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## Telomerase and DNA repair in glioma

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Both DNA repair (MGMT) and immortalisation (telomerase) have been linked to the root of cancer. In glioma, MGMT expression is negatively regulated through promoter methylation and its absence is associated with enhanced chemosensitivity. However, recent studies indicate that telomerase is positively regulated through methylation and its elimination enhances chemotherapy. These observations suggest that suppression of telomerase in combination with MGMT may have additional anti-proliferative and anti-apoptotic effects, which may lead to increased patient survival rates. However, different approaches maybe required to compliment the epigenetic events that regulate these genes. Nevertheless, given that median survival of glioma patients is less than a year, this review focuses on the recent approaches used to target MGMT and telomerase, with a view to increase life expectancy of patients while limiting side effects.

**Keywords:** Glioma; *hTERT*; *MGMT*, Chemotherapy

## Introduction

Glioma is the most malignant form of brain tumour, with median survival rate of less than a year [1] and thus consequently associated with the worst prognosis. Malignant gliomas are notoriously resistant to many of the currently available therapies such as cisplatin, carmustine (BCNU), lomustine (CCNU), vincristine (PCV) and temozolomide (TMZ) because these cancer cells cannot be induced to undergo apoptosis upon anticancer treatment [2,3]. Furthermore, gliomas almost always grow back even after complete surgical excision [4].

Gliomas can occur either spontaneously termed *de novo* (primary), or through malignant progression from a lower-grade lesion (secondary). Secondary gliomas tend to develop in younger patients in comparison to primary, through malignant progression from diffuse astrocytomas or anaplastic astrocytomas. The median survival rate of secondary glioma patients (7.8 months) is significantly longer than that of primary glioma patients (4.7 months), largely due to the younger age of secondary glioma patients in general [5]. The complex mutational pathways leading to primary or secondary gliomas entail different gene mutations, deletions, and amplifications (Fig.1) [6,7]. However this review will focus on the role of O<sup>6</sup> methylguanine-DNA-methyltransferase (MGMT) and telomerase in glioma.

### MGMT regulation in glioma

MGMT also known as O<sup>6</sup> alkylguanine-DNA-alkyltransferase, is a DNA repair gene that has been studied extensively in glioma. With its potential to increase anticancer efficacy of many alkylating agents *MGMT* inhibition is now at the forefront of glioma research. Most DNA repair enzymes are responsible for repairing double or single

strand breaks, nevertheless methylation of cytosine residues that proceed the guanine (G) residues, i.e. methylation of CpG (cytidine-phosphate-guanosine) dinucleotides are not excision of double or single DNA strands [10]. Methylation of cytosine is the normal way in which cells regulate gene expression, however, methylation of CpG dinucleotides leads to mispairing during DNA replication and is the by-product of DNA damage. *MGMT* transfers the alkyl/methyl groups to its active cysteine residue in a stoichiometric and autoinactivating process requiring resynthesis (suicidal repair enzyme) [11] thereby, averting the formation of lethal cross-links. One *MGMT* molecule is inactivated for each lesion repaired, thus *MGMT* itself is consumed due to its rapid degradation through the ubiquitin-proteasome pathway after receiving alkyl groups from DNA [12]. Furthermore, repletion of the cellular *MGMT* pool depends on the re-synthesis of the molecule.  $O^6$ -guanine lesions are produced by carcinogenic methylating alkylating agents used in therapy [13]. Thus, the repair of all  $O^6$ -alkyl/methyl-guanine-DNA lesions is essential for cell integrity and if un-repaired can result in mutagenesis.

