Summer 2013

# Role of Tyrosyl-DNA Phosphodiesterase I (TDP1) as a Prognostic and Predictive Factor in Malignant Glioma

Maha Said Abd-Alquader Al-Keilani
*University of Iowa*

# ROLE OF TYROSYL-DNA PHOSPHODIESTERASE I (TDP1) AS A PROGNOSTIC AND PREDICTIVE FACTOR IN MALIGNANT GLIOMA

by

Maha Said Abd-Alqader Al-Keilani

A thesis submitted in partial fulfillment
of the requirements for the Doctor of
Philosophy degree in Pharmacy
in the Graduate College of
The University of Iowa

August 2013

Thesis Supervisor: Assistant Professor Mahfoud Assem

Graduate College
The University of Iowa
Iowa City, Iowa

CERTIFICATE OF APPROVAL

_____

PH.D. THESIS

_____

This is to certify that the Ph.D. thesis of

Maha Said Abd-Alqader Al-Keilani

has been approved by the Examining Committee
for the thesis requirement for the Doctor of Philosophy
degree in Pharmacy at the August 2013 graduation.

Thesis Committee: _____
Mahfoud Assem, Thesis Supervisor

_____
Larry Fleckenstein

_____
Daryl Murry

_____
Gary Milavetz

_____
Aliasger Salem

This thesis is lovingly dedicated to my parents for believing in me, to my husband for supporting and unconditionally loving me, and to my son for filling my life with fun and excitement

The future belongs to those who believe in the beauty of their dreams

Eleanor Roosevelt

ACKNOWLEDGMENTS

My first and sincere appreciation goes to Mahfoud Assem, my advisor for supporting me during my PhD track in the past four years. Being patient, providing priceless advices and valuable suggestions, Mahfoud aided in my scientific progress. I also have to thank my committee members: Professors Larry Fleckenstein, Daryl Murry, Gary Milavetz, and Aliasger Salem for their time and effort in reviewing this work.

I am deeply and forever grateful to my parents for encouraging and believing in me in my whole life. I am also thankful for my sisters and brothers for their tenderness and love, and for my father in law and mother in law for being such a lovely encouraging second family for me.

I wish to express my gratitude to my friends Rowan Alejielat, Mohammad Saleh, Ahed Saleh, Rania Hamed and Mohammad Bataineh for their help and influence. A special thank goes to Tatian Kirresh for being such a decent and supportive friend.

A deep appreciation goes to the staff of the college of pharmacy especially Rita Schneider and Lois Baker for their cooperation and kindness with me.

I also want to deeply express my infinite thanks to the most wonderful, and amazing husband any woman would dream of, Ussama Massadeh. He provides me with the endless love that I want, he believes in me in the times that I may not even be able to believe in myself. He unlimitedly supported me during my good and bad times and he stood behind my back and I would not have made it this far without him.

I would like to convey my endless unconditional love to my sweet, naughty and funny son Hashem. He was the best gift I have ever received in my life. Waiting for him to come back from the daycare to give me the hugs and kisses that I was waiting for, was the best support for me.

# ABSTRACT

Glioma is the most common and aggressive type of primary intracranial tumors. The poor prognosis of glioma patients has not changed for decades despite the advancements in diagnostic tools and treatment strategies. The inability to accurately predict the survival and response to anticancer therapy emerges from several factors including the high heterogeneity of the tumor and the inadequacy of the currently applied World-Health Organization (WHO) classification system. Both factors result in high variability in the clinical outcome due to variable sensitivity to treatment. Thus, molecular classification represents an important strategy for better categorization of glioma patients and for their stratification to anticancer therapy. Our high-throughput screening analysis for the identification of genetic aberrations in the glioma study population revealed high frequency of chromosomal instabilities in glioma specimens. This indicates that DNA-repair mechanisms are defective which may have contributed to gliomagenesis and progression.

Furthermore, DNA-repair represents an integral interplayer in the determination of glioma response to anti-neoplastic agents due to the fact that the majority of the currently applied agents possess their cytotoxicity via DNA-damaging actions. Tyrosyl DNA-phosphodiesterase I (TDP1) has been implicated in the resistance to various types of anticancer agents *in vitro*, including radiation and topoisomerase poisons due to its ability to repair various types of DNA lesions. Moreover, it has been found to be overexpressed in different kinds of cancers, however, its relevance in glioma has not yet been studied.

In this work we show that TDP1 is overexpressed in patients with malignant glioma compared with non-tumor cases. An ascending increase of TDP1 protein expression with a correlation with glioma grade is evidenced in the astrocytic lineage and

glioblastoma multiforme samples expressed the highest levels. Moreover, we show an association between high TDP1 transcript levels and the poor prognosis of glioma patients. These findings suggest that TDP1 plays an important role in gliomagenesis; however, the underlying molecular mechanisms need to be identified.

For an exploration of the predictive value of TDP1 in malignant glioma the correlation between TDP1 level and the sensitivity of malignant glioma cell lines to anticancer therapy has been investigated. We show that manipulating TDP1 level alone in malignant glioma cell lines is not sufficient to modulate their response to treatment. TDP1 overexpression or knockdown resulted in changes in the transcript levels of several DNA-repair genes including O6-methylguanine DNA methyltransferase (MGMT), topoisomerases and the base excision repair genes poly [ADP-ribose] polymerase 1 (PARP-1), polynucleotide kinase phosphatase (PNKP) and x-ray repair cross-complementing protein 1 (XRCC1). This hindered the ability to characterize the role of TDP1 to modulate the *in vitro* sensitivity of malignant glioma cell lines to topoisomerase poisons and temozolomide. Nonetheless, this emphasizes the importance of the comprehensive role of several DNA-repair genes for a finalized DNA-repair process to determine the sensitivity of tumor cells to DNA-damaging anticancer agents.

Finally, we tested the ability of inhibiting TDP1 enzyme activity to potentiate or synergize the cytotoxicity of topoisomerase poisons using small-molecule ligands. We show that treatment of malignant glioma cell lines with a combinational therapy of a small-molecule TDP1 inhibitor and a topoisomerase poison enhances their sensitivity to the latter drug but with minimal efficacy.

As a conclusion, the characterization of TDP1 in glioma is a novel finding that can aid in enhancing the diagnosis and prognosis of patients. However, its role as a predictive biomarker for better stratification of patients to therapy needs further investigation.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

## LIST OF ABBREVIATIONS

| | |
|---|---|
| AAC | Anaplastic Astrocytoma; WHO grade III |
| ABC | ATP-Binding Cassette |
| ACG | Diffuse Astrocytoma; WHO grade II |
| aCGH | Array Comparative Genomic Hybridization |
| AOD | Anaplastic Oligodendroglioma; WHO grade III |
| AP-site | Apurinic/Apyrimidinic site |
| ATCC | American Type Culture Collection |
| ATM | Ataxia Telangiectasia Mutated |
| ATR | Ataxia Telangiectasia and Rad3-related protein |
| BBB | Blood Brain Barrier |
| BCNU | Carmustine |
| BER | Base Excision Repair |
| CBTRUS | Cancer Brain Tumor Registry of the United States |
| CCNU | Lomustine |
| Chk1 | Checkpoint kinase 1 |
| Chk2 | Checkpoint kinase 2 |
| Chr | Chromosome |
| CNA | Copy Number Alteration |
| DDR | DNA-damage response |
| DSB | Double-Strand Break |
| EGFR | Epidermal Growth Factor Receptor |
| EGFRvIII | Epidermal Growth Factor Receptor Variant III |
| EORTC-NCIC | European Organization for Research and Treatment of Cancer-National Cancer Institute of Canada |
| FISH | Fluorescent In Situ Hybridization |
| GAPDH | Glyceraldehyde 3-Phosphate Dehydrogenase |
| GBM | Glioblastoma Multiforme; WHO grade IV |
| HGG | High-Grade Glioma |

| | |
|---|---|
| HR | Homologous Recombination |
| IR | Ionizing Radiation |
| KPS | Karnofsky Performance Status |
| LGG | Low-Grade Glioma |
| Lig 3α | DNA-Ligase IIIα |
| LOH | Loss of Heterozygosity |
| MGMT | O6-Methylguanine DNA Methyltransferase |
| MMR | Mismatch Repair |
| MOA | Mixed Oligoastrocytoma |
| MRN complex | Mre11/Rad50/Nbs1 |
| NCI/DTP | National Cancer Institute/Developmental Therapeutic Program |
| NER | Nucleotide Excision repair |
| NHEJ | Nonhomologous End Joining |
| O6-meG | O6-methylguanine |
| ODG | Oligodendroglioma; WHO grade II |
| PARP-1 | Poly [ADP-ribose] polymerase 1 |
| PCR | Polymerase Chain Reaction |
| P-gp | P-glycoprotein |
| PLD | Phospholipase D |
| PNKP | Polynucleotide Kinase Phosphatase |
| ROS | Reactive Oxygen Species |
| RT-PCR | Reverse Transcription PCR |
| SCAN1 | Spinocerebellar Ataxia with Axonal Neuropathy Type 1 |
| shRNA | small-hairpin RNA |
| SIFT | Sorting Intolerant From Tolerant |
| siRNA | small-interfering RNA |
| SNP | Single Nucleotide Polymorphism |
| SSB | Single-Strand Break |
| T1 | NSC128609 |

| | |
|---|---|
| T2 | NSC120686 |
| TCGA | The Cancer Genome Atlas |
| TDP1 | Tyrosyl DNA-Phosphodiesterase I |
| TMZ | Temozolomide |
| TOP-I | Topoisomerase I |
| TOP-II | Topoisomerase II |
| TOP-IIβ | Topoisomerase II beta |
| TOP-poisons | Topoisomerase poisons |
| TOP I-poisons | Topoisomerase I poisons |
| TOP II-poisons | Topoisomerase II poisons |
| TRC | The RNAi Consortium |
| U251/mock | U251 cells transfected with empty vector |
| U251/TDP1 | U251 cells transfected with full length wild-type TDP1 |
| U87/mock | U87 cells transfected with empty vector |
| U87/TDP1/sh48 | U87/TDP1 cells transduced with TDP1/shRNA48 |
| U87/TDP1/sh50 | U87/TDP1 cells transduced with TDP1/shRNA50 |
| U87/TDP1/sh-ve | U87/TDP1 cells transduced with empty vector |
| U87/TDP1 | U87 cells transfected with full length wild-type TDP1 |
| XRCC1 | X-ray repair cross-complementing protein 1 |

LIST OF BUFFERS AND GELS

1- TBS-T buffer:

- 6.05 g Tris-base
- 8.76 g NaCl
- 800 ml dd$H_2O$
- 20 ml 2N $H_2Cl$
- 1 ml Tween 20

2- 10x TBE buffer:

- 108 g Tris-base
- 55 g boric acid
- 40 ml EDTA (pH 8.0)
- Finish volume to 1 L with dd$H_2O$

3- 1x TBE buffer:

- 100 ml 10x TBE
- 900 ml dd$H_2O$

4- 1% Agarose gel:

- 1 g agarose
- 100 ml 1x TBE
- 2 µl of 1 µg/ml ethidium bromide

5- 2% Agarose gel:

- 2 g agarose
- 100 ml 1x TBE
- 2 µl of 1 µg/ml ethidium bromide

6- 10% SDS-PAGE loading gel:

- 10 ml 29.1 30% Acrylamide
- 12.4 ml dd$H_2O$ water

- 7.5 ml of 1.5 M Tris 0.4% SDS (pH 8.8)
- 150 µl of 10% APS
- 10 µl Temed

7- Stacking gel for western blot:

- 1.33 ml 29:1 30% acrylamide
- 6.2 ml $ddH_2O$
- 2.5 ml 0.5 M Tris 0.4% SDS (pH 6.8)
- 50 µl APS
- 5 µl Temed

8- 1.5 M Tris 0.4% SDS (pH 8.8):

- 91 g Tris-base
- 400 ml $ddH_2O$
- Adjust pH to 8.8, 20 ml 10% SDS
- Finish volume to 500 ml with $ddH_2O$

9- 0.5 M Tris 0.4% SDS (pH 8.8)

- 30.5 g Tris-base
- 400 ml $ddH_2O$
- Adjust pH to 8.8, 20 ml 10% SDS
- Finish volume to 500 ml with $ddH_2O$

10- 10x Western blot running buffer (Tris-Glycine-SDS):

- 121.1 g Tris-base
- 576 g glycine
- 200 ml 20% SDS
- finish volume to 4 L with $ddH_2O$

11- 1x Tris-Glycine-SDS buffer

- 100 ml 10x buffer
- 900 ml $ddH_2O$

12- 10x Western blot transfer buffer (Tris-Glycine):

- 121.1 g Tris-base
- 576 g glycine
- Finish volume to 4 L with $ddH_2O$

13- 1x Transfer buffer:

- 700 ml $ddH_2O$
- 100 ml 10x buffer
- 200 ml methanol

14- Laemmli lysis buffer:

- 950 µl laemmli buffer
- 50 µl 2-mercaptoethanol

# CHAPTER I

# INTRODUCTION

## 1.1. Glioma

Gliomas are the most common type of primary intracranial tumors accounting for about 60% of cases (1). They develop from glial cells which are mainly responsible for supporting the neurons by holding them together and possessing other important functions including: providing nutrition to the neurons and maintaining the homeostatic environment of the CNS. The two main types of glial cells are astrocytes and oligodendrocytes. Thus, gliomas are classified based on the respective cell of origin into astrocytomas, oligodendrogliomas and mixed oligoastrocytomas.

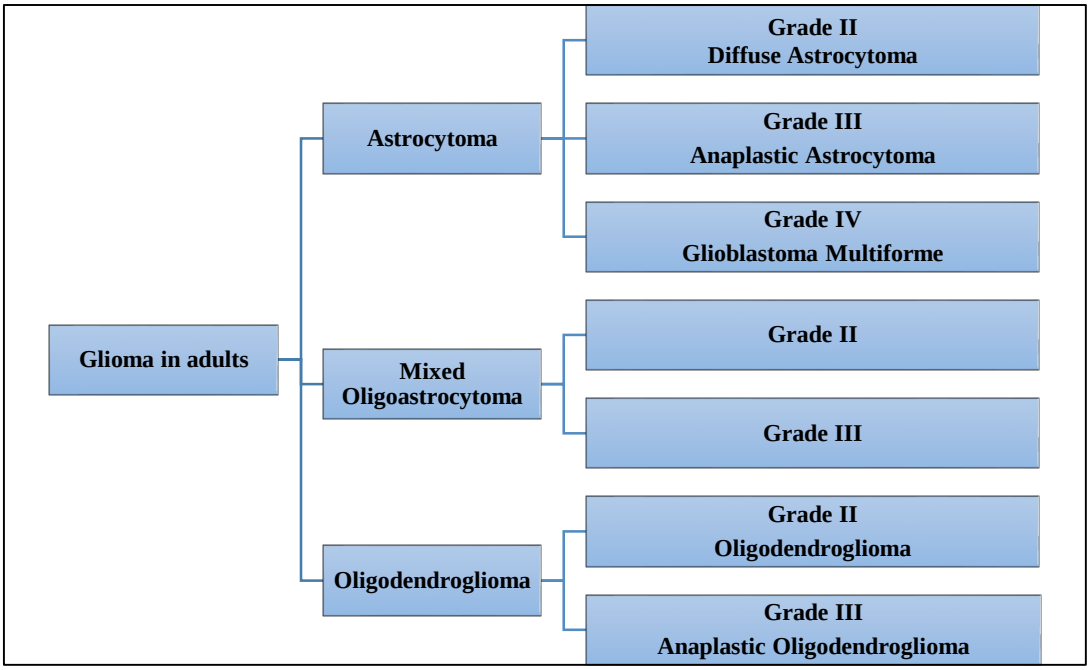


**Figure 1.1. Sub-classification of gliomas by the World Health Organization (WHO).** Based on the cell type of origin, gliomas are classified into astrocytomas, oligodendrogliomas, or mixed oligoastrocytomas.

According to the World health Organization (WHO) and based on the histological features of the tumor in terms of tissue necrosis (cell death), nuclear atypia (variant appearance of the nucleus), mitotic index and microvascular proliferation, gliomas are subclassified into four grades. Grade I glioma exclusively occurs in children. In adults, astrocytomas are subcategorized into three prognostic grades, grade II (diffuse astrocytomas; ACG), grade III (anaplastic astrocytomas; AAC), and grade IV (glioblastoma multiforme; GBM) (**Figure 1.1**).

As shown in **Figure 1.2** Astrocytomas are the most frequent type accounting for about 70% of all gliomas. GBM is the most common and aggressive type and it is contributing to almost two thirds of the glioma incidents. GBM can arise *de novo* (primary GBM) or after malignant transformation of grades II or III astrocytomas (secondary GBM). Primary GBM accounts for 95% of the GBM cases, 68% of them have a clinical history of only 3 months and mainly occurs in older patients and is associated with more resistance to therapy (2).

The WHO subclassification of oligodendrogliomas includes: grade II (well-differentiated oligodendrogliomas; ODG) and grade III (anaplastic oligodendrogliomas; AOD and mixed oligoastrocytomas; MOA) (**Figure 1.1**). Compared to astrocytomas, oligodendrogliomas account for less number of the cases (4%-15%), are slower growing and are more responsive to therapy. Thus the oligodendrocytic lineage gliomas are associated with prolonged overall survival (10 years for ODG and 5 years for AOD) (2).

## 1.2. Genetic alterations in glioma development and transformation

Based on aggressiveness, gliomas could be one of two categories; low-grade gliomas (LGG; grade II tumors) or high-grade gliomas (HGG; grade III and IV tumors). The latter are also called malignant gliomas. Low-grade gliomas are less common and are associated with better prognosis and response to therapy while high-grade gliomas

account for about 80%, are less differentiated and associated with poorer prognosis (3). It is believed that the anaplastic transformation process from low-grade to high-grade is driven by the occurrence of multiple genetic alterations (4). The degree of these aberrancies and their interactions correlate with the aggressiveness of the tumor. Thus, HGGs are characterized by the high genetic heterogeneity which plays an important role in the dreadful prognosis and the bad response to therapy.

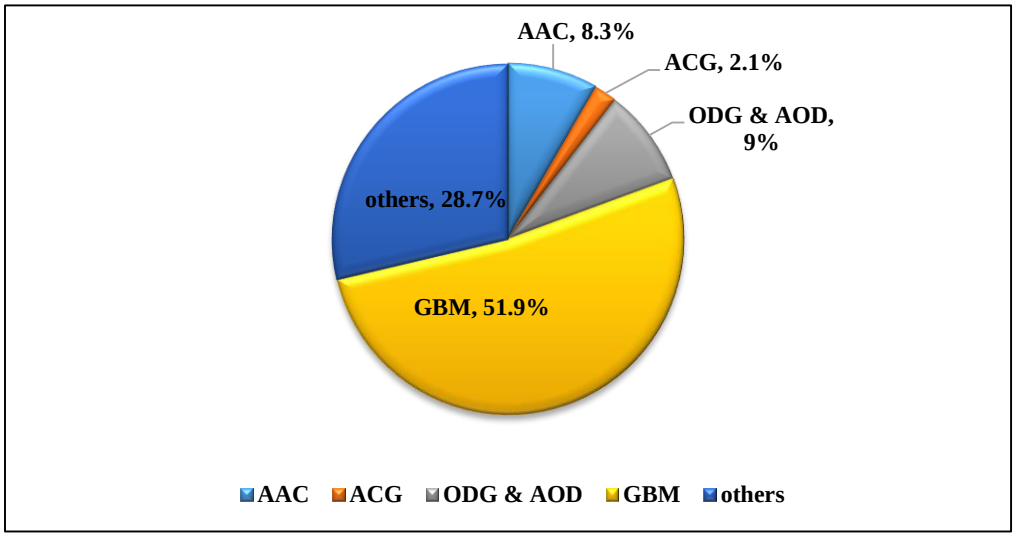


Figure 2. **A pie chart representation of the distribution of diffuse gliomas by histology (N=16,780).** ACG = diffuse astrocytoma, grade II, AAC = anaplastic astrocytoma, grade III, GBM = glioblastoma multiforme, grade IV, ODG = oligodendroglioma, grade II, AOD = anaplastic oligodendroglioma, grade III, others = other types of glioma.

Source: Central Brain Tumor Registry of the United States (CBTRUS) statistical report 2002-2003.

Different types of genetic alterations and their association with prognosis and survival of glioma patients have been identified. Typical oligodendrogliomas possess 1p/19q co-deletion in 83% of ODG and 63% of AOD cases, while they only occur in 39% of MOA (2) and in 10% of astrocytomas (5). This loss has been shown to be associated with better prognosis and response to therapy including ionizing radiation (IR) and PCV regimen (procarbazine, lomustine, and vincristine) in oligodendroglioma patients but has little impact in astrocytomas (2,5).

Epidermal growth factor receptor (EGFR) amplification and overexpression (in 50% of GBM) and mutant EGFRvIII (half of GBM with amplified EGFR) that results in constitutive activation of the receptor are hallmarks of primary GBM but rarely seen in secondary GBM and grade II and III gliomas (6). EGFR stimulation results in the downstream activation of different precursors such as phosphoinositide 3-kinase (PI3K) and RAC-alpha serine/threonine-protein kinase (Akt-1) thus promoting tumor survival and hence poor prognosis (6). On the other hand, tumor protein 53 (TP53; a tumor suppressor gene) mutations resulting in p53 inactivation are commonly seen in LGGs and is a hallmark of secondary GBM but not primary GBM (2,6). These mutations were associated with poorer prognosis (7).

