and thought to act alone to repair alkyl lesions of DNA. This protein however, is without a nuclear localization signal and yet found to be nuclear. In this study, utilising human Capan-1 cell system, BRCA2 is shown to be linked to the translocation of AGT. The generally nuclear AGT is shown to be sequestered in the cytoplasm, in Capan-1, in which the truncated BRCA2 is localized in the cytoplasm. We show that upon treatment with BCNU, an alkylating agent, both AGT and BRCA2 translocate into the nucleus. The degradation of the two proteins is also demonstrated to follow the same time line, suggesting the involvement of BRCA2 in AGT degradation. To assist further analysis cloning of AGT in a bacterial expression vector was achieved.

**Introduction**

Mutagens in the environment and some endogenous can generate electrophilic species that alkylates DNA (Marnett,1984 and Bartsch,1993). The alkylating agents attack the nucleophilic Nitrogens and Oxygens in the DNA bases. O\textsuperscript{6}-Methyguanine (O\textsuperscript{6}MG) is one such alkyl adduct, which is thought to be the most critical. O\textsuperscript{6}MG, if it is not repaired can cause point mutations (Toorchen,1983), sister chromatid exchanges (Kaina,1991), chromosomal aberrations , tumour initiation (Margison,1975), tumour progression and cell death (Meikrantz,1998 and Tominaga,1997). O\textsuperscript{6}-MG is repaired by O\textsuperscript{6}-Methylguanine-DNA Methyltransferase (MGMT or AGT), considered to be a unique DNA-repair enzyme that acts alone. Inspite of being carcinogenic, methylating agents generating O\textsuperscript{6}-MG are used to treat gliomas, melanoma, carcinoid tumours and Hodki’s lymphoma because of their cytotoxic properties. Chemotherapeutic agents, like Carmustine (BCNU), produce O\textsuperscript{6}- chloroethlyguanines lesions, which are converted into G-C interstrand crosslinks and leads to apoptosis.O\textsuperscript{6}-chloroethylguanine and O\textsuperscript{6}-ethylguanine lesions are also repaired by AGT. AGT repairs O\textsuperscript{6} alkyl adducts in one step, by transferring the alkyl group in the DNA to a cysteine residue in the catalytic pocket of AGT (cysteine at position 145 in human AGT). The alkylated AGT is rendered inactive and the inactivated AGT appears to be ubiquitinated (Srivenguopal,1996) and degraded by proteosome (Xu-Welliver,2002).

AGT is predominantly a nuclear protein; studies have however shown that AGT does not contain a nuclear localization signal. Cellular factors are thought to be involved in the nuclear targeting of AGT, however, the identity of such factors remain to be established. Recently, BRCA2, a double strand break repair protein, was shown to be involved in the function of AGT, in a mouse system (Philip S,2008). BRCA2 was demonstrated to be physically

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associated with AGT and degrades along with AGT, after exposure to alkylating agents. A mutant BRCA2 produced with a 29-amino acid mutation in an evolutionarily conserved region, does not bind to AGT and interestingly both the proteins are not degraded when the mouse embryonic fibroblasts are exposed to alkylating agents. To do a similar study in the human system, Capan-1 cells were used. Capan-1 cells are pancreatic cancer cells that carry a deletion of BRCA2 in one homolog, while the other codes for a truncated BRCA2 only 1981 amino acids long is shown to sequestered in the cytoplasm (Spain BH, 1999). This BRCA2 has only 6 BRC repeats as opposed to 8 BRC repeats found in full length BRCA2 (Chen PL, 1998). The objective of this study was to further analyse the interactions of AGT with BRCA2. Capan-1 system, which has a mutated BRCA2 was used to understand the importance BRCA2 in AGT-mediated alkylation repair. To further study the interactions of the AGT, recombinant AGT was cloned.

**Methods and Materials**

**Cell culturing:** Capan-1 cells, a pancreatic cancer cell line, were cultured in Iscove’s modified Dulbecco’s medium (IMDM) supplemented with 20% Fetal Bovine Serum (Hyclone, Thermal Fisher Scientific Inc.). While, Hela cells, a cervical cancer cell line, were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum. All the reagents used to culture the cells are from Invitrogen Corp.

**In vitro AGT degradation assay:** The total cell lysates were prepared in TDEG buffer. AGT degradation in the presence of 100 µM of O6-benzylguanine, was driven by 2mM ATP and 5mM MgCl2. Samples were taken after 0, 3, 6, 9, 12, 16 and 20 hours, proteins separated on a 12% denaturing SDS gel and analyzed by standard Western Blot technique.