The crystal structure of the *MGMT* protein shows that the transfer reaction takes place through the localisation of guanine in the *MGMT* pocket by the local amino acid side chain [14,15]. To initiate repair, it binds DNA and scans individual bases for evidence of DNA adducts [16]. *MGMT* directly removes DNA modifications from the  $O^6$  atom of guanine such as  $O^6$ -ethylguanine ( $O^6$ -EG), which is formed by ethylnitrosourea, and diethylnitrosamine and related compounds including chloroethyl, pyridyloxobutyl, butyl and benzyl adducts [14]. Unlike all other repair enzymes that work together forming multi-enzyme complexes to initiate and remove abnormalities within DNA [17], *MGMT*, exclusively works alone to repair DNA

adducts. Thus, implying that the MGMT repair reaction is remarkably simple and efficient, with more than 90% of adducts removed within 10 min of methylation [18]. Furthermore MGMT is a monomer that can act in the absence of cofactors and energy sources and is instrumental in DNA repair (Fig. 2).

Numerous brain tumours exhibit loss of heterozygosity (LOH) of chromosome 10, with *MGMT* located at 10q26 may subsequently be lost therefore the majority of gliomas only have one copy of the *MGMT* gene [19]. Deficiency of *MGMT* was shown to increase the chemosensitivity of the alkylating agents in brain tumours. Although in gliomas, *MGMT* is sometimes mutated or deleted, the major loss of *MGMT* gene expression is often a result of CpG island hypermethylation [10] commonly seen in solid tumours, specifically gliomas [20]. The predictive value of *MGMT* promoter methylation was tested in glioma patients treated with temozolomide (TMZ), this showed that promoter methylation was a surrogate marker for response to therapy. Patients with a methylated *MGMT* promoter survive up to 30 months longer than those without [17]. However, loss of *MGMT* increases the risk of mutations and malignant transformation, suggesting that *MGMT* promoter methylation is an important epigenetic event in tumourgenesis itself. MGMT protein plays a pivotal role in cellular defence against alkylating agents and thus its quantification maybe important both as a biomarker and for drug sensitivity testing [21].

### **Targeting MGMT predicts patient outcome**

*MGMT* repression has been associated with increase overall survival rates. 60% of glioma patients with an inactivated *MGMT* demonstrated a partial or complete response to drug therapy, compared with only 4% of those with intact *MGMT* capability [17]. Methylation of the *MGMT* promoter is associated with better

responsiveness to carmustine and increased overall survival, which is a better predictor of the outcome, than the grade of the tumour [11]. However, no correlation has been found to determine a link between promoter hypermethylation and MGMT protein expression in oligodendrogliomas [22]. The success of *MGMT* depletion as a strategy for therapy depends upon O<sup>6</sup>-alkylation being the determinant of cancer cell death [23] and whether the tumour cell expresses MGMT in the first instance (Fig. 1). A greater proportion of secondary gliomas have a methylated MGMT promoter in contrast to primary gliomas, therefore secondary glioma patients should be more responsive to alkylating agents. Nevertheless, this increase in chemosensitivity should be more preferential in oligodendrogliomas which have the highest rate of MGMT methylation rates [7].

Researchers have developed O<sup>6</sup>-benzylguanine (BzG), O<sup>6</sup>-4-bromothenylguanine (Patrin) and O<sup>6</sup>- bromothenylguanine-C8-β-D-glucoside [24] as alternative substrates of MGMT, which irreversibly inactivates the MGMT protein and thereby potentiates the effect of alkylating agents such as TMZ and the nitrosoureas to improve. Although, BzG and Patrin have gone through phase I and II clinical trials [25], the suboptimal nature of their pharmaceutical and pharmacokinetic properties suggests that other MGMT inhibitors (BzG analogues) may be required for the true potential of MGMT modulation [21,26]. More recently Hegi and colleagues reviewed the data supporting *MGMT* as a major mechanism of chemotherapy resistance (specifically TMZ) in malignant gliomas and found that some methods of inhibiting MGMT would benefit patients with an unmethylated MGMT promoter, albeit this may not guarantee success [27]. Furthermore, not all MGMT negative tumour patients show response to

therapy thus further work underpinning resistance-modulating mechanisms may be a necessity for the future.