Another important genetic aberration that has been identified is the O6-methylguanine DNA methyltransferase (MGMT) promoter methylation that is associated with gene silencing and thus reduced DNA-repair capacity. This epigenetic mutation was found to be more common in secondary GBM than primary ones (75% vs. 36%), which is consistent with the positive correlation between MGMT methylation and the presence of TP53 mutations in low-grade astrocytomas (8). Different studies have shown the positive prognostic value of hypermethylation of MGMT in glioma (2,9).

Beside the previously mentioned genetic changes, other important aberrations have also been identified such as platelet-derived growth factor receptor (PDGFR) amplification (mainly in oligodendrogliomas), phosphatase and tensin homolog (PTEN)

loss (in all gliomas) and isocitrate dehydrogenase (IDH 1and IDH2, higher frequency in low-grade gliomas and secondary GBM) mutations, all of which were associated with prognosis (2). Although considered potential therapeutic targets, the clinical relevance of the previously mentioned altered genes is still under investigation with no definite answer.

### 1.3. Glioma prognosis

Despite the advanced diagnostic tools and the aggressive therapy of glioma, the prognosis is still poor with only 10% of treated patients surviving for 5 years after diagnosis and most deaths occur in the first 2 years (10). The main prognostic factors currently implied to predict patients' survival are: 1- patient's age (prolonged survival in younger ages, cut-off value is 50-60 years) (11), 2- WHO histological grade (grade IV GBM are the most aggressive and the median survival is 11 months versus 27 months for AAC) (12), 3- Karnofsky performance status (KPS) (higher KPS indicates better prognosis with a cut-off value of 80) (13), 4- pretreatment tumor size (14) and 5- extent of tumor resection (less ability to resect the majority of the tumor mass indicates more infiltration and thus a more aggressive tumor; complete versus partial resection versus biopsy only) (11).

Although considered somehow useful in predicting survival, these prognostic variables are of modest reliability because of the intra- and inter-tumor molecular and the genetic heterogeneity of glioma. The WHO classification for example is problematic since it is subjective and highly depends on the part of the tumor that has been resected. Thus there is an urgent need for the identification of new, novel and more reliable prognostic factors that can classify gliomas into subgroups based on the genetic/molecular profile of the tumor.

Previous investigators tried to develop a prognostic scaling system of grade III glioma patients based on a combination of their clinical characteristics (including age,

gender, degree of tumor resection and adjuvant therapy) and a combination of molecular markers including: 1p/19q, IDH1/2, MGMT, EGFR, p-glycoprotein (P-gp), PTEN, matrix metallopeptidase 9 (MMP-9), p53, vascular endothelial growth factor (VEGF), and topoisomerase ii (TOP-II) (15). Based on the log-rank statistical analysis, they developed a scoring scale where age<50, 1p/19q co-deletion, IDH1/2 mutation, negative MGMT expression, and negative EGFR expression, all considered as positive prognostic biomarkers and significantly correlated with progression free survival and overall survival (15). Using this scoring system they were able to divide the patients into four levels with patients falling in level 1 having the worst prognosis (15). This highlights the importance of the identification of novel molecular biomarkers to aid in the classification of gliomas and hopefully in the treatment decisions.

The poor prognosis of glioma especially high-grade can be attributed mainly to the malignant location of the tumor which if not resected results in death due to increased intracranial pressure and cerebral edema. Nonetheless, the issue of resistance to therapy cannot be denied as a main contributor to the dismal prognosis of glioma. Resistance could be either intrinsic (*de novo*) which is characterized by inherent poor response to therapy or extrinsic (acquired) when the tumor cells develop after treatment and it is a main cause of tumor relapse (16). Examples of resistance mechanisms are: 1- upregulation of ATP-binding cassette (ABC) transporters in the blood brain barrier (BBB) such as P-gp which results in reduced intracranial concentration by increasing the drug's efflux, 2- TP53 mutations which result in anti-apoptotic properties and resistance to death (17), and 3- acquired MGMT overexpression as a result of treatment with DNA-alkylating agents such as temozolomide (TMZ), carmustine ( BCNU) and lomustine (CCNU).

The previously discussed genetic alterations and their prognostic value in glioma directed researchers to apply targeted molecular therapies in the treatment regimens and study their clinical effect on the survival of patients. Examples are tyrosine kinase

inhibitors (e.g. erlotinib (EGFR inhibitor), imatinib (PDGFR inhibitor) and bevacizumab (VEGF inhibitor)) and P-gp inhibitors. Tyrosine kinase inhibitors revealed limited efficacy due to several factors including the poor penetration across the BBB and the fact of the coactivation of different tyrosine kinases which hindered the usefulness of a single agent therapy. Moreover, increased intracellular concentrations of anticancer drugs by P-gp inhibition showed non-promising results which points at the presence of concurrent molecular alterations that renders glioma cells resistant to therapy.

Thus there is an inevitable need for a better understanding of the molecular biology of glioma which will provide insights into enhancement of the response to the currently available chemotherapeutic agents besides the development of clinically applicable targeted agents that can be used alone or in combination with conventional cytotoxic drugs to overcome glioma resistance.

Key players in the resistance of glioma to currently applied anticancer therapies are the DNA-repair proteins. "DNA-damage repair is a double-edged-sword in the cancer world", insufficient DNA-repair in normal cells is carcinogenic while in tumor cells it induces resistance to DNA-damaging anticancer therapy. An inverse correlation has been revealed between the DNA-repair capacity of high-grade gliomas and their sensitivity to therapy.

## 1.4. Treatment of glioma

### 1.4.1. Treatment of newly diagnosed glioma

For newly diagnosed gliomas, the gold standard treatment starts with maximal tumor resection by surgery when possible to reduce tumor size. This is also important for diagnostic purposes and molecular analyses. The role of surgery to improve survival of glioma patients is controversial. Surgery must be followed by chemoradiotherapy in GBM patients with controversy regarding the use of chemotherapy in astrocytomas,

oligodendrogliomas and mixed oligoastrocytomas because the benefit from chemotherapy is not yet established.

Radiation in a total dose of 60 Gy over a period of 6 weeks is the mainstay of therapy and has been shown to significantly prolong the survival of GBM patients (18). TMZ given concomitantly with IR followed by adjuvant TMZ alone improved the median survival of primary GBM patients from 12.1 months to 14.6 months and increased the 2 years survival rate by 16.1% (19).

Almost all malignant gliomas recur after therapy, and in general recurrent anaplastic gliomas are more responsive to treatment than recurrent glioblastomas. Up to date, there is no established treatment regimen for recurrent gliomas. TMZ has shown modest efficacy for treating glioma patients who already received the drug. Currently available salvage therapies consist of nitrosureas, procarbazine, carboplatin and topoisomerases I and II poisons such as irinotecan and etoposide respectively.

### 1.4.2. Anticancer therapy for recurrent gliomas

Almost all malignant gliomas recur after optimal treatment with surgery and adjuvant chemoradiotherapy. The median time to progression is about 7 months for GBM and 2-5 years for anaplastic gliomas (19,20). Recurrent gliomas are considered more aggressive and less responsive to therapy with a median survival of 3 to 9 months regardless of treatment option (21). Reoperation of the patients could be an option for debulking the tumor and decrease the symptoms, pathology confirmation and to enhance the response to chemotherapy; however, it results in a low survival benefit (20,22). Re-irradiation of patients could be also an option but with controversial results regarding its effect on 6-month progression free survival and overall survival (20,22). Moreover, to date there is no established chemotherapeutic regimen for treatment of recurrent gliomas. Retreatment with TMZ showed efficacy when used in an alternative dosing regimens. It

was associated with response rates and 6-month progression free survival of 35% and 46% and 5.4% and 21% in recurrent anaplastic gliomas and GBM respectively (20).

Other salvage therapies used as single agents or in combinations have been investigated include: alkylating agents such as nitrosureas (CCNU and BCNU) and procarbazine, carboplatin, cisplatin, topoisomerase I (TOP-I) poisons like topotecan and irinotecan, TOP II-poisons like etoposide and doxorubicin, and targeted agents such as bevacizumab and erlotinib.

*1.4.2.1. TOP-poisons as alternative anticancer therapies in glioma*

TOP-poisons, especially those targeting TOP-I enzyme, are promising anti-neoplastic agents in the treatment of malignant glioma. Irinotecan or topotecan single therapy has shown efficacy in patients with recurrent or progressive malignant glioma but with controversy. Investigators suggest the use of TOP-poisons in combination with other anticancer therapies in order to increase the antineoplastic activity and reduce the toxicity. *In vivo* and *in vitro* studies of the TMZ combination with TOP I-poison such as topotecan or irinotecan showed a synergistic effect between the two agents resulting in enhanced antitumor efficacy (23). This was explained by the generation of the TOP I-DNA cleavage complexes by the O6-methylguanine (O6-meG) adduct produced by TMZ which renders tumors more sensitive to TOP-I poisons. Clinically, this combination resulted in modest efficacy in newly diagnosed GBM patients and was associated with higher toxicity compared to TMZ alone (24).

Bevacizumab-irinotecan combinational strategy was also investigated in patients with recurrent glioma. A study by Cecchi *et al.* revealed an improvement in the rates of progression free survival and overall survival at 6-months. Compared to the patients' group treated with bevacizumab alone, the combination of irinotecan + bevacizumab resulted in an increase by 10.3 months and 4.3 months in the progression free survival

and overall survival, respectively (25). Moreover, meta-analysis of the available phase II trials' findings suggest the promising efficacy of this combinational therapy in recurrent glioma (21).

On the other hand, treatment of newly diagnosed GBM patients with a combination of topotecan with IR has resulted in an increase in the 6-month progression free survival by 16% compared to IR alone. However, this effect vanished after 8 months (26), which may indicate the development of acquired resistance against topotecan. A phase II trial of topotecan in combination with IR was well tolerated and resulted in reducing or stabilizing the disease in one third of the patients with non-resectable GBM but with no effect on the overall survival (27).

Beside TOP I-poisons, TOP II-poisons-based combinational therapies have been also investigated as second-line therapies of recurrent gliomas. *In vitro* and *in vivo* studies revealed a synergistic effect between carboplatin and etoposide. This combination has proven efficacy in children with low-grade gliomas and in adults with recurrent malignant gliomas (12,28,29). Furthermore, etoposide combined with TMZ was effective in adults with recurrent or treatment-induced malignant gliomas (23).

## 1.5. DNA-damaging anticancer therapy and DNA-damage response in glioma

### 1.5.1. DNA-damage response (DDR)

The majority of the anticancer agents used in the treatment of glioma have DNA-damaging properties. Upon occurrence of the DNA-damage, the cells respond by inducing signal transduction cascades as a DDR (**Figure 1.3**). The response depends on the type of the DNA damage and is mainly regulated by two signal transduction cascades, ataxia telangiectasia and Rad3-related protein/checkpoint kinase 1 (ATR/Chk1) in response to DNA single strand breaks (SSBs) and ataxia telangiectasia

mutated/checkpoint kinase 2 (ATM/Chk2) in response to DNA double strand breaks (DSBs) (30).

The cascades start with the activation of PI3K-related kinases ATM and ATR which results in the subsequent phosphorylation and activation of checkpoint kinases Chk1 and Chk2 effector kinases and cellular checkpoints such as p53 (31). Then based on the severity of the DNA damage, the cell is directed to a cell-cycle arrest to allow for enough time for the DNA-repair to occur or if the DNA-damage is very severe it will result in cellular death by apoptosis (32).

SSBs (affect one strand of the DNA) and DSBs (affect both strands of the DNA) are the two major types of DNA lesions produced in the cell. SSBs if not properly repaired convert to DSBs by collision with replication forks. DSBs are more toxic and lethal to the cells. The cells possess different DNA-repair processes that are redundant and intertwined. Base excision repair (BER), nucleotide excision repair (NER) and mismatch repair (MMR) are the main processes for the fixation of SSBs while DSBs are repaired by homologous recombination (HR) and nonhomologous end joining (NHEJ) pathways. Direct reversal is another option to repair certain types of DNA lesions.

### 1.5.2. DNA-repair mechanisms

#### *1.5.2.1. Base excision repair (BER)*

The BER involves two pathways, the short-patch and the long-patch (34). The decision between the two is hypothesized to be cell-cycle and ATP-dependent. The short-patch BER is more defined and as shown in **Figure 1.4**, involves the following steps: 1- The recognition and excision of the damaged DNA residue by DNA-3-methyladenine glycosylase (MPG) leaving an apurinic/apyrimidinic site (AP-site), 2- cleavage of the AP-site by AP endonuclease 1 (APE-1) leaving a 3'-phosphate terminus, 3- recognition of the SSB by Poly [ADP-ribose] polymerase 1 (PARP-1) which in turn recruits the BER proteins: the scaffolding protein x-ray repair cross-complementing protein 1 (XRCC1)

and polynucleotide kinase phosphatase (PNKP) enzyme that removes the 3'-phosphate and leaves a 3'-hydroxyl end and 4- Strand extension by DNA-polymerase β (pol β) and then ligation by DNA-ligase IIIα (Lig 3α) (34).

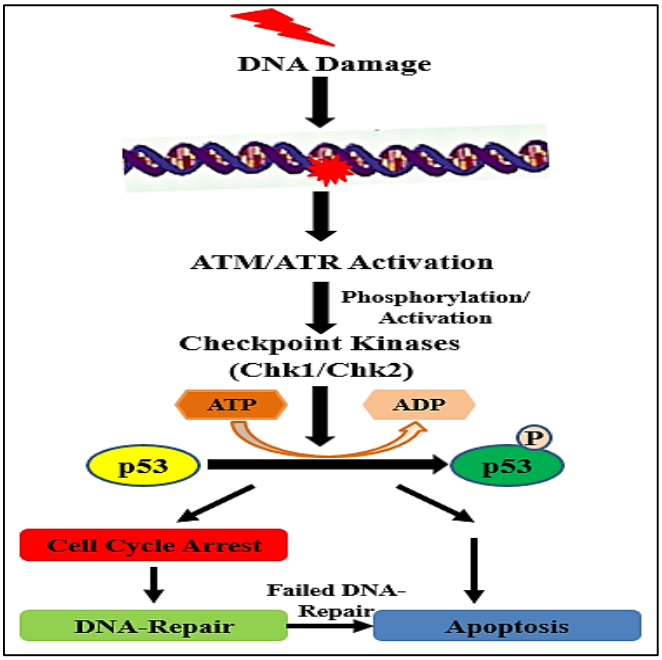


**Figure 1.3. DNA-damage response. DNA damage is caused by various endogenous or exogenous factors.** The DNA-damage response involve the activation of kinases ATM or ATR depending on the type of the damage. This is followed by activation of checkpoint kinases and cell cycle checkpoints such as p53. Based on the severity of the DNA damage, p53 activation leads to cell cycle arrest to allow for DNA repair, or to apoptosis when the damage is very severe.

Source: Adapted and modified from "The p53 network: cellular and systemic DNA damage responses in aging and cancer", Reinhard, 2012 (33).

*1.5.2.2. Nucleotide excision repair (NER)*

This pathway is responsible for the removal of bulky DNA base adducts resulting eventually in the excision of the damage-containing oligonucleotide (35) **Figure 1.5**. NER involves four steps. First, the recognition of the damage via the xeroderma pigmentosum complementation group C/UV excision repair protein Rad23 homolog B (XPC/HHR23B) complex which is important for the recruitment of the NER proteins (35). Second, the demarcation of the lesion by unwinding of the DNA surrounding the lesion. This is achieved by the action of different proteins including xeroderma pigmentosum complementation group A (XPA) and transcription factor II human (TFIIH) (35). Third, the excision of the oligonucleotide containing the lesion with a size of 24-32 nucleotides through the excision repair cross-complementing rodent repair deficiency complementation group 1/xeroderma pigmentosum group F complementing protein (ERCC1/XPF) complex which cuts at the 5'-end and xeroderma pigmentosum group G complementing protein (XPG) which cuts at the 3'-end (35). Finally, the gap left after excision is filled by DNA polymerase σ under the effect of replication factors such as single-strand DNA-binding protein (RPA), replication factor C (RFC) and proliferating cellular nuclear antigen (PCNA), followed by ligation by DNA-ligase I (Lig I) (35).

*1.5.2.3. Mismatch repair (MMR)*

As shown in **Figure 1.6**, this process starts with recognition of the mismatch via the mutator Sα (MutSα; a heterodimer of MutS protein homolog 2 (MSH2) and MutS protein homolog 6 (MSH6) that recognizes mismatches of 1-2 nucleotides) or mutator Sβ (MutSβ; a heterodimer of MSH2 and MutS protein homolog 3 (MSH3) that recognizes longer mismatches) (37). Both MutSα and β are also important for MMR initiation. This is followed by the excision of the mismatched bases by mutator Lα (MutLα; a heterodimer of MutL homolog 1(MLH1) and postmeiotic segregation increased 2

(PMS2)). Finally, under the activation of different replication factors such as PCNA and RFC the gap produced is filled by DNA polymerase σ (37).

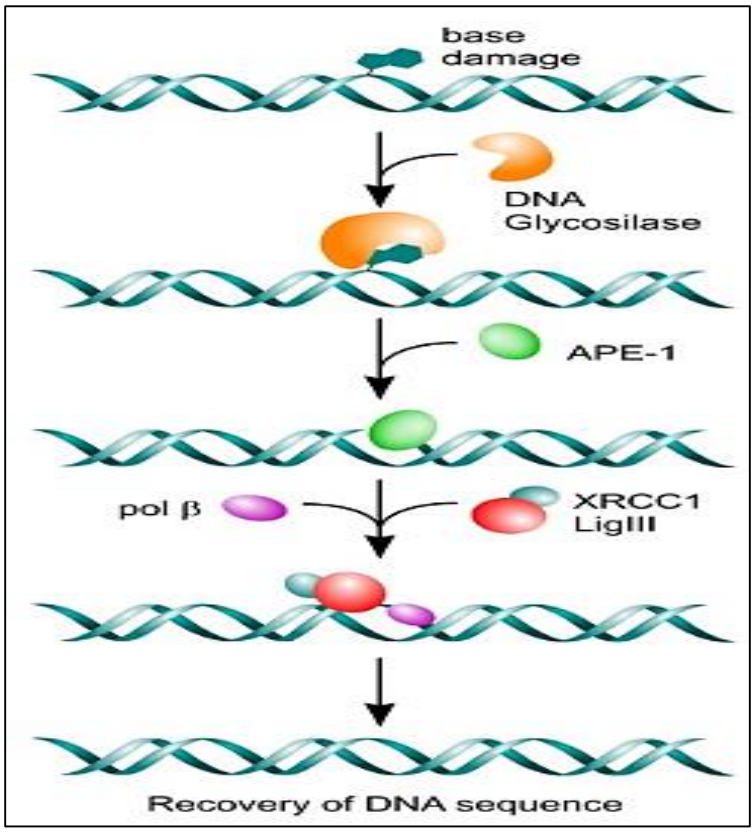


**Figure 1.4. Schematic representation of the short-patch base excision repair process.**

Source: Emil Mladenov and George Iliakis (2011). The pathways of double-strand break repair-on the pathways to fixing DNA damage and errors.

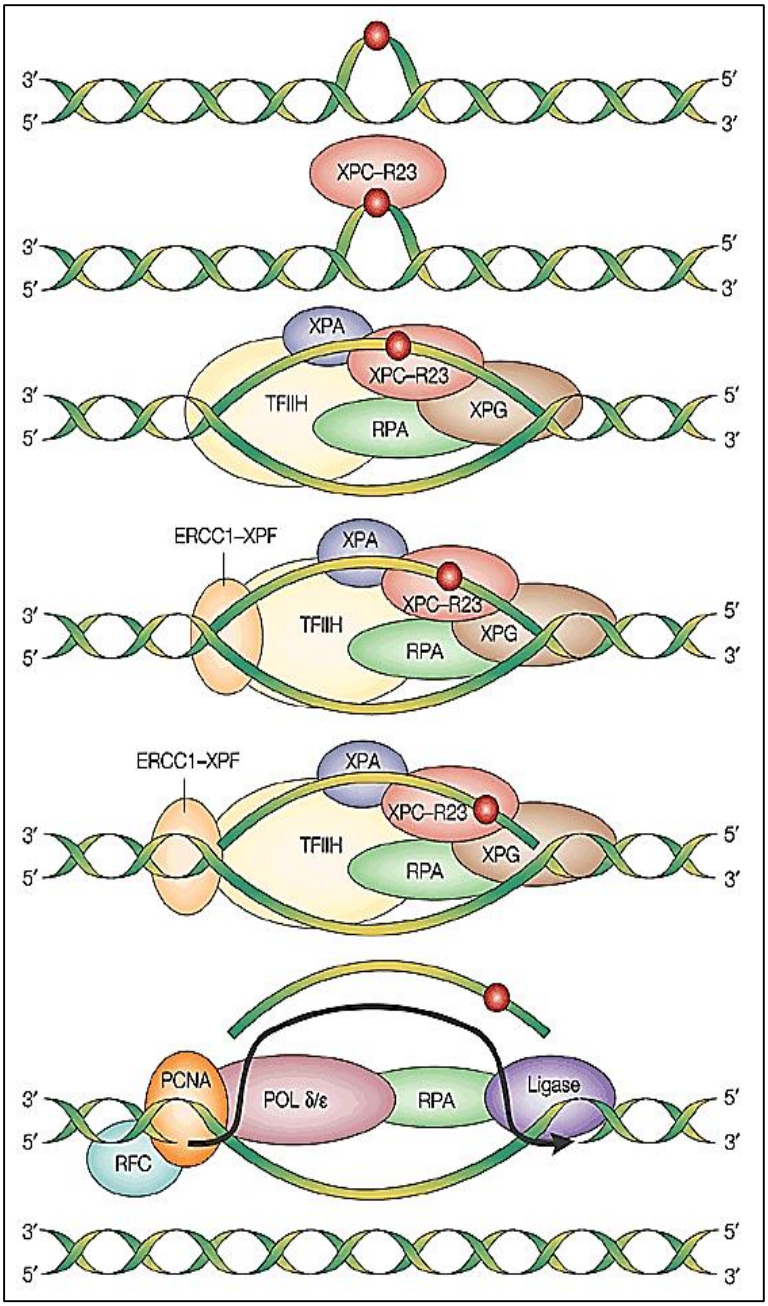


**Figure 1.5. Schematic representation of nucleotide excision repair pathway.**

Source: How nucleotide excision repair protects against cancer. Friedberg, 2001(36).