**In vivo AGT degradation assay:** 150 µM of BCNU was added to the Capan-1 cells. The cells were extracted after 16 and 20 hours and the nuclear and cytoplasmic fractions were separated using the NE-PER Nuclear-Cytoplasmic extraction kit (Thermo Scientific Pierce Protein Research Products). The proteins extracted were separated on a denaturing 4-12% Tris-glycine gradient gel and analysed by Western Blot.

**Immunofluorescence:** Cells were plated on coverslips at 50% - 60% confluency and treated with alkylating agent-(64 µM BCNU; IC 50) for 2 hours and allowed to recover for varying time periods. The cells were fixed with cold methanol and permeabilized with 0.1% Triton X-100 solution and blocked with 5% goat serum before staining with primary antibodies (mouse anti-human AGT; rabbit anti-BRCA2 from AbCam Ltd.) in a humidifying chamber at 4°C overnight. The cells were then washed and stained with secondary antibodies [Alexa Fluor 488 and 568]. The nuclei were counter-stained with DAPI and the cells were then visualized using a fluorescent microscope.

**Cloning:** pET-21b Vector from Novagen, was prepared by digesting the plasmid sequentially with BamH I and Xho-I restriction enzymes. Human AGT gene (accession number M29971) was amplified from the cDNA extracted from Hela cells by PCR reactions. PCR utilized primers containing BamH I and Xho I restriction sites to amplify the human AGT gene. The insert and the vector, with their sticky ends were ligated using Ligafast Rapid DNA ligation system (Promega Corp.). The vector and insert were ligated in ratio of 1:3. The ligated plasmid was then transfected into chemically competent DH5α cells. The bacterial colonies were screened for the plasmid using Ampicillin resistance. The plasmids extracted from the PCR positive clones were digested with vector and insert specific restriction enzymes to check for the presence of the AGT fragment in the pET-21b system. The cloned AGT fragment was then sequenced with T7
promoter primer. All the restriction enzymes used were from New England Biolabs Inc. All the common salts and chemical reagents used were from Sigma-Aldrich Co.

**Results and Discussion**

Capan-1 is a BRCA2 mutated, pancreatic cancer cell line derived from a patient. It has a truncated BRCA2, only 1981 aminoacids long, sequestered in the cytoplasm due to the lack of nuclear localising signal. Our earlier studies revealed that Capan-1 cells show exponential mortality rate when treated with alkylating agents. It was observed that AGT, a universally nuclear protein, is sequestered in Capan-1 in the cytoplasm along with BRCA2 [Figure 1(Left)]. AGT degradation from the total cell lysates *in vitro*, of Capan-1 and Hela, was driven by exposure to O⁶-Benzylguanine, a non-toxic inhibitor of AGT. The rate of degradation was observed to be almost the same indicating that the AGT is functional in Capan-1 [Figure 1(Right)].

Capan-1 cells were treated with BCNU, an alkylating agent, and the localisation of AGT and BRCA2 was studied using Immunofourescence and Western Blot analysis. The immunoflourescence studies indicate that AGT translocates into the nucleus of the Capan-1 cells by 16 hours, and by 20 hours it starts to degrade (Figure). Western Blot confirms the translocation of AGT into the nucleus by 16 hours (Figure). However, AGT degradation was not seen by 20 hours due to the resynthesis of AGT. Interestingly, BRCA2 is also shown to get translocated into the nucleus along with AGT, and its degradation rate also parallels that of AGT. These observations indicate that Association of truncated BRCA2 and AGT in Capan-1 cells causes cytoplasmic sequestering of repair enzyme AGT. This leads to sensitivity to alkylating DNA lesions in these cells. The association allows for stimulus induced nuclear localisation of truncated BRCA2 and AGT and simultaneous processing and destruction of associated proteins after lesion repair.

**Figure 1:** (Left) Image shows Western blot analysis of AGT in Capan-1 cells and Hela cells. (Right) Images shows the rate of in vitro degradation of AGT, when Capan-1 cells were exposed to O⁶-Benzylguanine (an inhibitor).

**Figure 2:** (Left) Images showing IF study of AGT degradation in Capan-1 cells on treatment with BCNU. AGT is stained in green, Nucleus stained in blue (DAPI)(Right)AGT degradation in vivo, on treatment with BCNU.
Our recent studies have shown that full-length BRCA2 physically associates with AGT, and this association gets stronger on alkylation. Exon-11 region is implicated in binding and AGT processing. Capan-1 BRCA2, truncated in its exon-11 region, interacts strongly with AGT and can support AGT processing once localised into the nucleus. To further analyse the interactions between BRCA2 and AGT, recombinant AGT was cloned into pET-21b system. This protein can be used to study various protein-protein interactions. It can be used as a competitive inhibitor in reactions. It can also be used to produce antibodies against alkylated AGT. Furthermore, it can be used to study the effects of various post translational modifications such as phosphorylation.

References
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