While increasing evidence shows that TMZ, the most efficacious treatment, is the way forward with regards to glioma therapy, a reliable and routine method of determining MGMT status in patients is required to enhance survival outcome. Additionally, treatment that caters for patients with either positive or negative MGMT expression would be the way forward for glioma treatment. Recent data from our laboratory involving the combination of 5-Aza-2'-deoxycytidine (5azadC) and paxitaxol as a treatment showed reduced cell viability by two fold of two glioma cell lines, suggesting an alternative method of targeting gliomas that do not rely on TMZ [28]. Greater than 30-50% of glioma patients have an unmethylated *MGMT* promoter, thus increasing the need for a new therapy approach that does not rely on alkylating agents. Careful tailored treatment dependant on MGMT profile, utilizing RT-PCR analysis directly following tumour tissue removal had led to a 71% two year survival rate of glioma patients [29]. This by far exceeds the general survival rate of glioma patients. However, careful consideration must be taken to assess only the tumour region of the brain as the non-neoplastic brain components such as lymphocytes, vascular endothelial cells, and macrophages/microglia, may contribute to the overall MGMT expression detected in tumour homogenates resulting in overestimation of tumour MGMT expression [30] leading to misjudgement of treatment.

### **Telomerase activity in malignant gliomas**

Nevertheless, according to the literature many other genes are activated/ silenced to maintain tumour development and progression. These include genes to promote



angiogenesis, (growth of new blood vessels to support new tumour tissue), genes to promote mobility (cancer can spread) and genes to "immortalise" (allow cells to proliferate indefinitely, without replicative senescence) [31]. The ability of cancer cells to become immortal is one of the main characteristics that distinguish them from their normal counterparts. To escape replicative senescence important cell cycle genes are lost or activated which include *p53*, *p21*, *p16INK4a*, *Rb* and telomerase [32]. The complex transcriptional regulation of telomerase with its intercalating pathways, has led us to evaluate its epigenetic regulation, through the methylation status of the hTERT gene. Given that immortalisation not only requires telomerase but needs imbalance/inactivation of DNA repair functions, further supports the need to understand MGMT and telomerase interactions through promoter methylation. Most genes are switched off when the promoter is methylated, in contrast, hTERT (telomerase active subunit) expression in some tissues (colorectal, ovarian and cervical cancer) seems to undermine this general rule and positive correlations between hypermethylation of the hTERT promoter, hTERT mRNA expression and telomerase activity have been reported [33,34]. Moreover, mammalian telomeres have enriched epigenetic regions, without which regulation of telomerase would be uncontrollable [35,36] thus enhancing evidence for epigenetic regulation controlling the expression of telomerase in general.

Researchers have studied telomerase in glioma over 10 years with the earliest reported study in 1995 which assessed telomerase activity as a marker for malignancy [37]. The study showed a significant correlation between pathological diagnosis and telomerase, while 75% of gliomas have detectable telomerase activity, only 10% of the anaplastic astrocytomas have been found to be telomerase positive [37-39].

Another report showed that the level of telomerase activity and *hTERT* expression is significantly higher in secondary gliomas in comparison with primary and is correlated with *p53* mutations during the early stage of carcinogenesis [40] thus confirming that senescence activated by *p53* is due to telomere dysfunction [41, 42]. Moreover, low expression levels of telomerase predicted better survival and correlated positively to the prognosis and treatment not only in glioma's, but also in genitourinary and colorectal cancers [1,43,44].

### **Targeting telomerase**

There are currently a number of techniques used to target telomerase, these include 2-5 antisense systems, viral constructs expressing antisense transcripts, hammerhead ribozymes, peptide nucleic acids (PNAs) dominant negative *hTERT*, immunotherapy and reverse transcriptase inhibitors [45-47]. Although, various translational approaches have been adopted to target telomerase, some of which are in the final stages of preclinical development and others currently in clinical trials, telomelysin trials have recently been accessible for various solid tumours with no reference to gliomas [47].