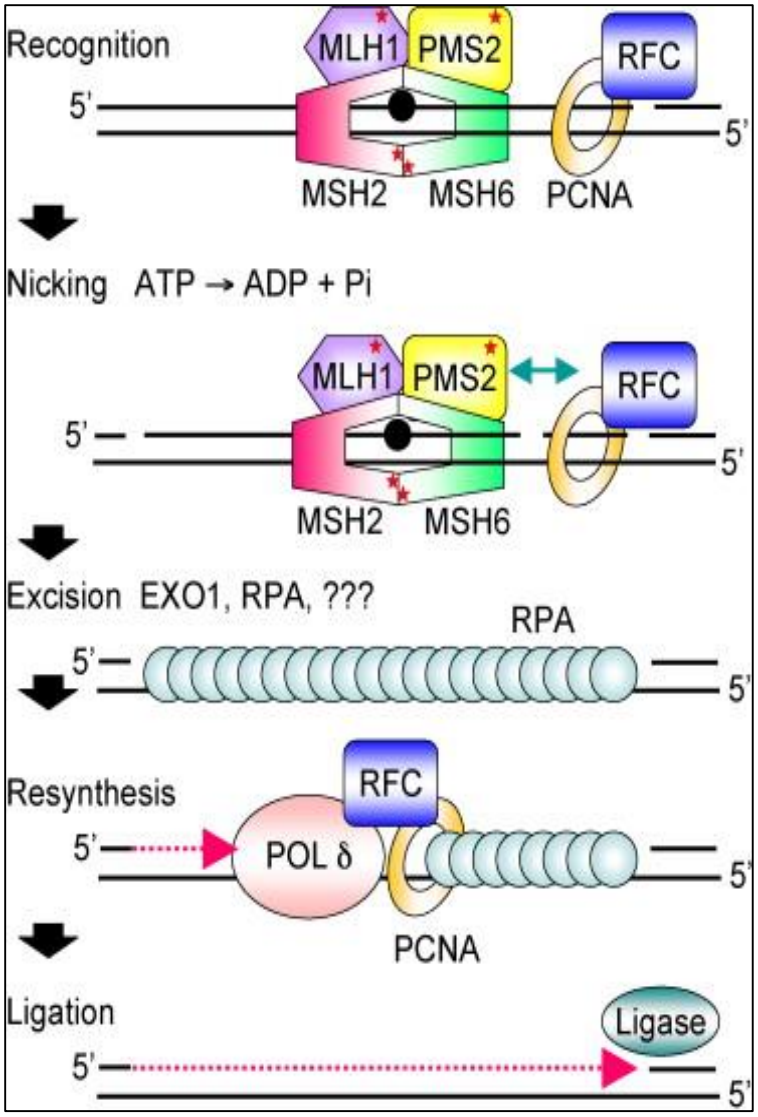


**Figure 1.6. Schematic representation of the mismatch repair pathway.**

Source: DNA mismatch repair: molecular mechanism, cancer, and ageing. Hsieh, 2008 (38).

*1.5.2.4. DNA-DSB repair*

DNA DSBs are the most lethal type of DNA lesions. The cells employ mainly two types of DSB repair processes, NHEJ or HR (**Figure 1.7**). The NHEJ is the predominant mechanism in mammalian cells; however the choice between the two depends on the following factors: 1- cell cycle phase, NHEJ operates in all phases but mainly in the G1 phase while the HR is active only in the S/G2 because it requires the presence of a complementary DNA strand (sister chromatid), 2- Type of the DSB, NHEJ repairs DSBs involving ≤ 4bp while HR repairs longer lesions, and 3- The degree of expression of the repair proteins and cofactors involved in both pathways (39).

1.5.2.4.1. Nonhomologous end joining (NHEJ)

The first step in the pathway is the recognition and binding of the DNA end of the DSB by Ku complex {a heterodimer of Ku70 encoded by x-ray repair cross complementing 6 (XRCC6) and Ku80 encoded by x-ray repair cross complementing 5 (XRCC5)} by forming a ring around the lesion (40). Then DNA-dependent protein kinase, catalytic subunit (DNA-PKcs) is recruited which results in activation of its kinase activity causing the phosphorylation of different proteins and the DNA-PKcs itself (40). This leads to the trimming of the DNA end by the MRN complex (Mre11/Rad50/Nbs1) and the recruitment of the x-ray repair cross complementing 4/ligase IV (XRCC4/Lig IV) complex that is important for the religation process (40).

1.5.2.4.2. Homologous recombination (HR)

DNA damage signaling is induced by the MRN complex that leads to the activation of the DDR proteins and checkpoints (39,41). RPA and Rad52 are then recruited and coat the 3'-single strand ends to protect them from further degradation. Rad51 then wraps the ends for homology recognition and strand exchange (39,41). Then, Rad54 promotes homologous DNA pairing and activates DNA recombination, DNA polymerases extend the 3'-end of the strand and the 3'-endonuclease XPF/ERCC1

complex removes flaps formed after extension and the strand ends are annealed by Lig I (39,41).

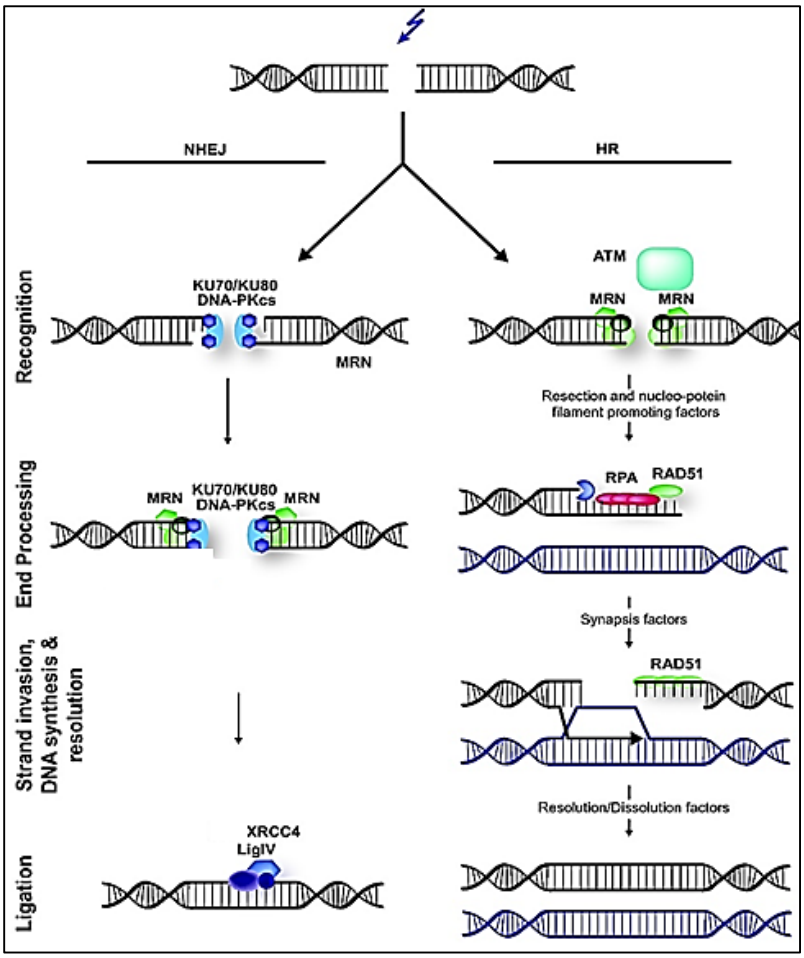


**Figure 1.7. Schematic representation of the double-strand break repair pathways.** To the left is the non-homologous end joining (NHEJ) pathway and to the right is the homologous recombination (HR) pathway.

Source: Adapted from "ATP-dependent chromatin remodeling in the DNA-damage response". Lans, 2012 (42).

### 1.5.3. Radiotherapy resistance and DNA-repair in glioma

IR exerts its anticancer effects mainly by attacking the DNA resulting in base damages, and DNA strand breaks consisting of DNA SSBs and DSBs in a ratio of 25 to 1 (43). However, DSBs are considered more severe and lethal to the cells and thus are the main DNA lesions responsible for the cytotoxicity of IR. Gliomas are intrinsically radioresistant and about 90% of them recur after radiotherapy.

Different studies tried to address the radioresistance due to induced DNA-repair. A study by Mukherjee *et al.* revealed a positive correlation between EGFRvIII mutant and the repair of IR-induced DSBs through the increase in the DNA-PK activity and thus activated NHEJ process (44). Moreover, PTEN loss that is commonly seen in GBM has shown to induce the PI3K/Akt-1 signaling and enhance DNA-repair as a result and thus induce resistance to IR (45). Additionally, inherent radioresistance of glioma due to glioma stem cells characterized by CD133 positive expression was attributed to the enhanced DNA DSB repair (46).

Although DSBs are the main contributors to radiation cytotoxicity, SSBs and abasic sites are produced in significantly higher amounts and if not repaired can be converted to DSBs thus enhance the radiosensitivity. These lesions are repaired through the BER process. APE-1 was found to be activated in human glioma samples and HGGs showed about 3 times more active enzyme compared to LGGs (47). Another study revealed an inverse correlation between APE-1 expression level and sensitivity to IR, where high APE-1 level was associated with elevated DNA-repair capacity and thus lower IR-induced tumor cell death (48). PARP-1 has been also implicated in radioresistance. It was found to be overexpressed in GBM patients samples compared to normal brain tissues that showed undetectable protein levels (49). Moreover, *In vitro* and *in vivo* studies showed that the inhibition of PARP-1 via small-molecule inhibitors was associated with enhanced radiosensitivity and decreased GBM xenograft size (50).

### 1.5.4. Temozolomide resistance and DNA-repair in glioma

DNA-alkylating agents are considered the mainstay chemotherapy of malignant glioma. They include TMZ, nitrosureas like BCNU and CCNU, and procarbazine. TMZ is the first chemotherapeutic agent to be approved for the treatment of high-grade gliomas and currently it is the preferred alkylating agent in glioma treatment and is widely used because of: 1- 100% oral bioavailability, 2- highly lipophilic and small molecular size so readily penetrate the BBB with CNS levels that are 30% of blood concentration, 3- is well tolerated by patients, 4- spontaneous physiological activation (hepatic activation is not required) and 5- broad anti-tumor activity (51).

Once in the CNS, TMZ is spontaneously converted to the active metabolite MTIC which in turn dissociates producing methyl groups that are added to the DNA. Four major types of base alkylation products are formed. N7-guanine position is highly nucleophilic thus N7-methylguanine (7meG) adducts are produced in large amounts (70%), N1 methyladenine (1meA) account for about 15%, N3 methyladenine (3meA) for 10% and 5% represents O6-meG (52). The latter two are the most cytotoxic and are the main DNA adduct responsible for the cytotoxicity of TMZ. Adducts, 7meG, 1meA and 3meA are repaired via the BER process. Direct reversal via MGMT is the responsible mechanism for the removal of methyl group from O6-G (53). MGMT is a ubiquitous DNA-repair protein that is also called a suicide enzyme because through its mechanism it transfers the methyl group to its active site resulting in the irreversible inactivation of the enzyme without resulting in the formation of transient DNA strand breaks (54).

If not repaired via MGMT, O6-meG will induce MMR (recognition of the mismatches and initiation of the process by MutSα complex) to mispair the alkylated base with thymidine (53). The recurrent addition and removal of mispaired bases will eventually induce a futile cycle of MMRs that will result in the formation of SSB (55) which collide with replication forks leading to DSB formation, apoptotic activation and cell death (23). Consequently, sensitivity to TMZ is highly dependent on DNA-repair and

an increase in MGMT or BER levels or a deficiency in MMR is expected to induce resistance to TMZ.

Epigenetic silencing of MGMT by promoter hypermethylation is associated with decreased MGMT expression. A study by Esteller *et al.* showed an enhanced sensitivity of malignant astrocytomas with MGMT promoter methylation to carmustine in terms of overall and progression-free survival (56). Another study by Hegi *et al.* revealed that the benefit from combining IR and TMZ compared to IR alone was only observed in GBM patients with MGMT promoter methylation (57). Although it shows a promising tool for stratifying patients for TMZ therapy, MGMT methylation status is not yet approved as a predictive biomarker for TMZ therapy because of the following reasons: 1- complete concordance between MGMT methylation and protein expression levels is not established (58,59), 2- an enhanced response to TMZ could not be observed in half of the GBM patients harboring this epigenetic mutation (60), 3- some GBM patients with unmethylated MGMT were still sensitive to TMZ (19,60), 4- measurements of MGMT methylation status and expression levels are highly dependent on the surgical site and the assessment methodology (58) and 5- The clinical Use of MGMT inhibitors such as O6-(4-bromothenyl)guanine did not show efficacy (52).

The differential response to TMZ in some patients regardless of the MGMT expression level or methylation status indicates the involvement of other resistance mechanisms. Regardless of MGMT activity, MMR deficiency may lead to tolerance to TMZ, thus a functional MMR was shown to be required for TMZ-induced toxicity. Hypermuation of MSH6 (MMR initiator) was revealed in recurrent GBM samples and contributed to TMZ resistance and glioma progression (61). Moreover, immunohistochemistry analysis of MMR proteins including MSH2 and MSH6 in recurrent GBM samples showed a downregulation of these proteins (62). *In vitro* knockdown of MSH6 in GBM cell lines was associated with increased resistance to TMZ (63). Nevertheless, variable expression of MMR proteins (MSH2 and MLH1) in GBM

patients' samples was not associated with differential response to TMZ (64). MMR deficiency is not enough to induce resistance to TMZ due to the following possible explanations: 1- Microsatellite instability (MSI), a damaged DNA due to DNA-repair defects and could be used a surrogate marker for MMR deficiency, is rare in high-grade gliomas (62), 2- MMR deficiency manifested by MSH6 mutations did not correlate with MSI in recurrent GBM samples (64), and 3- some GBM samples expressed low MGMT and a proficient MMR were still resistant to TMZ; highlighting the complexity of mechanisms that determine cellular sensitivity to TMZ. The major adducts produced by TMZ are 3meA and 7meG and they are readily repaired by the BER, so they contribute a little to the antitumor activity.

Thus, targeting BER is an attractive option to enhance the cytotoxicity of TMZ regardless of the MGMT or MMR status. MPG expression was found to vary among astrocytomas patients' samples indicating a tumerogenic potential of the enzyme (65). Another study by Agnihotri *et al.* found a variable protein expression of MPG (APNG) among human GBM cell lines with TMZ-resistance was evident in those expressing both MPG and MGMT (60). In the same study, analysis of MPG expression data from the European Organization for Research and Treatment of Cancer-National Cancer Institute of Canada (EORTC-NCIC) trial showed that MPG presence inversely correlated with the overall survival of patients. This was only evident after TMZ therapy and only in patients expressing the unmethylated MGMT, indicating the negative prognostic value of positive MPG expression and that patients who will benefit more from TMZ are those with MPG negative and methylated MGMT. On the other hand, MPG overexpression enhanced the sensitization of GBM cell lines to TMZ when used in combination with BER inhibitors (52).

As mentioned before, APE-1 was found to be overexpressed and activated in malignant glioma (47). APE-1 knockdown in a GBM cell line resulted in enhanced sensitivity to different alkylating agents including TMZ (66). Furthermore, stabilization

of the AP-site by methoxyamine and prevention of APE-1 binding potentiated the cytotoxicity of TMZ in GBM cell lines (67).

A currently under intensive investigation to enhance the efficacy of TMZ are PARP-1 inhibitors. Preclinical studies showed a promising effect of different small-molecule PARP-1 inhibitors to increase the cytotoxicity of TMZ toward malignant glioma (especially those which are resistant due to lack of MMR) (52,68-70). A phase I/II trial is ongoing to investigate the combinational therapy of BSI-201 (PARP-1 inhibitor) and TMZ in newly diagnosed malignant glioma patients (71). Pol β mRNA expression was also found to vary between GBM samples and normal brain tissue and its downregulation correlated with enhanced sensitivity to TMZ under BER inhibition via methoxyamine (52). PTEN mutations have been also shown to affect TMZ efficacy. This gene is lost in about 36% of GBM and was found to be associated with sensitivity to a TMZ analogue due to accumulation of alkylation-induced DSBs caused by impaired HR process (72).

### 1.5.5. Topoisomerase-poisons and DNA-repair

#### *1.5.5.1. Topoisomerase enzymes*

Topoisomerases are ubiquitous DNA-repair enzymes that regulate the topology of DNA during important cellular mechanisms including DNA replication, transcription, recombination and chromatin remodeling (73).

Two major families of topoisomerases have been identified in humans, TOP-I and TOP-II, both of which act by introducing a break in the DNA to relieve the superhelical tension, then producing an intermediate where the tyrosine residue of topoisomerase enzyme is linked to the site of the break at the 3'- or 5'- termini in the DNA making a TOP-DNA cleavage complexes (74). After topological changes occur topoisomerases religate the breakage with a faster rate of religation than the rate of cleavage making the TOP-DNA intermediates reversible and short-lived. For the religation to occur the 3'-

hydroxyl end of the DNA must be aligned with the tyrosyl-phosphodiester bond between the DNA and TOP enzyme in order for a nucleophilic attack to occur (75). However, if the cleavage occurred near certain DNA lesions such as methylated guanine, mismatched bases, abasic sites (76), DNA nicks (77), modified bases (78) or modified sugars (79) or in the presence of DNA-damaging anticancer agents such as TOP-I poisons and TOP-II poisons (act directly on the cleavage complexes) (80,81) and IR (produces oxidized bases and abasic sites thus indirectly acting on the cleavage complexes) (82) , the religation process is blocked leading to the accumulation of the TOP-DNA protein complexes which is called "trapping" because the 5'-hydroxyl end of the DNA is misaligned so the two DNA ends cannot be resealed together forming what is called "dead-end covalent complexes".

TOP-I enzyme acts to relax the superhelical structure of DNA by making a transient SSB. The catalytic mechanism of TOP-I involves two transesterification processes. First the Tyrosine residue (Tyr723) of TOP-I binds to the 3'-phosphate of the DNA forming TOP-DNA cleavage complex resulting in breakage of the single strand which will then rotate around the other strand in a specific way to relieve the tension of the DNA double helix. After the DNA has relaxed, religation of broken single strand occurs by the second transesterification process where the free 5'-hydroxyl of the DNA acts as a nucleophile and attacks the 3'-phosphotyrosyl linkage of TOP I-DNA covalent complex; as a result the DNA original double helical structure is restored (83).

On the other hand, TOP-II acts to remove negative or positive DNA superhelical tension by the application of energy resulting in the cleavage of the two strands of the DNA double helix causing transient DNA DSBs and producing TOP II-DNA cleavage complexes (84). Thus, the cleavage complexes formed are temporary and reversed via the TOP enzymes themselves, but since they are ubiquitous they represent good targets for anticancer therapy.

TOP-poisons (not TOP inhibitors because they do not inhibit the enzyme itself) are DNA-damaging anticancer drugs that act by stabilizing the TOP-DNA cleavage complexes. TOP-I poisons mainly act via intercalating between the DNA bases in the covalent complex forming a ternary complex that results in misalignment of the 3'-hydroxyl end of the DNA "misalignment model" that prevent the religation process (85). TOP-II poisons exert their function via different postulated mechanisms. One of them is the "misalignment model". TOP-I poisons lead to the formation of persistent irreversible covalent complexes that if collide with replication forks lead to DSBs or if collide with transcription factors lead to the formation of SSBs that then convert to lethal DSBs. TOP-II poisons result in the formation of DSBs directly (86). This means that compared to TOP-I poisons cytotoxic effect that is replication dependent, TOP-II poisons cytotoxicity is replication independent (85). Both types of TOP-poisons induce cell death by mainly triggering cellular apoptosis (87).

*1.5.5.2. Irreversible topoisomerase-DNA cleavage complexes and DNA-repair*

While the repair of the irreversible TOP I-DNA cleavage complexes is well understood, the repair of the stalled TOP II-DNA cleavage complexes is not yet well characterized. There are three important steps in the repair of the DNA-damage induced by TOP-poisons, first, the recognition of the TOP-DNA cleavage complexes as a DNA lesion not as a reversible intermediate of the TOP enzyme function. Second, the removal of the protein linked to the DNA. Third, the activation of the DDR previously discussed and the recruitment of the appropriate DNA-repair factors and enzymes.

Since the produced TOP-DNA covalent complexes trap the replication fork and transcription factors, the recognition step is attainable. The removal of the bounded protein in TOP II-DNA cleavage complexes is accomplished through two possible mechanisms, first, the removal of the TOP-II plus part of the DNA that is attached to it

via an enzyme called 5'-tyrosyl DNA phosphodiesterase II (TDP2) or also called TTRAP (88). Second the direct removal of the TOP-II via proteolytic degradation by 26S proteosome. The removal of the TOP-II enzyme is followed by the DSB repair processes HR and NHEJ (85).

For the repair of irreversible TOP I-DNA cleavage complexes, the removal of the bounded protein is dependent if the cleavage complexes were trapped by replication forks or by transcription factors. If associated with transcription then degradation of the TOP-I protein by 26S proteosome is the main pathway. However, if the trapping was by collision with replication forks then the removal of the TOP-I protein is attained mainly via two alternative pathways (**Figure 1.8**). The first pathway is the 3'-tyrosyl DNA phosphodiesterase/phosphatase pathway TDP1/PNKP which hydrolyzes the phosphodiester bond in the cleavage complex releasing the TOP-I enzyme and thus induces the BER subsequently (XRCC1/PARP-1/PNKP/pol β/lig 3α complex). The second pathway is via the 3'-endonuclease XPF/ERCC1 which removes the TOP-I enzyme and part of the DNA bound to it, and is associated with subsequent NER pathway where MRN (Mre11/Rad50/Nbs1) complex is a main component (75,81). The DSBs produced by TOP I-poisons are repaired via HR and NHEJ pathways as discussed earlier (81).

## 1.6. Tyrosyl DNA-phosphodiesterase I (TDP1)

TDP1 is the only known DNA-repair enzyme to be responsible for the hydrolysis of the phosphodiester bond in the TOP I-DNA cleavage complexes. It was first identified in *Saccharomyces cerevisiae* by Nash and colleagues in 1996 to have an ability to catalyze the hydrolysis of the phosphodiester bond between a DNA 3'-phosphate and an O-4 tyrosine residue (89).

TDP1 is highly conserved from yeast to human, which indicates its importance. Homo sapiens TDP1 gene locates in chromosome 14 (14q32.11) and consists of 15

coding and two 5' noncoding exons with a total size of 88,863 bases. The gene encodes a 608 amino acids protein with a molecular weight of about 68 kda that has been described as a member of the phospholipase D (PLD) superfamily (90).

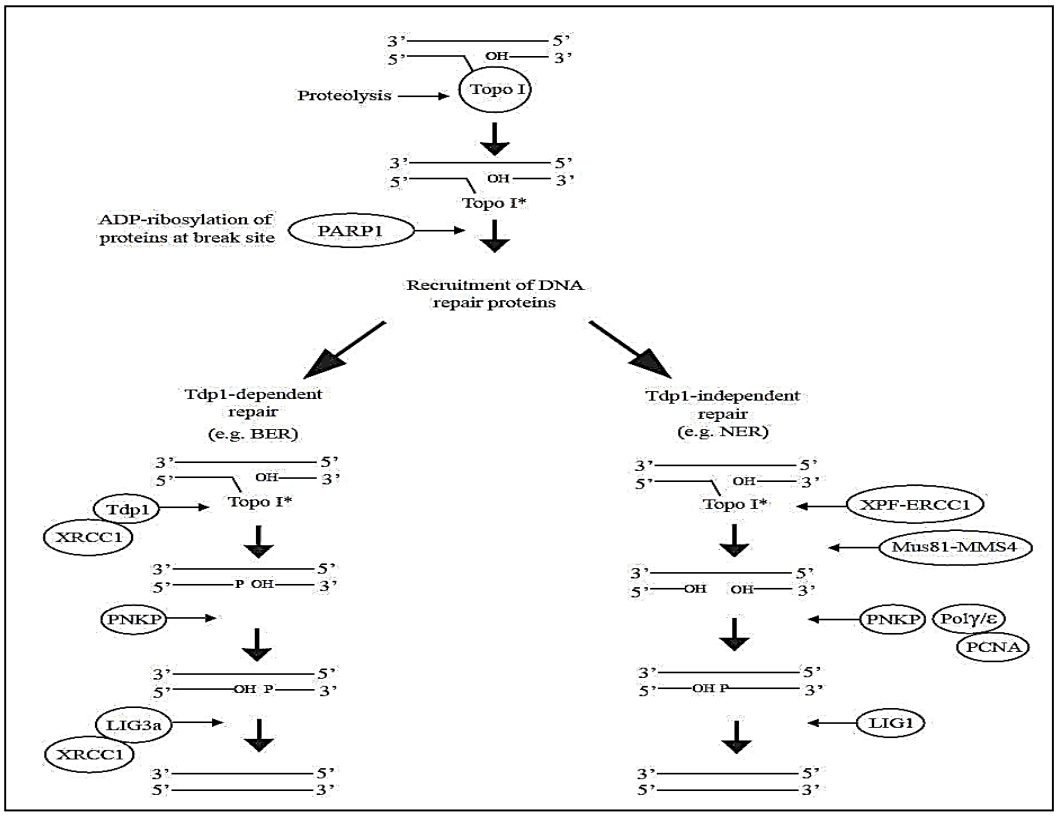


**Figure 1.8. DNA-repair of topoisomerase I-DNA covalent complexes.** Topoisomerase I (TOP-I) enzyme is degraded via proteolysis followed by its removal from the DNA by a TDP1-dependent pathway (right scheme) or a TDP1-independent pathway (left scheme).

Source: Spinocerebellar Ataxia with Axonal Neuropathy (SCAN1): A disorder of Nuclear and Mitochondrial DNA Repair. Hok Khim Fam, Miraji K. Chowdhury and Cornelius F. Boerkoel, 2012. ISBN: 978-953-51-0542-8.

This superfamily includes a group of enzymes that are responsible for catalyzing the hydrolysis of phosphodiester bonds in 3—phospho-DNA adducts. PLD enzymes possess two H(X)K(X4)D motifs (HKD) in the catalytic site which in TDP1 present in the N- and C- terminal domains and contain histidine and lysine residues (263His/Lys265 and 493His/Lys495) but lacks aspartate residues present in other HKD motifs (**Figure 1.9**). The C-terminus (351-608) is highly conserved and it is important for the enzymatic activity, while the N-terminal domain (1-350) is poorly conserved, has no role in the enzymatic activity, and is believed to have regulatory functions such as protein-protein interactions (91).

There are two transcript variants of TDP1 that differ in their 5'-UTR (**Table 1.1**). The region from 70-293 bps is deleted in TDP1 transcript variant 2. However, both encode the same protein isoform consisting of 608 amino acids. The functionality of transcript variant 2 is still unknown.

TDP1 protein has been shown to be localized both in the nuclei and mitochondria; with the mitochondrial TDP1 (mtTDP1) expression is dependent on the nuclear gene because the mtDNA does not encode for DNA-repair proteins (82).

### 1.6.1. Catalytic mechanism of TDP1

The hydrolysis of the phosphodiester bond catalyzed by TDP1 involves two steps (**Figure 1.10**) (92). The first step is the formation of a 3'-phosphohistidine Tdp1 covalent intermediate by the nucleophilic attack of the phosphodiester bond by H263 then H493 act as a proton donor to the tyrosyl moiety of the leaving group. The second step is the hydrolysis of the 3'-phosphohistidine intermediate by a nucleophilic attack by a water molecule. This hydrolysis reaction is activated by H493. A 3'-phosphate product (usually a DNA strand) and a free TDP1 is the final result.

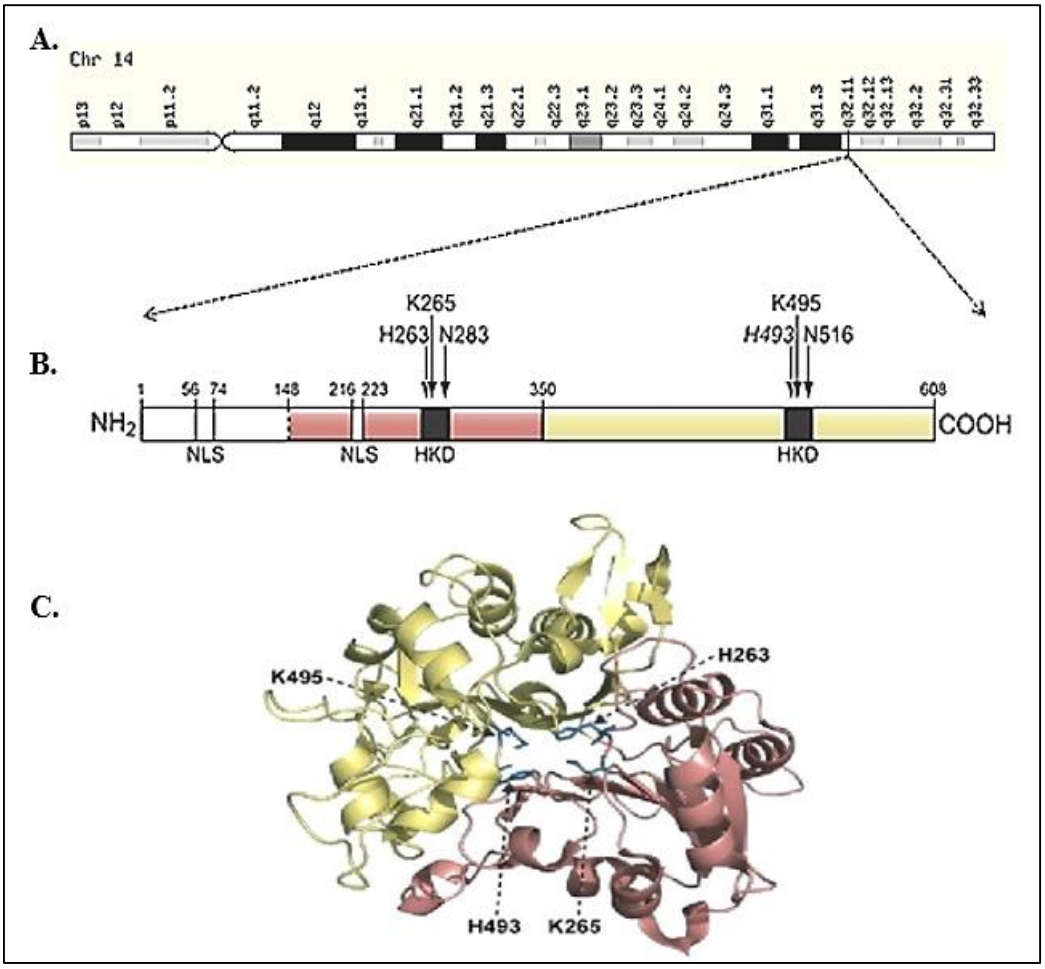


**Figure 1.9. Modified representation of:** (**A**), the chromosomal arrangement of 14q32.11, (**B**), Tyrosyl-DNA-Phosphodiesterase I (TDP1) protein structure, including the N-terminal domain (amino acids 1-350; red) and the C-terminus (amino acids 351-608; yellow). HKD motifs present in the catalytic site of TDP1 in the N- and C- terminal domains and contain active site residues, histidine and lysine residues (263His/Lys265 and 493His/Lys495) indicated by arrows in C. (**C**), Three dimensional representation of human TDP1 protein.

Source: Inhibitors of human tyrosyl-DNA phosphodiesterase (hTdp1) developed by virtual screening using ligand-based pharmacophores. Weildlich, 2010 (91).

**Table 1.1. Tyrosyl-DNA-phosphodiesterase I (TDP1) transcript variants**

| TDP1 Transcript Variant | Refsequence accession # | mRNA length (bps) | 5'-UTR Region | Coding Region |
|---|---|---|---|---|
| 1 | NM_018319 | 3763 | 1-299 | 300-2126 |
| 2 | NM_001008744 | 3540 | 1-76 | 77-1903 |

### 1.6.2. DNA lesions targets of TDP1

TDP1 has been described as the only enzyme capable of catalyzing the phosphodiester bond in irreversible TOP I-DNA cleavage complexes such as those produced by TOP I-poisons. Besides hydrolyzing the 3'-phosphodiester linkage in TOP I-DNA covalent complexes produced in the nucleus, TDP1 has been found to be important in the repair of these complexes formed in the mitochondria since they possess their own TOP-I enzyme (mtTOP-I) (82). Additionally, TDP1 has been found to catalyze the removal of other different types of moieties from the 3'-end of the DNA. Such adducts include 3'-abasic sites, 3'-nucleosides, and 3'-ribonucleosides (93).

Furthermore, TDP1 has shown the ability to hydrolyze the phosphodiester bond between 3'-end of DNA and glycolate (3'-phosphoglycolate) (94). This substrate is the result of oxidative DNA damage produced either exogenously by exposure of cells to ionizing radiation, tobacco or xenobiotic agents (e.g. bleomycin, and mitomycin C) or endogenously as a result of naturally occurring endogenous reactive oxygen species (ROS). TDP1 is responsible for the removal of 3'-phoshpgylcolate lesions associated with SSBs or 3'-overhangs or blunt ends of DSBs (82). TDP1 has been suggested to be the only enzyme capable of hydrolyzing the protruding 3'-phosphoglycolate in DSBs and 3'-overhangs (94,95). This activity against ROS-induced DNA lesions has shown significance in the mitochondria since they possess high oxidative stress and the mtDNA

needs to be preserved because almost all of it is translated into proteins that are important for the function of the cells (82).

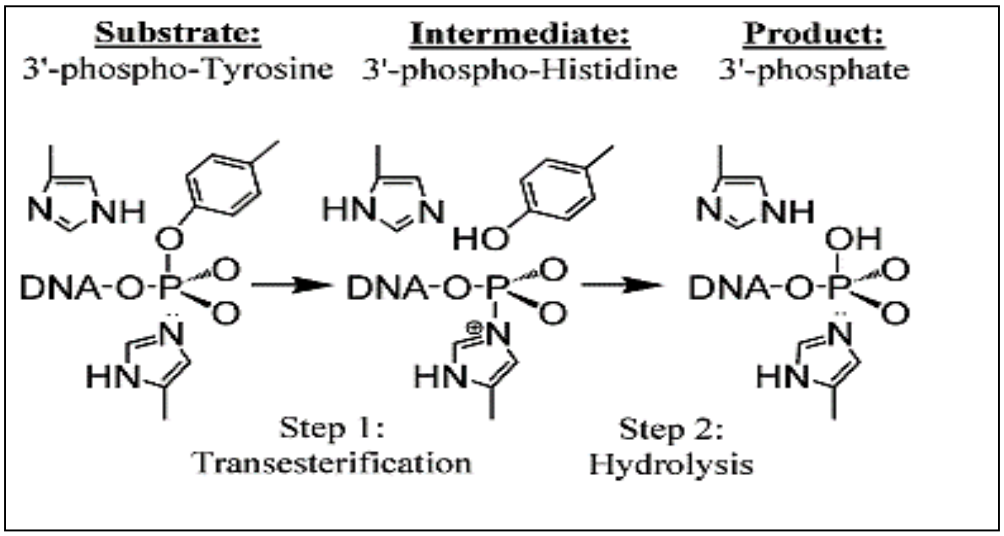


**Figure 1.10. Tyrosyl DNA-Phosphodiesterase I (TDP1) catalytic activity**. The hydrolysis of the phosphodiester bond catalyzed by TDP1 involves two transesterification reactions. First, a nucleophilic attack of the phosphodiester bond by H263 resulting in the formation of a 3'-phosphohistidine TDP1 covalent intermediate. Second, hydrolysis of the TDP1-DNA intermediate resulting in a 3'-phosphate DNA and a free TDP1.

Source: Analysis of human tyrosyl DNA-phosphodiesterase I catalytic residues. Raymond, 2004 (92).

In addition, TDP1 has the ability to hydrolyze the phosphoamide linkage in the phosphohistidine intermediate that is produced during the TDP1 catalytic cycle. TDP1 also has a limited DNA and RNA 3' exonuclease activity resulting in the removal of a single nucleoside from the 3'-hydroxyl end of the substrate. Furthermore, studies on yeast showed that this enzyme is capable of hydrolyzing the 5'-phosphotyrosyl linkage

between TOP-II and DNA. However, this activity could not be proven in human cells and was attributed to a different phosphodiesterase enzyme called TDP2 (88).

### 1.6.3. TDP1 and DNA strand breaks repair

TDP1 has revealed DNA repair activities against both SSBs and DSBs. TOP I-DNA cleavage complexes as discussed earlier induce both SSBs and DSBs as shown in the **Figure 1.11**. TDP1 is involved in the SSB repair of TOP I-DNA cleavage complexes through the BER process. It has a 3'-endonuclease activity inducing the cleavage of the AP-sites (the initial steps in the BER process) and has been found to exist in a multiprotein complex with BER proteins including XRCC1, PARP-1, PNKP, Lig 3α, and pol β. A prerequisite for the hydrolysis of the TOP I-DNA cleavage complex by TDP1 is the proteolysis of TOP-I by 26S proteosome (96). After the hydrolysis of the phosphodiester bond, DNA with a 3'-phosphate end results which is removed by PNKP. On the other hand, the role of TDP1 in the repair of the DSBs produced by TOP I-poisons is not yet well established.

A homozygous nonsynonymous mutation A1478G in the active site of TDP1 gene resulting in a histidine to arginine mutation (H493R) has been identified as the cause of an autosomal recessive disease which is called spinocerebellar ataxia with axonal neuropathy (SCAN1). This mutation has been associated with a 25 fold decrease in the TDP1 activity compared to the normal levels. Thus, extensive studies have been performed on SCAN1 cells to assess the effect of TDP1 mutation on the response to anticancer therapy.

The ability of TDP1 to hydrolyze the 3'-phosphoglycolates contributed to the TDP1-dependent repair of SSBs induced by IR. It has been shown that SCAN1 lymphoblastic cells are defective in the repair of 3'-phosphoglycolate termini (95,97) and they are less able to repair SSBs induced by hydrogen peroxide or IR when compared to

normal cells (98,99). This disease as mentioned earlier is due to a mutation in the active site of the TDP1 resulting in an enzyme product that is 25x less active than the wild-type.

Although TDP1 is more efficient in the repair of SSBs, its role in the repair of DSBs has been investigated in different studies. Compared to normal cells and after treatment with different DSBs-inducing anticancer agents (IR, bleomycin, and chalicheamicin), low TDP1 levels due to mutation as in SCAN1 cells or due to protein knockdown in murine embryonic fibroblasts, *Hela* cells or vertebrate chicken erythrocytes have been shown to be associated with lower DSB repair rate (95,97,100,101). This was explained by the lower ability to remove the 3'-phosphoglycolates associated with 3'-overhangs of DSBs. A study by Das *et al.* has shown that in response to the DSBs produced by either IR or camptothecin, the DDR cascade induced by ATM followed by an activation of DNA-PK (NHEJ initiator) has resulted in the phosphorylation of TDP1 at serine 81. This was not required for TDP1 enzymatic activity but resulted in its stabilization and enhanced its interaction and the recruitment of XRCC1 at damage sites, which is consistent with the role of the TDP1 N-terminus in regulating protein-protein interactions. In the case of DSBs produced by camptothecin, at the damage sites, γH2AX were also present; suggesting the role of both TDP1 and XRCC1 in the repair of the DSBs produced by camptothecin (102).

Several studies investigated the role of TDP1 in the repair of the DNA damage induced by TOP II-poisons resulted in controversial findings. A study by Barthelmes *et al.* showed that TDP1 overexpression in HEK293 cell lines resulted in reduction in the DSBs produced by etoposide compared to cells expressing mutant TDP1 which were less able to remove the damage (103).