Human glioma cell lines (T98G, LN-229, LN-18, U87MG and LN-308) were subjected to penclomedine, an alkylating agent previously reported to inhibit telomerase activity in human cervical carcinoma cells, found that telomerase activity was unaffected [48], concluding that telomerase activity may not be a suitable target to enhance chemosensitivity of malignant gliomas given its complex regulation. Furthermore, quantification of telomerase in brain tumours showed that there was no correlation between downregulation of telomerase and patient survival [49]. These

differences maybe due to the methods used in analysing telomerase or a genuine difference in the expression. However, this finding was not in agreement with a study in 2006 which showed that median survival time of patients with and without telomerase activity were 21 and 34 months respectively, indicating that telomerase activity is an important prognostic factor in predicting progression and survival of patients with brain tumours [50]. Glioma cells sensitive to low doses of cisplatin died as a consequence of telomere loss, a phenomenon that could contribute to the success of cisplatin as an anticancer agent specifically for glioma [51,52].

Vaccination with pseudotype baculovirus expressing *hTERT* in animals with brain tumours induced protective anti-tumour immunity to *hTERT* [53]. The level of *hTERT* transcript appeared to predict survival in glioma patients (low *hTERT* expression, better survival) [54]; therefore the development of anti-telomerase treatment for malignant gliomas and *hTERT* analysis is essential. Intravenous injections of hTERT-Ad suppressed the growth of subcutaneous tumours in nude mice and prolonged the survival of mice with gliomas, and the transfected cells underwent autophagic cell death via inhibition of the mTOR signalling in telomerase positive tumours [55]. Telomerase itself plays an additional role in protecting chromosomes by regulating the overall configuration of the chromatin state and its depletion affects chromatin structure and impairs activation of DNA damage responses [56]. The significant role of telomerase in glioma pathogenicity can be a potential therapeutic target for gliomas.

Until recently it was unknown whether hTERT expression in gliomas was regulated via hypo or hyper methylation, in addition, data regarding demethylation treatment

assessing the expression of hTERT was limited. Many drugs have been used to down regulate telomerase methylation. Recently 5azadC treatment in glioma cell lines reduced telomerase expression and activity, however given the complex regulation of telomerase it was not clear by what mechanism this was happening [28].

Indirect evidence showed that promoter methylation may be inhibiting telomerase expression in glioma cells, thus by inhibiting the methyltransferase gene DNMT1 using targeted siRNA, it was clearly demonstrated that not only was the DNMT1 expression reduced, as a knock on effect telomerase expression was also reduced. Furthermore combining DNMT1 siRNA and chemotherapeutic drugs (taxol and TMZ) enhanced the efficiency of the drug by up 7 and 4 fold, respectively in glioma cells [57]. This in itself proposes that inhibition of telomerase can be used to complement a direct cytotoxic agent, possibly offering an alternative treatment by the use of lower concentrations of the drug, hence reducing side effects and improving the life expectancy of patients. However, its clinical implications will require further elucidation of these findings with regards to tumour grade and response. Inhibition of telomerase whether by means of gene knockdown, chemical interactions or via immuno therapy, has the potential to increase the efficiency of many anticancer agents such as cisplatin, TMZ and PCVs, therefore it could be used in combination therapies. The importance of studying telomeres in combination with telomerase inhibition as a therapeutic target was shown to be a prognostic tool in paediatric glioma patients [58].

## Conclusion

Despite the technological advances in molecular biology that have assisted in enhancing our understanding of cancer, there still remain gaps in our understanding of the mechanisms governing glioma. Research directed towards gene therapy with particular focus on telomerase and MGMT will further aid development of new diagnostic and therapeutic approaches to human gliomas. Given that malignant gliomas are predominantly telomerase positive and normal somatic cells are negative, targeting telomerase should fundamentally lead to minimum side effects. Combining inhibition of both telomerase and MGMT could further enhance glioma treatment and patient care.

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**Fig. 1** Genetic pathways in the evolution of primary and secondary gliomas (adapted and revised from [7-9]).

**Fig. 2** MGMT at work. MGMT scans double-stranded DNA for alkylation at the O<sup>6</sup> position of guanine and covalently transfers it to its active-site cysteine, inactivating the MGMT and restoring the guanine to normal (adapted from [14] Gerson, 2004).