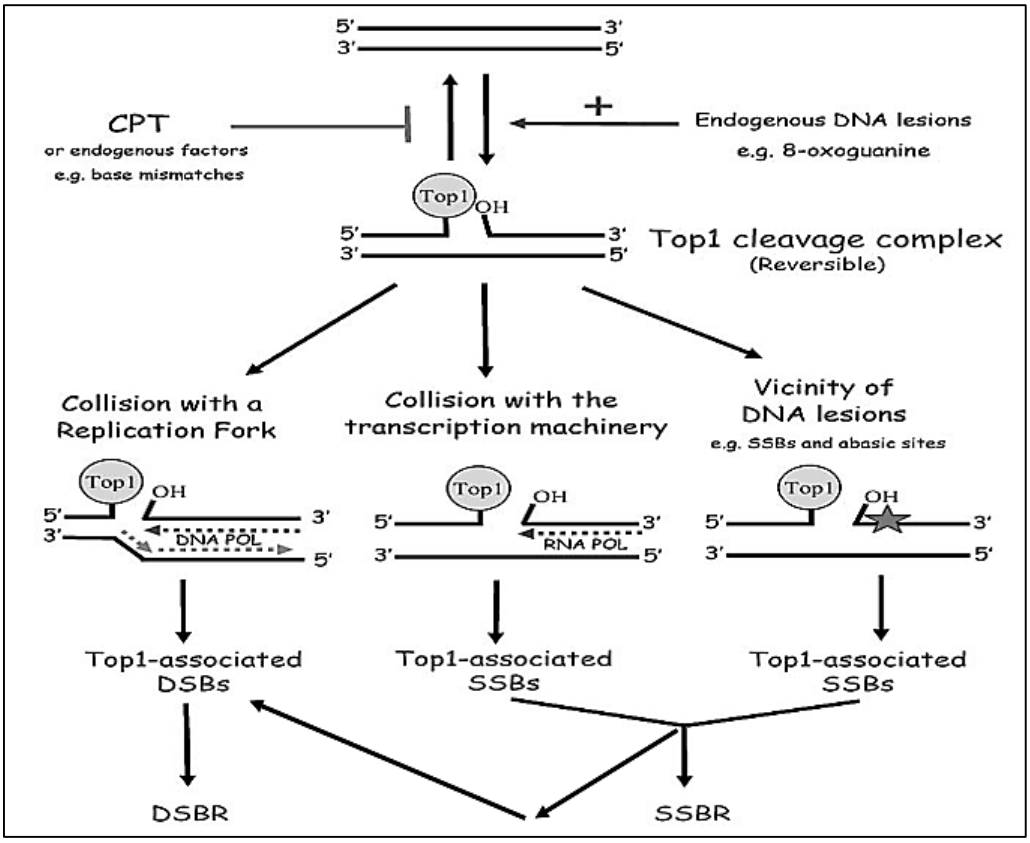


**Figure 1.11. Schematic representation of the DNA-repair pathways of the DNA damage associated with irreversible topoisomerase I-DNA cleavage complexes.** As part of its mechanism of action, topoisomerase I enzyme produces temporary DNA single-strand break (SSB) through binding to the 3'-end of the DNA via a phosphodiester bond resulting in the formation of reversible intermediates called TOP I-DNA cleavage complexes. Endogenous DNA lesions or exogenous factors such as TOP I-poisons (e.g. camptothecin) stabilize these complexes making them long-lived and irreversible. Vicinity to endogenous DNA-lesions such as abasic sites or collision with transcription factors converts stalled TOP I-DNA cleavage complexes to permanent SSBs, while collision with replication forks results in DNA double-strand breaks (DSBs) formation. This results eventually in the activation of SSB and DSB repair processes.

Source: TDP1-dependent DNA single-strand break repair and neurodegeneration. El-Khamisy and Caldecott, 2006 (86).

### 1.6.4. Correlation between TDP1 level and sensitivity of cells to anticancer therapy

The effect of TDP1 level on response to different DNA-damaging agents has been investigated with promising results. SCAN1 cells have shown hypersensitivity to camptothecin when compared to a heterozygous cell line and wild-type cell lines (cell lines containing wild-type TDP1) (104). Another study on mice has shown that cells derived from the TDP1-knockout mice were hypersensitive to topotecan (105). The ability of TDP1 to resolve 3'-phosphglycolates induced by oxidative stress was translated into hypersensitivity of TDP1-konckout mice and TDP1-knockout chicken DT40 cells to bleomycin, with however no effect on response to IR in SCAN1 cells (95,99,101). Studies on yeasts have demonstrated that TDP1 knockdown has resulted in the hypersensitivity to etoposide. However, this finding was not observed in SCAN1 lymphoblastoid cells and TDP1 knockout mice (104,106), but was evidenced to be true in TDP1-knockout chicken DT40 cell lines (101).

On the other hand, overexpression of wild-type in HEK293 cell lines has been associated with resistance to etoposide and camptothecin (103). Moreover, a study by Nivens *et al.* has shown that bone marrow progenitor cells retrovirally overexpressing TDP1 are not sensitive to the cytotoxic effects of camptothecin (107).

All the previous findings were on normal cell lines (non-neoplastic), only one study by Perego *et al*., tried to identify the correlation between TDP1 level and the response to TOP I-poisons like gimatecan, topotecan and camptothecin and to TOP II-poisons like etoposide and doxorubicin. Ovarian carcinoma cell lines (IGROV-1) resistant to gimatecan were developed by continuous exposure to gradient concentrations of the drug showed an overexpression of TDP1 compared to parental cell lines. Nevertheless, knockdown of TDP1 using siRNAs or miRNAs in U2-osteosarcoma (U2-OS) cells did not enhance their sensitivity to gimatecan. Moreover, TDP1 overexpression

in the same type of cell line did not induce their resistance to many TOP I and TOP II-poisons.

In order to produce a completed DNA repair process this requires the removal of the phosphate produced by TDP1 and the recruitment of other DNA-repair pathway interplayers. Thus, in the study by Perego *et al*., manipulation of several DNA-repair gene levels in U2-OS was performed to investigate their effect on response to gimatecan. Co-silencing of TDP1 and PARP-1 or Rad17 (DDR checkpoint protein) was not associated with enhanced sensitivity compared to parental U2-OS. On the other hand, knocking-down both TDP1 and XRCC1or breast cancer type 1 susceptibility protein (BRCA1; component of the HR pathway) enhanced U2-OS sensitivity to gimatecan. This indicates the complexity of the response to the DNA-damaging therapy and that it is not solely dependent on one DNA-repair protein.

Since almost all the previous studies support the efficient role of TDP1 in the repair of the DNA damage induced by different anticancer agents, TDP1 represents a novel therapeutic target. Using specific inhibitors may potentiate/synergize the cytotoxicity of DNA-damaging antineoplastic therapy. The identification of TDP1 inhibitors is under investigation with the focus on small-molecule inhibitors that can trap the TDP1-DNA intermediate. There is also a current effort on finding molecules that can inhibit both TOP-I and TDP1 enzymes (108,109).

### 1.6.5. Clinical relevance of TDP1 in cancer

Analysis of the protein expression and enzyme activity in cancer has shown an overexpression of TDP1 in more than half of 30 non small-cell lung cancer samples (NSCLC) compared to 8 normal lung tissues and an increased enzymatic activity was evident in all tumor samples (110). Suggesting a negative prognostic value of TDP1 in NSCLC and that this overexpression could be responsible for the resistance of this tumor

to TOP I-poisons. Moreover, overexpression of TDP1 in colorectal cancer tissues compared to normal ones has been also reported (111,112).

## 1.7. Clinical relevance of TDP1 in glioma

TOP I-poisons are currently a promising second-line option given in combination with other anticancer agents in the treatment of glioma. The pivotal role of TDP1 in the repair of the DNA damage induced by TOP I-poisons encouraged us to study the clinical relevance of TDP1 in glioma. Moreover, the critical role of TDP1 in the repair of ROS-induced DNA SSBs and DSBs indicates that TDP1 could be of critical value in the brain tissue. Normally, the brain tissue encounters high levels of oxidative stress that needs efficient DNA repair mechanisms to maintain the genome integrity besides the fact that IR is the mainstay of therapy in gliomas; both lead to the accumulation of DNA strand breaks resulting from oxidation. It is also known that IR induces the formation of TOP I-DNA cleavage complexes, emphasizing the role of TDP1. This may be evidenced by the high expression levels of TDP1 in normal brain and by the neurodegeneration, cerebellar atrophy and mental retardation associated with SCAN1 disease. Moreover, among the DNA-repair processes, BER has been found to be the most vital in the brain. And since more than 70% of the DNA-lesions produced by TMZ rely on BER for reversal, there is also a possible role of TDP1 in modulating the response of brain cells to TMZ via the BER pathway.

## 1.8. Statement of the problem

Glioma is an aggressive type of CNS tumor with the property of dreadful prognosis has not changed since decades. Although the available WHO classification system is useful, it does not explain the large variability in clinical outcome and response to therapy among patients of the same histological group. Consequently, a more comprehensive and accurate approach is required. Molecular classification represents a vital strategy that aids in the identification of molecular signatures that provide more

accurate diagnosis, better prediction of survival and may represent novel therapeutic targets for personalization of medicine. Part of these signatures are the ones related to the DNA-repair pathways.

DNA-repair capacity is an important determinant of the response of cancer diseases to antineoplastic agents. It has a magnified role in glioma because the majority of the approved chemotherapeutic agents and the irradiation used in the treatment of glioma possess their anticancer properties via damaging the DNA. Tyrosyl DNA-phosphodiesterase I (TDP1) is one newly discovered DNA-repair enzyme that has shown relevance in affecting the response to various anticancer agents including but not limited to topoisomerase I poisons and topoisomerase II poisons, all of which are main second-line agents used in the treatment of glioma.

TDP1 repairs various types of DNA-lesions and plays an important role in the repair of DNA single-strand and double strand breaks produced by topoisomerase I-poisons and ionizing radiation. Moreover, TDP1 is a vital component of the base excision repair process that is important for the repair of about 70% of the DNA lesions produced by temozolomide. Although ionizing radiation and temozolomide are the mainstay therapies for glioma, there is a non-neglectable number of patients who are resistant to these therapies and the majority of the tumors recur after treatment. The identification of the causes of resistance is not yet well established. Additionally, the relevance of TDP1 in gliomagenesis and determining the response to anticancer therapy has not yet been studied.

To pave the way for molecular classification, several steps and confirmatory investigations are to be followed which can be referred as translational research. The first step is the screening for genetic aberrations and the build of gene expressions profiles from human tumor specimens. This made feasible by the evolvement of high-throughput screening assays such as microarray, array comparative genomic hybridization, fluorescent in situ hybridization, and SNP array. This must be followed by analytical tests

for correlations between significant alterations or differential gene expression levels and tumor aggressiveness and clinical outcome of patients. The second step is to characterize the underlying molecular mechanisms of the significant correlations identified. This is obtained from preclinical studies including *in vitro* and *in vivo* by utilizing cancer cell lines and mouse models with molecular modifications that are representative to tumor microenvironment. The third step is to translate the results of these preclinical investigations into clinical studies where patients can be stratified to therapy based on the tumor molecular profiles. Moreover, this can aid in the development of novel targeted agents. This is to be followed by intensive preclinical and clinical investigations for a new therapeutic agent to be applied in the clinic as part of the treatment regimen of patients with glioma.

## 1.9. Project goals and hypotheses

*Goal 1: To identify genetic alterations associated with DNA-repair genes and their relevance in glioma diagnosis, prognosis, and treatment prediction*. Due to the critical role of molecular classification in providing better understanding of cancer diseases, we wanted to analyze chromosomal instabilities such as copy number alterations (CNAs) and loss of heterozygosity (LOH) that are associated with DNA-repair genes including TDP1 and assess their differential frequency in glioma biopsies compared to control samples.

*Hypothesis: Glioma biopsies show various types of genetic alterations in major DNA-repair genes with differential frequency among glioma samples compared to controls and each of these alterations present in certain glioma subtypes and correlate with the clinical outcome of patients.* To address this hypothesis we performed a SNP array analysis to identify CNAs, LOH regions and single nucleotide polymorphisms (SNPs) in 60 glioma biopsies. Moreover, we performed several statistical analyses to identify their correlation with glioma diagnosis and the clinical outcome of patients.

*Goal 2: To analyze TDP1 expression at both mRNA and protein levels in glioma samples and correlate them with tumor aggressiveness and patient's survival.* The relevance of TDP1 in gliomagenesis and prognosis has not yet been studied. Thus, we will identify TDP1 relevance in glioma in terms of improving the diagnosis and predicting the clinical outcome of patients.

*Hypothesis: TDP1 is overexpressed in malignant glioma tissues compared to controls and that TDP1 level correlates with tumor aggressiveness (an increase in expression from grade II to grade IV) which thus is associated with poorer survival.* To address this hypothesis we analyzed TDP1 protein level by western blot in glioma biopsies and compared it to that in control samples. Furthermore, we quantified TDP1 mRNA expression level in glioma samples and performed survival analyses to identify its correlation with the survival of patients.

*Goal 3A: To study in vitro the effect of TDP1 level in determining the response of glioma cell lines to anticancer therapy.* Several studies suggested the role of TDP1 level in affecting the response to various anticancer agents mainly topoisomerase I and II poisons. Low or mutated TDP1 resulted in enhanced cytotoxicity of topotecan and etoposide while high TDP1 levels yielded opposite results. However, until now, there are no studies investigating the effect of TDP1 level on the cytotoxicity of antineoplastic agents toward malignant glioma cell lines.

*Hypothesis: Malignant glioma cell lines expressing high TDP1 levels are resistant to anticancer therapy compared to those expressing low TDP1 levels.* To address this hypothesis we utilized U87 and U251 malignant glioma cell lines. U87 and U251 cells express very low levels of TDP1, thus we overexpressed the full length TDP1 gene and we measured the viability of the cells overexpressing TDP1 after treatment with topotecan, etoposide, doxorubicin or temozolomide.

*Goal 3B: To assess the effect of TDP1 inhibitors to potentiate/synergize the cytotoxicity of topoisomerase-poisons.* Since TDP1 has been shown to play a role in the

repair of the DNA damage produced by topoisomerase I and II poisons, targeting TDP1 could be a promising strategy to enhance the cytotoxicity of topoisomerase-poisons via inhibiting the repair of the DNA damage produced by these agents.

*Hypothesis:* *A combinational therapy of topoisomerase-poisons and a TDP1 inhibitor is associated with a significantly higher cytotoxic effects compared to treatment with a topoisomerase-poisons alone.* To address our hypothesis, we utilized phosphotyrosine mimitics as TDP1 inhibitors that act as false substrates for the enzyme. We treated U87 cell lines with topotecan, etoposide or doxorubicin alone or in combination with a TDP1 inhibitor. Then we measured tumor-cell viability and performed comparison tests for the potentiation effect.

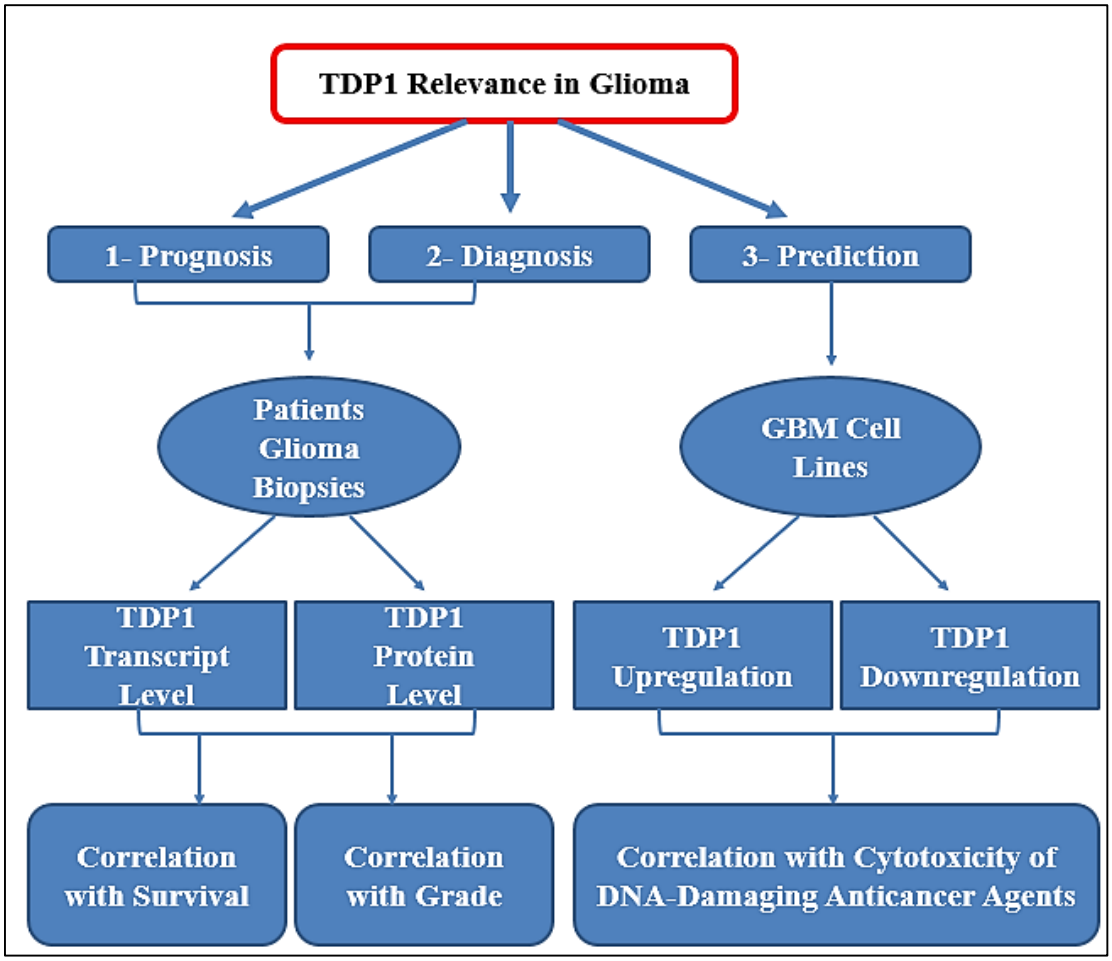


**Figure 1.12. Chart representation of the thesis objectives.** Tyrosyl DNA-phosphodiesterase 1 (TDP1) relevance in glioma includes the identification of its role 1- To predict the clinical outcome of patients (prognosis) by showing correlation between TDP1 level and the overall survival , 2- To assist in glioma diagnosis by showing differential expression levels among glioma subtypes, and 3- To predict the response of glioma patients to anticancer therapy (prediction) by showing correlation between TDP1 level and the cytotoxicity of DNA-damaging anticancer drugs.

# CHAPTER II

# TYROSYL DNA-PHOSPHODIESTEARASE I (TDP1): A PROMISING PROGNOSTIC FACTOR IN GLIOMA

## 2.1. Introduction

The poor prognosis of malignant glioma is an inevitable truth that has not changed since decades despite the advances in the diagnostic tools and the treatment strategies including surgery, radio- and chemotherapy. Practitioners rely on the world health organization (WHO) classification to predict the patients' outcomes. This classifying system is pathologically related to the respective cell type of origin and divides gliomas into astrocytomas, oligodendrogliomas and mixed oligoastrocytomas. Further grading into WHO grades I-IV is based on the aggressiveness of the tumor in terms of mitosis, necrosis and microvascular proliferation. Although the WHO classification is considered the only clinically applied strategy for classifying patients into diagnostic and prognostic groups and for the stratification to therapy, it is inadequate to explain the variability in the clinical outcome between patients in the same pathological group. Even with the application of other clinical prognostic factors such as age and Karnofsky Performance Status (KPS), the prognostic variability can be partially explained. Multivariate Cox proportional hazards modeling performed by Vitucci *et al.* revealed that a proportion of 20—30% of prognostic variability is not explained by the currently adapted clinicopathological prognostic factors {WHO grade, patient's age, KPS, and 1p/19q co-deletion (mainly for oligodendrogliomas)} (113). Therefore, the molecular classification and the identification of genetic signatures represent an important strategy for the subclassification of gliomas. By shading the light on molecular biomarkers and critical cellular pathways that play a role in the tumor growth and progression, part of the prognostic variability can be explained. There are two types of molecular markers; 1- prognostic, they predict the survival of the patient regardless of treatment, and 2- predictive, they predict the response of the patient to a certain therapy.

As a consequence, molecular classification advantages are: 1- dividing patients into more homogeneous diagnostic groups, 2- providing prognostic information, 3- identification of new therapeutic targets and 4- the stratification of patients to treatment and individualization of therapy. The latter two are considered the most critical and play a vital role in enhancing the response of gliomas to the currently applied anticancer treatments, and the development of novel chemotherapeutic agents that can be used alone or in combination with other chemical drugs. The advantageous role of molecular classification in terms of targeted therapy is evident in different types of cancers. For example, in lung adenocarcinoma, epidermal growth factor receptor (EGFR) mutation predicted better response to erlotinib (EGFR inhibitor), and proto-oncogene B-Raf (BRAF) mutation predicted better response to vemurafenib (BRAF inhibitor) (114).

In an attempt to address the unequivocal need for more informative categorization of glioma, various studies have been conducted with some promising results regarding prognostication but not prediction. All of the up to date available molecular markers identified in glioma have a prognostic value with controversial results regarding their predictive benefits. Abnormalities associated with EGFR (amplification or EGFRvIII) are commonly seen in high-grade astrocytomas but rarely in oligodendrogliomas (6,115); however targeted therapy toward EGFR failed to show clinical efficacy and did not correlate with EGFR status (116). 1p/19q co-deletion is a common feature of oligodendrogliomas and has been associated with increased survival and enhanced response to the PCV regimen (procarbazine, lomustine and vincristine) (2,5). In a survey study by Abrey *et al*., about half of the practitioners recommend chemotherapy as the first-line option rather than ionizing radiation (IR) for anaplastic oligodendrogliomas (AODs) harboring the co-deletion (117). O6-methylguanine methyltransferase (MGMT) methylation has been found to act as a positive prognostic factor in gliomas and was associated in some studies with enhanced response to temozolomide (TMZ) and IR (19). Nonetheless there are no definitive answers regarding stratifying patients to PCV or TMZ

based on the 1p/19q co-deletion or MGMT methylation statuses. This enlarged the circle of molecular classification from being based on a single gene to be dependent on multiple genetic signatures. Moreover, clinical targeting of a single gene in glioma was associated with negative results, explained by the heterogeneic nature of this aggressive tumor and the complexity and redundancy of the cellular pathways. This indicates the necessity of a comprehensive examination of multiple genes rather than relying on a single gene signature.

An initial step in understanding glioma is the clarification of the causative or risk factors. However, there is little known about the etiology of glioma with exposure to large doses of IR or ultraviolet light are the only established risk factors and they account only for limited number of cases. An assumptive fact is that cancer is a genetic disease and that the accumulation of genetic aberrations results in tumor formation and its malignant transformation. The cancer cells can acquire one of two major types of genetic alterations/imbalances including: 1- numerical abnormalities (aneuploidy; complete loss or gain of a chromosome), and/or 2- structural abnormalities (insertion, deletion, translocation, or point mutation). Examples are loss of heterozygosity (LOH) and copy number alterations (CNAs; deletions or amplifications). LOH is a common phenomenon in glioma and is mainly related to tumor-suppressor genes (TSGs). According to Knudson's two-hit model the first hit is a point mutation resulting in loss of one allele and the second hit is usually a deletion resulting in gene silencing. In opposite to hereditary cancers where the first hit is inherited, in glioma both mutations are acquired (118).

Single nucleotide polymorphisms (SNPs) are normal variations in a single base of the DNA. They are the most common type of DNA variation and their correlation with glioma risk has been extensively studied (119-121). CNAs are abnormal gains or losses of DNA regions larger than 1 kilobases (1 kb). This type of genetic aberration is believed to play a major role in tumorigenesis and transformation. Various CNAs have been

identified in glioma such as amplifications in EGFR, alpha-type platelet-derived growth factor receptor (PDGFRA), cyclin-dependent kinase 4 (CDK4), mouse double minute 2 homolog (MDM2), and MDM2-like p53-binding protein (MDM4) or deletions in cyclin-dependent kinase inhibitor 2A (CDKN2A), phosphatase and tensin homolog (PTEN) and neurofibromin 1 (NF1) (122,123).

Conclusively, cytogenetic analysis techniques are of critical value in providing large genomic datasets by applying high-throughput screening in order to be able to comprehensively study the tumor samples and thus provide information that can aid in the understanding of the etiology of glioma besides the identification of novel molecular therapeutic targets. Such techniques include but not limited to fluorescent in situ hybridization (FISH), array comparative genomic hybridization (aCGH) and the most important is the microarray analysis.

An example of a study that utilized several high-throughput screening assays and helped in the better understanding of glioma is The Cancer Genome Atlas (TCGA). It can be considered the biggest ongoing project to characterize the genomic changes that accompany cancer thus can enhance the ability to diagnose and treat patients. Through this project four high-grade glioma subtypes have been identified: proneural, mesenchymal, neural and classic (124-126). Each subtype revealed differential expression of certain genes and the first two subtypes have been associated with survival. The proneural subtype was associated with better survival compared to the mesenchymal one and the latter two (neural and classic) did not show impact on survival but they seem to be important for treatment selection (126).

Besides gene expression profiling and the identification of genetic aberration, analyzing protein levels represents another important strategy to identify molecular markers. Although genomic analysis provides informative data regarding the molecular changes associated with cancer, but it cannot predict protein concentration or activity. Since proteins are the final products of genes, different factors beyond the transcriptomic

level modulation or genetic alterations are responsible for the tumor growth, progression and response to therapy. Examples of small-scale methods that are used for protein quantification are immunohistochemistry and western blot, both of which provide rapid and accurate results. An evidence of the importance of protein expression analysis is the findings regarding MGMT prognostic and predictive value in glioma. Although MGMT promoter methylation is associated with gene silencing, a concordance between this epigenetic silencing of the gene and MGMT protein level could not be proved (127-129). This hindered the MGMT applicability as a predictive biomarker for TMZ therapy in glioma.

Despite the critical role of DNA-repair genes in the tumorigenesis and progression of tumors and their response to therapy, there was a limited focus on investigating their differential expression or the presence of certain genetic alterations in gliomas (121). Most of the studies have focused on studying mainly tumor suppressor genes and oncogenes due to their direct relationship with gliomagenesis and proliferation (125,130). DNA-repair processes constitute inevitable mechanisms of resistance, especially due to the fact that almost all of the approved anticancer agents used in the treatment of glioma possess their cytotoxic properties by damaging the DNA. Thus, through genome-wide analysis, the multiple DNA-repair pathways and their respective genes that are responsible for drug resistance can be identified.

Several studies tried to explore the correlation between genetic alterations associated with DNA-repair genes and the risk of glioma especially aided by the availability of the large genomic datasets. For example, there was an attempt to investigate the role of SNPs as potential risk factors for glioma. Various studies focused on SNPs associated with DNA-repair genes such as MGMT and those involved in nonhomologous end joining (NHEJ), nucleotide excision repair (NER), and base excision repair (BER) (121,131,132). An interesting finding among many studies is the strong correlation between certain SNPs in BER genes and glioma risk, supporting the

importance of this DNA-repair pathway in the brain tissue (121,131,133). However, being one of the main focuses of one project, investigating all CNAs, SNPs and LOH associated with DNA-repair genes was not, up to our knowledge, studied before.

Moreover, gene expression profiling via microarray technique has been employed in different studies to identify the contribution of the DNA-repair genes in the resistance of glioma cell lines to various anticancer agents. A study by Otomo *et al.* analyzed the differential expression of 638 genes including both cancer-related and housekeeping genes in U87 compared to the more radiosensitive A172 glioblastoma multiforme (GBM) cell line post-radiotherapy (134). Of the commonly upregulated genes, those implied in DNA-repair such as topoisomerase IIα (TOP-IIα), x-ray repair cross complementing 5 (XRCC5; codes for Ku80 which plays a role in NHEJ), and Rad52 (plays a role in HR) revealed the highest fold differences (higher expression in U87 cell line) (134). Another study by Morandi *et al*., U87 cell lines resistant to camptothecin were developed in order to identify genes implicated in the resistance process (135). Among the 1403 genes probed, 6 DNA-repair genes were expressed differentially after the development of camptothecin resistance. Those genes were XRCC5 (NHEJ gene), Rad50 (NHEJ gene), excision repair cross-complementing rodent repair deficiency complementation group 1 (ERCC1; NER gene), poly ADP-ribose polymerase 2 (PARP2; BER gene) and topoisomerase I (TOP-I) (135).

In an attempt to explore chromosomal abnormalities associated with gliomas (including genes involved in several DNA-repair pathways) and their value in providing information that may assist in accurate diagnosis, prognosis and prediction of therapy choice and response, we have performed microarray analysis for 60 glioma samples using SNP Mapping 6.0 array (123). This technique allows genotyping of 1.8 million genetic markers including more than 906,600 SNPs and more than 946,000 probes for CNAs detection.

Furthermore, we investigated a clinically relevant DNA-repair gene that has not yet been studied in glioma which is tyrosyl DNA-phosphodiesterase 1 (TDP1). Despite its high expression level in normal brain tissue and the previous preclinical studies that support targeting the enzyme product of this gene besides the pivotal role of BER process in the brain and that TDP1 is a key player in this pathway, there are no studies investigating TDP1 role in glioma. Knowledge of the clinical relevance of TDP1 in glioma may help providing molecular data for better classification of glioma besides addressing some of the resistance issues due to DNA repair. Thus, adding to the portfolio of the prognostic/predictive biomarkers of this aggressive tumor.

The goals of this chapter were: 1- to identify the genetic alterations in major DNA-repair genes including TDP1, and 2- to analyze TDP1 expression at both mRNA and protein levels in glioma samples and correlate them with tumor aggressiveness and patient's survival. We performed DNA microarray analysis for sixty glioma biopsies and we analyzed the TDP1 gene and protein expression levels in 43 samples and compared the results to controls.

This work hypothesizes that genetic alterations are present in the probed DNA-repair genes and are significantly different in frequency between glioma samples and controls. This will aid in identifying new molecular biomarkers in glioma. The work also hypothesizes that TDP1 is overexpressed in malignant glioma tissues compared to controls and that TDP1 level correlates with tumor aggressiveness (an increase in expression from grade II to grade IV) which thus is associated with poorer clinical outcome.

## 2.2. Materials and methods

### 2.2.1. Study population

Glioma and non-neoplastic brain tissue specimens were harvested at the University of Iowa Department Of Neurosurgery according to institutional review board

(IRB) regulations and guidelines (IRB#200707727). Biopsies were snapped frozen within 1 to 2 hours following surgery. Patients had not received chemotherapy or radiotherapy prior to surgery. After surgery, 72% of patients received IR, 50% received TMZ. Alternative therapies including bevacizumab, PCV, procarbazine, lomustine (CCNU), vincristine, thiotepa, cisplatin, carboplatin and etoposide were also used in minority of the patients.

**Table 2.1. Study Population Demographics & clinicopathological properties.**

| **Total Number of Patients** | **60** |
|---|---|
| Age, years | |
| Median | 51 |
| Range | 18 – 79 |
| ≤ 51 (%) | 52 |
| > 51 (%) | 48 |
| Sex, No. | |
| Female | 31 |
| Male | 29 |
| Ethnicity, No. | |
| Caucasian | 55 |
| African American | 1 |
| Unknown | 4 |
| Survival, months | |
| Median | 31 |
| Range | 1 – 180 |
| WHO Histological Type & Grade, No. | |
| 1- Astrocytoma | |
| • Diffuse Astrocytoma (ACG; grade II) | 5 |
| • Anaplastic Astrocytoma (AAC; grade III) | 13 |
| • Glioblastoma Multiforme (GBM; grade IV) | 29 |
| 2- Oligodendroglioma | |
| • Oligodendroglioma (ODG; grade II) | 6 |
| • Anaplastic Oligodendroglioma (AOD; grade III) | 7 |

Histology, diagnosis, pathology, predictive clinical, fluorescence *in situ* hybridization (FISH), and outcome data were recorded. About half of the patients experienced tumor progression and recurrence. Analyses were performed for a total of 60 glioma biopsies (**Table 2.1**) which included 11 low-grade {5 diffuse astrocytomas (ACGs) & 6 oligodendrogliomas (ODGs); WHO grade II} and 49 high-grade (malignant) gliomas {13 anaplastic astrocytomas (AACs) & 7 anaplastic oligodendrogliomas (AODs); WHO grade III & 29 GBM; WHO grade IV}. Almost all of the patients were Caucasians with a male to female ratio of 1:1 and a median age at time of diagnosis of 51 years. For CNA confirmations, 5 ml blood samples for DNA extraction were collected for 24 patients and used as controls. Of the 60, a total of 43 biopsies (4 ACG, 3 ODG, 8 AAC, 7 AOD & 21 GBM) were analyzed for TDP1 mRNA and protein expression levels. 4 non-neoplastic brain tissues obtained from patients with gliosis were used as controls.

### 2.2.2. DNA extraction

Genomic DNA was isolated from the samples with DNAeasy genomic DNA purification kit (Qiagen, Germany). Briefly, about 25 mg of the tissues was cut, crushed and then lysed with proteinase K (for protein breakdown), ATL buffer (for tissue lysis) followed by AL buffer (for cells lysis). Ethanol was added to precipitate DNA. The previous mixture was then pipetted into DNeasy mini spin columns and centrifuged at 8000 rpm (≥6000 g). The DNA was bound to the DNeasy membrane while the contaminants passed through and accumulated in the collection tube. The DNA was then washed with two types of buffers, AW1 and AW2 both of which acted to remove any non-specific inhibitors and salts bound to the DNeasy filter. Finally, the DNA was eluted using the AE buffer. Integrity and quality of the extracted DNA was assessed by gel electrophoresis on a 1% agarose gel in 1x TBE buffer prior to use. The DNA bands were stained with ethidium bromide which fluoresces when intercalates with DNA and the fluorescence was visualized via UV-light. The DNA was quantified using Nanodrop 2000

spectrophotometer (ThermoScientific, Huntsville, AL) in ng/µl units. This technique measures absorbance at 260 nm and a conversion factor of 50 per one unit of absorbance is used and represents an estimated concentration of 50 µg/µl.

### 2.2.3. High-throughput SNP array screening

Genomic DNA from the 60 selected human gliomas and the control samples were Genotyped by the Affymetrix GeneChipR Human SNP Mapping 6.0 array according to the manufacturer's protocol (Affymetrix, Santa Clara, CA). This microarray allows genotyping of >1.8 million genetic markers including > 906,600 SNPs and > 946,000 probes for the detection of copy number variation with median inter-marker distance of around 1 kb.

The Genome-Wide Human SNP *Nsp/Sty* Assay Kit 6.0 was used to amplify the digested DNA fragments. Briefly, 250 ng of DNA was digested using either *Sty*I or *Nsp*I (New England Biolabs, Ipswich, MA). The cohesive four-base pairs overhangs of the DNA fragments formed were ligated to *Sty*I or *Nsp*I adaptors using T4 DNA ligase (New England Biolabs) regardless of the fragment size. This was used as a template in PCR amplification using Titanium *Taq* (Clontech, Mountainview, CA) and a single primer complementary to the adaptor sequence. After purification from excess primers and salts with column filtration, PCR products were fragmented using DNase I and a sample of the fragmented product was visualized in a 4% agarose gel to confirm that the average fragment size was about 200 bp. Subsequently and before hybridizing each sample to the Human Genome wide 6.0 SNP arrays, the DNA fragments were end-labeled with biotin using terminal deoxynucleotidyl transferase.

The Human Genome wide 6.0 SNP arrays were hybridized for 16 h at 50°C. Then, the arrays were washed and stained using Affymetrix Fluidics Station 450 and R-phycoerythrin (Life technologies, Carlsbad, CA) respectively. Raw microarray data were produced using a GCS3000 high-resolution scanner. The Affymetrix Gene Chip

Operating Software (GCOS v1.4) was used to process and analyze the raw fluorescence data.

### 2.2.4. Data analysis

Partek Genomic Suite (PartekGS) software (Partek, St. Louis, MO) and Nexus (BD Biosystems, El Segundo, CA) were used for the analysis and the determination of copy number abnormalities and for the identification of LOH status. For tumor samples without a corresponding blood DNA, 270 normal HapMap samples (www.HapMap.org) for PartekGS and NCBI built 36.1 for Nexus were used as a reference to create baseline files for DNA copy number estimates.

Data were normalized using a Hidden Markov Model (compares to unpaired samples). Deletions and amplifications were detected by genomic segmentation algorithm of the PartekGS. Comparisons were generated using blood samples (24 samples) for baseline and pairwise comparisons between paired blood and tumor samples.

### 2.2.5. RT-PCR and real-time q-PCR

RNA was isolated from frozen glioma biopsies using TRIzol (Life Technologies, Carsland, CA). The samples were lysed using 1 ml trizol, followed by pipetting up and down several times for complete homogenization. 200 µl chloroform was then added followed by centrifugation at 12,000 g for 15 min at 4$^{o}$C for phase separation (upper aqueous phase carrying RNA, middle phase carrying the DNA, and a lower organic phase carrying the proteins). 400 µl of the aqueous phase was pipetted in another tube and mixed with 500 µl isopropanol, followed by incubation for 10 min at room temperature and then centrifugation at 12,000 g for 10 min at 4$^{o}$C for precipitation of the RNA. The RNA pellet was washed with 200 µl 70% ethanol and then centrifuged at 7500 g for 5 min at 4$^{o}$C. The liquid was pipetted out and then the RNA pellet allowed to dry at room temperature for 2-3 min. RNA integrity and quality was assessed on a 1% agarose gel, and quantified by spectrometry at 260 nm/280 nm and Nanodrop method (Thermo

Scientific). The RNA was quantified using Nanodrop 2000 spectrophotometer (ThermoScientific, Huntsville, AL) in ng/µl units. This technique measures absorbance at 260 nm and a conversion factor of 40 per one unit of absorbance is used and represents an estimated concentration of 40 µg/µl.

For cDNA synthesis, oligo(dT)-primed reverse transcriptase of 5 µg total RNA was done using SuperScript III reverse transcriptase (Invitrogen, Carsland, CA). 5 µg of each RNA was mixed with 1 µl of 50 µM OligodT. The mixtures were heated at 65$^{o}$C for 7 min followed by an incubation on ice for 2 min. MasterMix consisting of the following components was then added to each tube: 4 µl 5x first-strand buffer, 1 µl 0.1 DTT, 2 µl of 10 mM dNTP mix (10 mM each dATP, dGTP, dCTP, dTTP in DNase/RNase ultrapure water) and 0.25 µl Superscript III enzyme. The volume was completed to a total of 20 µl with DNase/RNase ultrapure water. The mixture was then incubated at 50$^{o}$C for 90 min followed by an inactivation step at 70$^{o}$C for 15 min. The quality of the resulted cDNA was assessed in 1% agarose gel.

For RT-PCR or real-time PCR purposes, the resulting single stranded cDNA was amplified at the appropriate number of cycles. The primers used are listed in **Table 2.2**. GAPDH was used as the housekeeping gene for data normalization. RT-PCR products were visualized in 2% agarose gel. Quantitative real-time PCRs were performed on an ABI StepOne machine (Applied Biosystems, Foster City, CA).

### 2.2.6. TDP1 sequencing

Genotyping for TDP1 cDNA, promoter, and the splice variants was performed. The amplification of the full length TDP1 was performed using Expand Long Template PCR system (Roche Diagnostics, Indianapolis, IN). The primers used for the amplification covers the region from 5′ and 3′ UTRs (**Table 2.3**) and were designed using Primer3 program. High-throughput sequencing of PCR-amplified gene products was performed using 8 internal sequencing primers covering different regions of TDP1 gene

(**Table 2.3**), at the University of Iowa DNA Core Facility using a BigDye Terminator v3.1 Cycle sequencing kit (Applied Biosystems, Foster City, CA) and ABI 3730x1 DNA analyzer (Applied Biosystems, Foster City, CA). DNA Baser v2 programs was used to align and assemble the sequences.

**Table 2.2. Primers used in reverse-transcription PCR and real-time PCR reactions**

| Gene | Gene Symbol | Refseq accession number | Forward Primer | Reverse Primer | Amplicon size (bp) |
|---|---|---|---|---|---|
| Tyrosyl-DNA Phosphodiesterase 1 | TDP1 | NM_018319 & NM_001008744 | 5'-ACATCTCTGCTCCCAATGAC-3' | 5'-CTCTCCAGATTTGTGGGTTC-3' | 599 |
| O6-Methylguanine Methyltransferase | MGMT | NM_002412 | 5'-CCTGGCTGAATGCCTATTTC-3' | 5'-GATGAGGATGGGGACAGGATT-3' | 239 |
| Epidermal Growth Factor Receptor | EGFR | NM_005228 | 5'-CCACCAAATTAGCCTGGACA-3' | 5'-CGCGACCCTTAGGTATTCTG-3' | 118 |
| EGFR variant 3 | EGFRvIII | NM_201283 | 5'-GAGCTCTTCGGGGAGCAG-3' | 5'-GTGATCTGTCACCACATAATTACCTTTCT-3' | 131 |
| Glyceraldehyde 3-Phosphate Dehydrogenase | GAPDH | NM_002046 & NM_001256799 | 5'-ACCACAGTCCATGCCATCAC-3' | 5'-TCCACCACCCTGTTGCTGTA-3' | 452 |

**Table 2.3. List of primers used in human Tyrosyl DNA-Phosphodiesterase 1 (hTDP1) full length cDNA amplification and sequencing**

| | Forward Primer | Reverse Primer |
|---|---|---|
| **hTDP1 Amplification Primers Amplicon size = 3036** | hTDP1 37: 5'-ATCCGAGGCAAGCGTTGGTTC-3' | hTDP1 3072: 5'-AGAGGAGAAGCCTCGTAAAGC-3' |
| **hTDP1 Sequencing Primers** | **Forward Primer** | **Reverse Primer** |
| hTDP1 643 | 5'-ACATCTCTGCTCCCAATGAC-3' | 5'-GTCATTGGGAGCAGAGATGT-3' |
| hTDP1 1222 | 5'-GAACCCACAAATCTGGAGAG-3' | 5'-CTCTCCAGATTTGTGGGTTC-3' |
| hTDP1 1866 | 5'-GGAGCATTGGAGAAGAATGG-3' | 5'-CCATTCTTCTCCAATGCTCC-3' |
| hTDP1 2407 | 5'-AATGCTTGGGACCAGAAGTG-3' | 5'-CACTTCTGGTCCCAAGCATT-3' |

### 2.2.7. Western blot analysis

Total proteins were extracted from 20 frozen glioma biopsies aliquots and 4 non-neoplastic brain tissues control. The protein concentrations were measured using Bradford protein assay (Bio-Rad, Hercules, CA). This technique is a colorimetric assay that is based on the conversion in color of the Coomassie blue G-250 dye in Bradford reagent from red-brown before reacting with proteins (acidic condition) to blue color after reacting with protein, thus the absorbance maximum is shifted. The experiment was performed as following, Bradford reagent was diluted 5x with DNase/RNase ultrapure water. 100 µl of the diluted Bradford was mixed with 1 or 2 µl of the protein homogenate. Bovine Serum Albumin (BSA) was used as the standard at concentrations 1,

2, 5, 10, and 20 µg/µl. The absorbance measurements were determined at 595 nm using Spectramax Plus384 spectrophotometer (Molecular Devices, San Francisco, CA, USA).

An aliquot corresponding to 25 µg from each of the human tissue extracts were loaded onto 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). The gel electrophoresis was run overnight at 8 mA current. The proteins were then transferred to a 0.45 µM nitrocellulose membrane (Bio-Rad, Hercules, CA) at 300 mA current for 8 hours. Successful protein transfer was checked with Ponceau S stain (Fisher Scientific, Pittsburgh, PA). The membrane was blocked with 10% fat-free milk overnight at 4$^{o}$C. Then it was incubated with a 1:200-fold dilution in TBS-T buffer of polyclonal rabbit antibody against human TDP1 (H-300, Santa Cruz Biotechnology, Santa Cruz, CA) at room temperature for 3 hours. The housekeeping gene human GAPDH was used as the loading control.

The membrane was incubated with 1:50,000-fold dilution in TBS-T buffer of monoclonal mouse antibody against GAPDH (Abcam, Inc., Cambridge, MA) for 2 hours at room temperature. After the incubation with primary antibodies, the blots were incubated with secondary antibodies (anti-rabbit IgG for TDP1 or anti-mouse IgG for GAPDH) at 1:5000-fold dilution in 10% fat-free milk for 90 min. at room temperature. The blots were visualized using peroxidase substrate system (ECL Western blotting detection reagents, Amersham Biosciences, Salt Lake City, UT). Image Studio Lite software (LI-COR biosciences, Lincoln, NE) was used to quantify TDP1 and GAPDH protein expression levels.

### 2.2.8. Statistical analysis

Survival analysis was performed using Kaplan-Meier method and the differences between survival curves were assessed with log-rank test. Pearson and Spearman correlation tests were used for correlation analyses. Significant correlation between genetic alteration and different diagnoses was tested using Chi-square test. Two-tailed

student's t-test with unequal variances was used for the analysis of protein quantification and data normalization was performed in excel. Likelihood-ratio test was used for comparing the two survival groups of glioma patients. Statistical significance was considered as $p<0.05$. Statistical analyses were performed using PartekGS software (Partek, St. Louis, MO), R-project and SAS 9.3.

## 2.3. Results

### 2.3.1. Clinical data

The study population consisted of 5 ACGs, 13 AACs, 29 GBMs, 6 ODGs and 7 AODs. To identify clinical factors that correlated with the final outcome of the patients, Kaplan-Meier survival analysis was conducted. The patients' clinical outcome correlated significantly with the WHO diagnosis and grade (p = 0.0376; Log-rank test, **Figure 2.1.A**) with the median survival times for patients with ACG, AAC, GBM, ODG and AOD were 180, 84, 18, 71, and 29 months respectively.

Moreover, the survival of patients also correlated with their age (cut-off value= 51 years; p = 0.0016, Log-rank test, **Figure 2.1.B**). The median survival for patients ≤51 years was about 5.5 times higher than that for patients >51 years (71 months vs. 13 months).

### 2.3.2. DNA extraction

Several factors affect the quality of the DNA extracted from patients' biopsies. Examples include: the pathological condition of the tumor sample, specimen type, preexcision hypoxia, storage conditions, preservation method, and multiple freezing and thawing of the specimens. For instance, tissue necrosis is associated with damage and degradation of the DNA thus hindering the usefulness of the DNA extracted from these tumor samples. Thus, we needed to extract the DNA from hundreds of biopsies in order to obtain the 60 DNA samples of good quality and quantity to be used for further

analyses in this project. A good quality DNA is represented by a single band at the top of the agarose gel (**Figure 2.2.A, B; thick arrows & C**) which corresponds to about 3200 mega basepairs. DNA degradation occurs due to the mentioned factors and the contamination of the samples with DNase enzyme. A degraded DNA appears as a smear which represents DNA fragmentation into 100-200 basepairs (**Figure 2.2.A & B**; thin arrows), or as a single band at the lower part of the gel which represents a full degradation of the DNA (accumulation of the 150 bp DNA segments, **Figure 2.2.A & B**; dashed arrows).

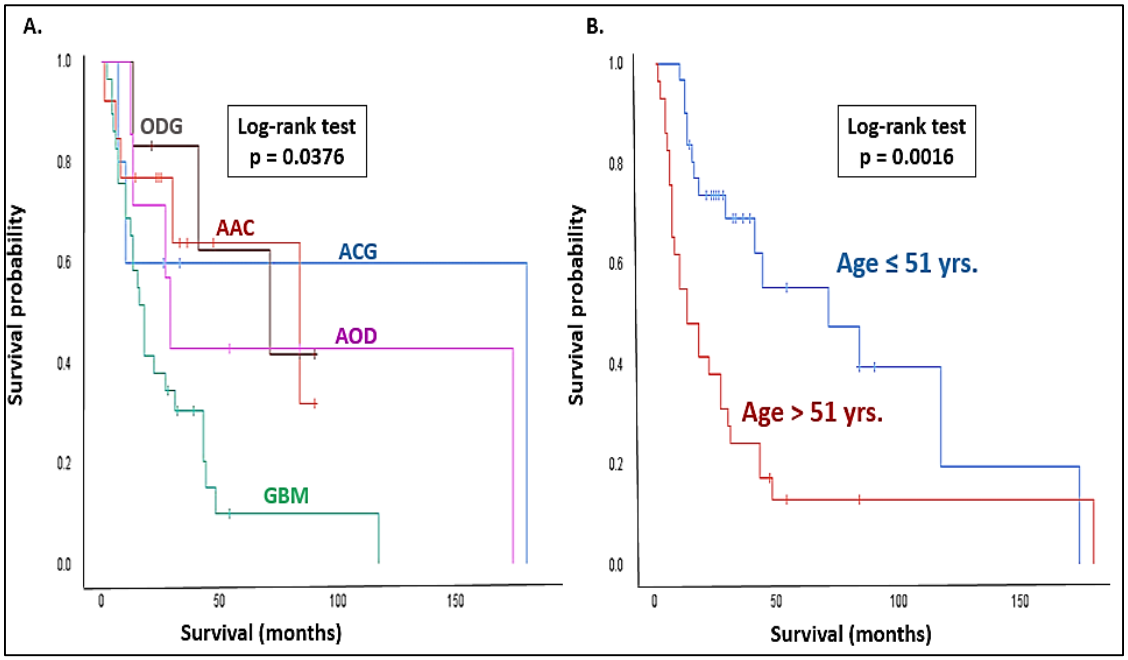


**Figure 2.1. Kaplan-Meier estimates of probability of survival stratified according to diagnosis or age. (A),** WHO diagnosis and grade {1=ACG (diffuse astrocytoma, grade II), 2=AAC (anaplastic astrocytoma, grade III), 3=GBM (glioblastoma multiforme, grade IV), 4=ODG (oligodendroglioma, grade II), & 5=AOD (anaplastic oligodendroglioma, grade III)}. (**B**), Age (cut-off value = 51 years). Log-rank test was used to measure significant difference between curves.

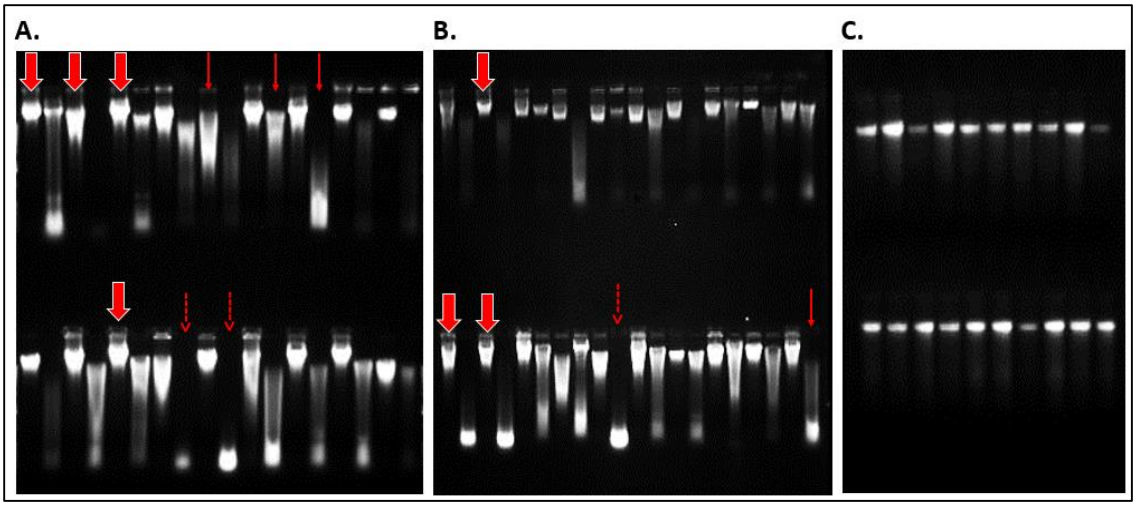


**Figure 2.2. Example of three sets of DNA samples extracted from glioma biopsies.** DNA was extracted from glioma biopsies using Qiagen DNeasy kit. Quality of the DNA was detected in 1% agarose gel. (**A**) & (**B**) represent a mixture of good quality DNA which appears as a single band at the top (thick arrows), and degraded DNA which appears as a smear (thin arrows) or as a single band at the bottom (dashed arrows). (**C**) DNA extracted from all samples was of good quality (single band at the top).

### 2.3.3. RNA extraction

Tumor specimen collection, handling, processing and storage conditions significantly affect the quality of the RNA extracted from glioma biopsies. Thus the isolation of RNA from several hundreds of samples was needed in order to obtain the 35 RNA samples with a good quality and enough quantity for further experiments. In an intact RNA, the two ribosomal RNA (rRNA) bands 18S (lower 2kb band) and a twice more intense 28S (upper 5kb band) appear on the agarose gel. Partial degradation of RNA appears as a smeared band, less intense 18S and 28S bands, more intense 18S band, or equally intense18S and 28S bands. Completely degraded RNA appears as a smear of very low molecular weight. **Figure 2.3.A** shows a subset of RNA samples that have been

extracted from patients' biopsies and it reveals a mixture of intact (e.g. lane 8), partially degraded (e.g. lane 11), and completely degraded (e.g. lane 2) RNA. RNA quality determination and quantification was also performed using Nanodrop 2000 spectrophotometer (ThermoScientific, Huntsville, AL). This technique measures the absorbance at 230, 260, and 280 nm and provides A260/A280 and A260/A230 ratios. For a good quality and non-contaminated RNA, the ratios fall in the range of 1.8-2.0. A ladder consisting of RNA fragments which are evenly spaced, is used for size estimation of the single-stranded RNA (**Figure 2.3.B**). For a high quality RNA, the typical profile is shown in **Figure 2.3.C,** where there are two high peaks for 28S rRNA and 18S rRNA. However, the quality of the RNA can be limited by the previously mentioned factors resulting in partial or complete degradation of the RNA (**Figure 2.3.D & E**, respectively).

### 2.3.4. Identifying copy number alterations (CNAs) in several DNA-repair genes

To identify CNAs occurring during gliomagenesis, microarray analysis of the DNA extracted from glioma samples was performed. Due to the fact of genetic heterogeneity of glioma, high-throughput screening provides wide genetic dataset that can assist in the diagnosis and the identification of molecular markers that are important for treatment selection and thus individualization of therapy.

Since only 24 of the glioma samples, blood DNAs were available, HapMap samples and NCBI built 36.1 data were used as controls. The validity of the latter two as controls was confirmed by comparison analyses that revealed similar sensitivity compared to the pairwise comparison performed for the 24 glioma samples with corresponding blood DNA. Due to probing a huge number of markers or genes, the possibility of false-positive results cannot be denied. To resolve this issue, multiple algorithms for statistical analyses (Partek and Nexus) were used. Stringency of the

analyses was determined if well-known CNAs that have been detected using FISH, were also identified using these algorithms.

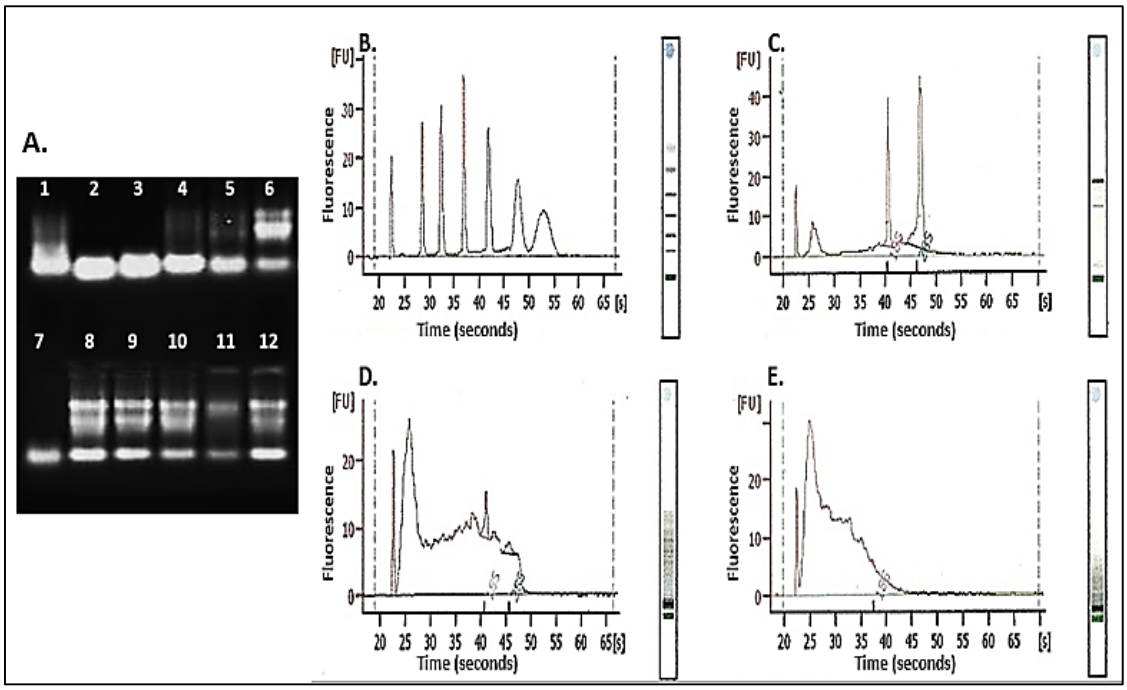


**Figure 2.3. RNA quality and quantity detected and measured by** (**A**) gel electrophoresis and (**B-E**) Nanodrop method. (**A**) 2 µl of RNA was loaded in 1% agarose gel. Lanes 6, 8, 9, 10 represent an intact RNA, 5 & 12 represent partially degraded RNA; and 1-4, 7 & 11 represent completely degraded RNA. (**B**) RNA 6000 Nano Ladder, contains 6 RNA fragments with sizes range from 0.2 to 6 kb (0.2 kb, 0.5 kb, 1.0 kb, 2.0 kb, 4.0 kb, and 6.0 kb) at a concentration of 150 ng/µl. (**C**) good RNA, two high peaks for 18S and 28S ribosomal RNA. (**D**) RNA is partially degraded. (**E**) RNA is completely degraded.

The analyses revealed >4000 CNAs ($p < 0.0001$, ANOVA test) involving several genes of the entire genome (**Figure 2.4**). They included focal CNAs ranging in size from

100 bp to large chunk of chromosomes. The average frequency of the identified CNAs was higher in high-grade gliomas (AAC, AOD & GBM). These CNAs included complete deletions of the short arm of chromosome (Chr.) 1p and/or the long arm of Chr. 19, or partial loss of the short arm of Chr. 9 (9p11-9pter). Also a complete gain of Chr. 7 and a partial gain of the long arm of Chr. 9 (9q21-9qter) were also identified. Complete or partial loss or gain of chromosomes 10, 13, 14, and 22 were also evident.

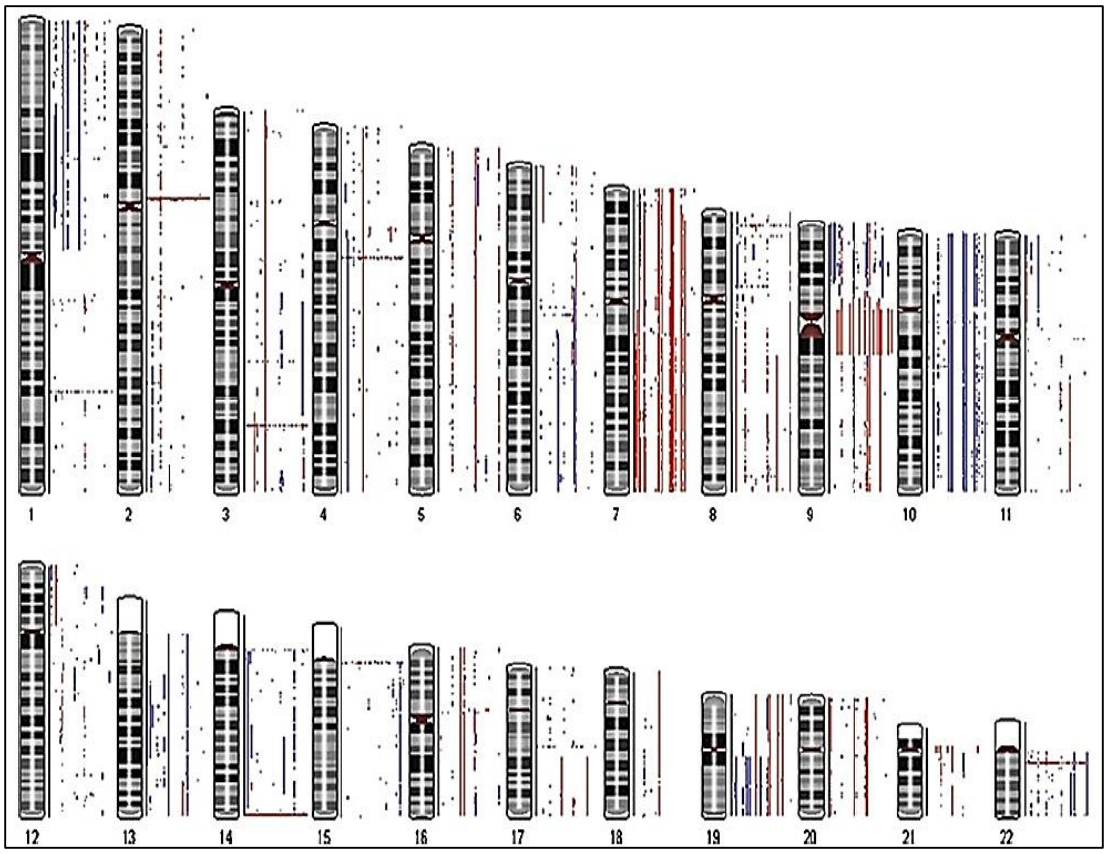


**Figure 2.4. Graphical representation of major chromosomal abnormalities detected in the glioma study population ($p < 0.0001$)**. Blue color represent copy-number losses and red color represent copy-number gains identified by SNP array.

Chi-square statistical analysis to identify the correlation between the presence of certain CNAs of the major CNAs found and the glioma diagnosis was performed. It revealed that certain CNAs in chromosomes 1, 7, 9, 10, 19 & 20 present in some glioma types but not the others and also revealed differential frequency among them (**Figure 2.5**).

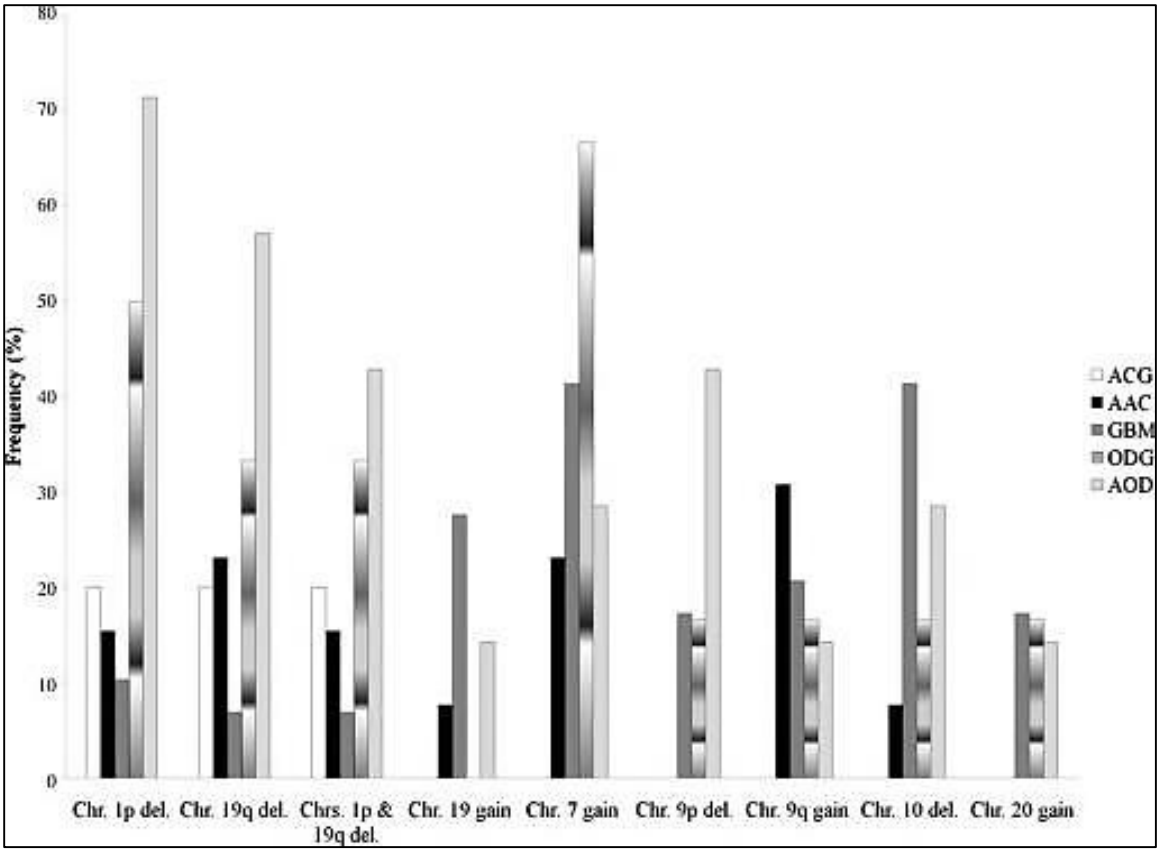


**Figure 2.5. Frequency of major CNAs in chromosomes 1, 7, 9, 10, 19 & 20 in the five glioma diagnoses**. The most statistically significant CNAs per glioma subtype was determined using chi-square test. ACG = Diffuse astrocytoma grade II, AAC = Anaplastic astrocytoma grade III, GBM = Glioblastoma multiforme grade IV, ODG = Oligodendroglioma grade II, & AOD = Anaplastic oligodendroglioma grade III.

Consistent with previous studies, Chr. 1p/19q co-deletion was more evident in oligodendroglioma than astrocytoma (61%, p= 0.026 vs. 17%, p= 0.0048; Pearson test, **Figure 2.5**). Dividing patients of grades II and III based on Chr. 1p/19q co-deletion status correlated significantly with their survival and was a better predictor compared to the pathological category (p = 0.0025, Log-rank test, **Figure 2.6**). Hence, indicating the important role of molecular classification in glioma diagnosis and prognostication.

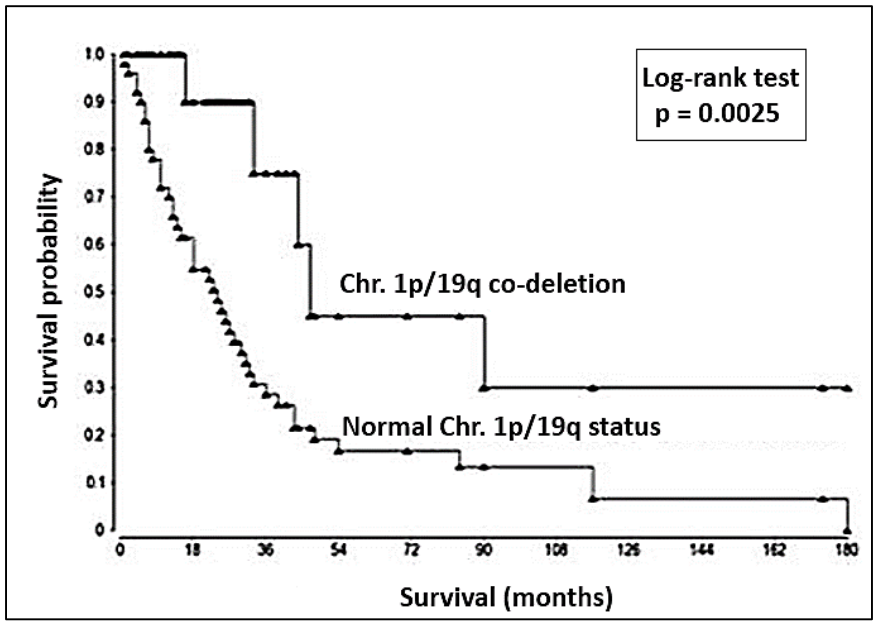


**Figure 2.6. Kaplan-Meier survival estimates of probability of survival according to chromosomes (Chr.) 1 & 19 statuses.** Patients were stratified based on Chr. 1p/19q co-deletion status. Log-rank test was used to measure significant difference between survival curves.

More than 2% of the focal CNAs identified involved various genes of the DNA damage response (DDR) such as ATR and ATRIP and different DNA-repair pathways including BER, NER, MMR, HR and NHEJ ($p<0.0001$, ANOVA test; **Table A.1**). The CNAs sizes extended from few thousands of base pairs to a complete loss or gain of a chromosome. The frequency ranged from 2-40% with the highest mean percentage corresponded to those involved in the NER (mainly ERCC1). CNAs in BER genes included CNA gain of one allele in PARP1, PARP3, PNKP, XRCC1 and TDP1 in frequencies 2, 4, 10, 10 and 8% respectively; and CNA loss of one copy in APE1, PARP2, PNKP and XRCC1 in frequencies 6, 6, 14, and 16% respectively. A representative picture of CNAs associated with Chr. 14 which hosts TDP1, our gene of interest at 14q32.11 position is shown in **Figure 2.7**.

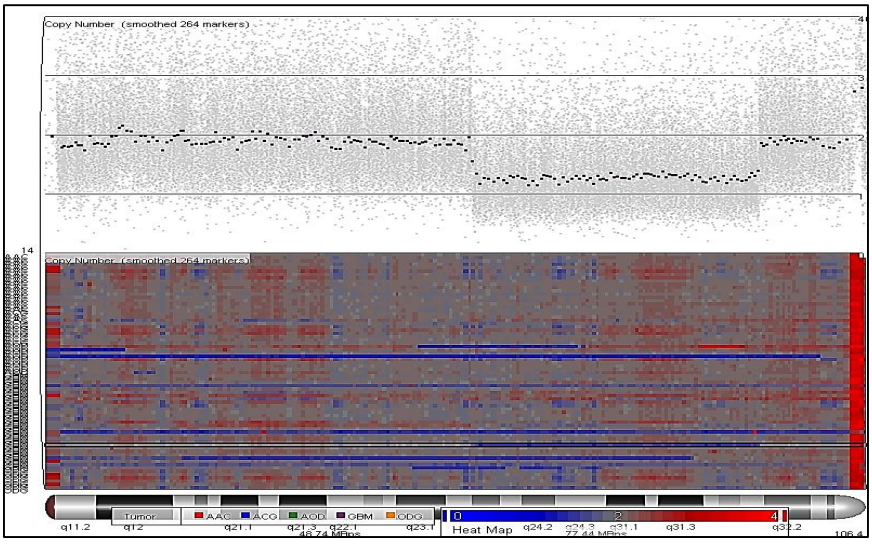


**Figure 2.7. Representative picture of copy number alterations observed in chromosome 14 in a glioma sample.** Red indicates gain events and blue indicates loss events.

CNAs affecting NER genes included: CNA gain of at least one copy of ERCC1 (20%), ERCC2 (10%) and ERCC8 (18%) and CNA loss in ERCC1 (40%), ERCC2 (20%), ERCC5 (8%), ERCC6 (20%) and ERCC8 (6%). Chromosomal regions containing MMR genes were also affected. CNAs in MLH1, MLH3, MSH2, MSH3, MSH4, PMS1, and PMS2 were evident in less than 16% of glioma specimens. DSB repair genes affected by the CNAs included BRCA2, LIG4, XRCC2, 3, 4, 6, RAD50, MRE11A (part of the MRN complex) and ERCC4.

Amplification of TOP enzymes including TOP I, mtTOP I, TOP2β, TOP3α and TOP3β was also evident with a CNA gain of one or two copies.

### 2.3.5. Correlation between CNAs and gene transcription levels

It is conceivable that copy number alterations associated with loss events will result in concordant reduction in gene transcription while gain events will be associated with an upregulation of the affected genes. Thus, to validate our SNP array data and to confirm the concordance between some of the major identified CNA involving genes with a well-known role in glioma and their gene transcript level, RT-PCR analysis was performed for EGFR, EGFRvIII and MGMT. As shown in the figure below, in the majority of the samples that showed Chr.7 gain, there were an amplification of EGFR and few of the samples showed expression of EGFRvIII (**Figure 2.8.A**). All of the 7 glioma samples that have shown loss of Chr.10, MGMT transcripts were absent. However, few tumors with intact Chr.10 have no MGMT which is explained by the promoter methylation that results in gene silencing (**Figure 2.8.B**). Chr.7 gain and Chr.10 loss events were more evident in astrocytoma lineage (**Figure 2.5**).

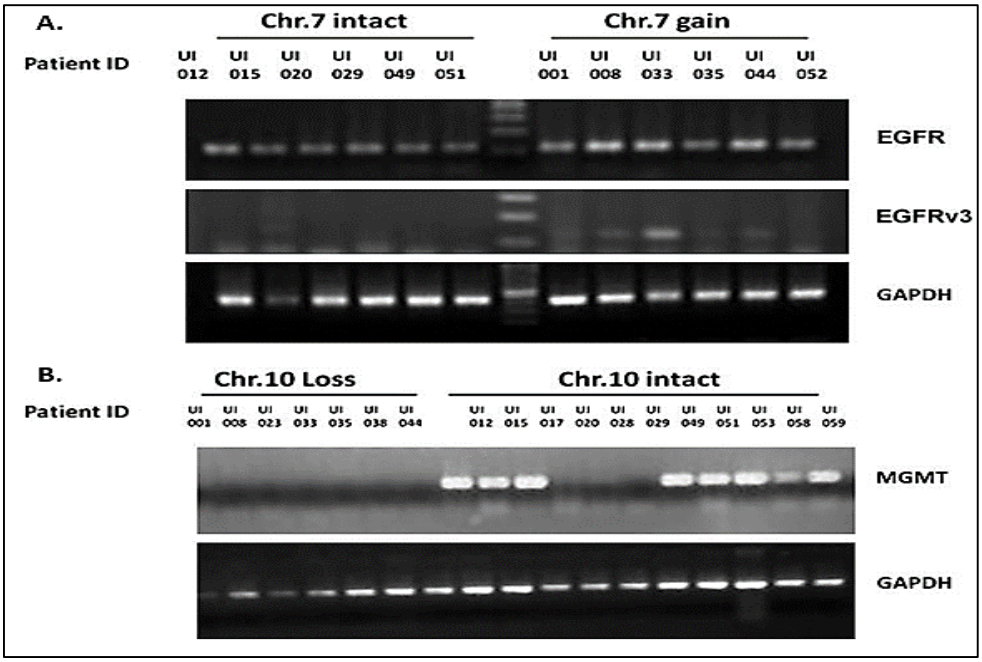


**Figure 2.8. RT-PCR analysis for EGFR, EGFRvIII, and MGMT gene expressions.** Chr. = chromosome. EGFR, EGFRvIII and MGMT transcript levels are dependent on Chr.7 and Chr.10 statuses. GAPDH serves as a housekeeping gene for loading control. (**A**), Concordance between Chr.7 gain and amplification of EGFR and the expression of EGFRvIII was seen. (**B**), Concordance between Chr.10 loss and absent MGMT transcripts was evident.

### 2.3.6. LOH analysis

The identification of large chromosomal abnormalities indicates that DNA-repair pathways are severely altered. DNA-repair genes especially MMR genes can be considered as a unique subtype of TSGs since they maintain the genetic integrity of the cells and thus prevent carcinogenesis by preventing mutations in oncogenes and TSGs. Consequently, the identification of LOH can shed the light on important DNA-repair genes that are critical for gliomagenesis and transformation. SNP array compared to other genotyping methods allows for the detection of several types of genetic alterations (allelic

imbalances, CNAs, and homozygous deletions) and without the need for matching control samples (136). This program uses Hidden Markov Model to identify LOH from tumor samples with unpaired normal specimens by considering SNP intermarker distances, genotyping error rate, SNP-specific heterozygosity rates, and the haplotype structure of the human genome (137). Several LOH regions in various chromosomal regions carrying different DDR genes and DNA-repair genes has been revealed. They included TP53 and ATM, and DNA DSB repair genes BRCA1 (HR; in 27 tumors) and XRCC5 (NHEJ; in 33 tumors), MMR genes (MSH2, 5, 6), NER genes (ERCC1, 2, 3, 5, 6 and 8), BER genes (LIG3 and POLβ), TOP IIα, and TDP1 (in 30 tumors). The most statistically significant were those involving POLβ (8p11.21) and TDP1 (14q32.11) (**Table A.1).**

### 2.3.7. SNPs in TDP1 gene

To identify SNPs associated with TDP1 gene and to confirm the specificity of our SNP array analysis, genotyping was performed using DNA sequencing. SNPs are the most common type of genetic variation affecting single nucleotides and they represent plausible candidates that may correlate with the glioma grade or the clinical outcome of the patients. Most of the SNPs occur in the non-coding regions (introns, 3'-UTR or 5'-UTR) of the DNA rather than the coding region (exons). . Representative cycle sequencing graphs for four SNPs are shown in **Figure 2.9.** As shown in **Table 2.4**, a total of 12 SNPs have been identified; 1 intronic, 4 in the 3'-UTR of the gene and 7 in the coding region, 6 of them were synonymous (code for the same amino acid) and 1 nonsynonymous. SNP10 and SNP11 have not been mentioned in the NCBI website. SNP9 (nonsynonymous: missense) results in Ala133Thr. This mutation was analyzed in SIFT (Sorting Intolerant From Tolerant) software (J. Craig Venter Institute) to identify its effect on TDP1 enzymatic activity and it showed that it is tolerated (does not affect

protein activity). Statistical analyses to analyze the correlation between the SNPs and the diagnosis or the clinical outcome of glioma patients showed insignificant results.

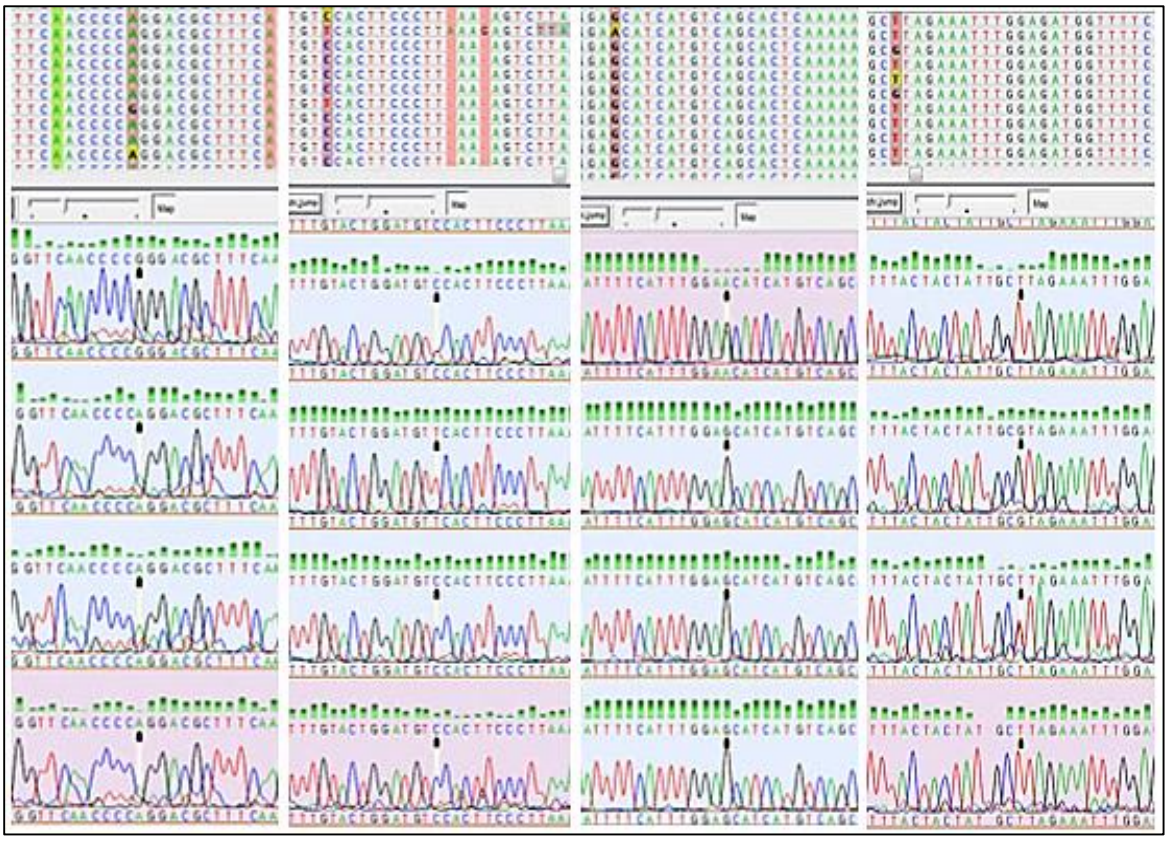

**Figure 2.9. Representative chromatograms of sequence traces of four mutations in the coding region of TDP1 transcripts from glioma patients' biopsies.** DNA Baser v2 software was used for sequence assembly and alignment. The reference sequence NM_018319 was used as a template.

**Table 2.4. Information about 11 genotyped SNPs of TDP1.**

| SNP No. | dbSNP rs# | Allele Variant | Chromosomal Position | mRNA Position | Amino Acid Position | Function | Genotype: Frequency (%) | SIFT |
|---|---|---|---|---|---|---|---|---|
| SNP1 | rs17126522 | A/G | 90451500 | 1376 | 359 | synonymous | AA: 81, AG: 16, GG: 35 | Tolerated |
| SNP2 | rs190397530 | C/G/A | 90455335 | 1517 | 406 | synonymous | CC: 56, CG: 13, CA: 30, CT: 1 | Tolerated |
| SNP3 | rs9488 | C/T | 90509564 | 2203 | 3'-UTR | 3'-UTR | CC: 70, TC: 16, TT: 14 | |
| SNP4 | rs10151377 | T/C | 90451530 | 1406 | 369 | synonymous | TT: 97, TC: 3, CC: 0 | Tolerated |
| SNP5 | rs17126522 | A/G | 90451500 | 1376 | 359 | synonymous | AA: 86, AG: 11, GG: 3 | Tolerated |
| SNP6 | rs7150480 | T/C | 90509880 | 2519 | 3'-UTR | 3'-UTR | TT: 96, TC: 4, CC: 0 | |
| SNP7 | rs35090050 | G/A | 90510026 | 2665 | 3'-UTR | 3'-UTR | GG: 96, GA: 4, AA: 0 | |
| SNP8 | rs3825663 | A/G | 90429749 | 590 | 97 | synonymous | AA: 68, AG: 10, GG: 22 | Tolerated |
| SNP9 | rs28365054 | G/A | 90429858 | 699 | 133 | Missense | GG: 85, AG: 7, AA: 8 | Tolerated |
| *SNP10 | New | C/G | ---------- | 1121 | 274 | synonymous | GG: 5, CG: 92, CC: 3 | Tolerated |
| *SNP11 | New | T/G | ---------- | --------- | --------- | Intron | TT:48, GT:37, GG:15 | |

### 2.3.8. Quantitative analysis of TDP1 gene expression in gliomas

In an attempt to identify the prognostic significance of TDP1 in glioma, real-time quantitative PCR was performed for the RNA extracted from glioma biopsies and TDP1 transcript levels were correlated with patients' survival using Kaplan-Meier survival analysis. A strong correlation between TDP1 gene expression level in 35 glioma biopsies (4 ACG, 6 AAC, 18 GBM, 3 ODG, and 4 AOD) and the overall survival of patients was revealed.

The patients were classified into two groups based on their expression of TDP1 (cut-off value = median TDP1-level = 852.66) and they were stratified based on the glioma lineage (astrocytoma vs. oligodendroglioma) to ensure that the obtained significant effect of TDP1 level on survival is not due to lineage difference. The data indicated that patients expressing TDP1 levels lower than the median had three times better survival compared to those with TDP1 levels higher than the median (median survival 41 vs. 11.5 months, $p$ = 0.006; Log-rank test) (**Figure 2.10**). This suggests that TDP1 acts as a negative prognostic biomarker in glioma.

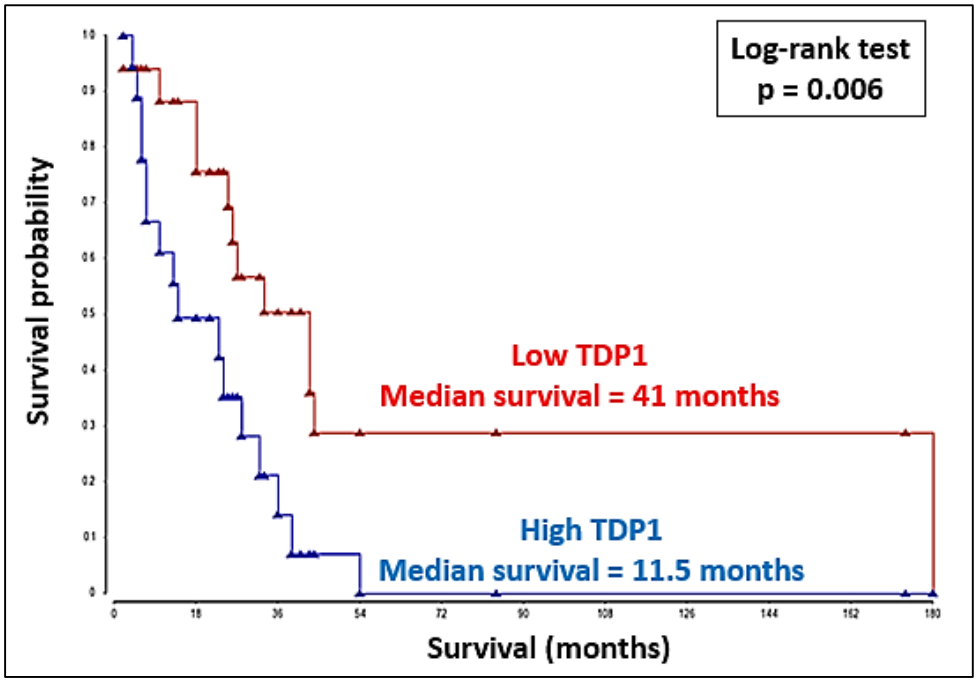


**Figure 2.10. Kaplan-Meier estimates of probability of survival stratified according to TDP1 mRNA expression levels** in 35 glioma biopsies of 3 ODG (oligodendroglioma, grade II), 4 AOD (anaplastic oligodendroglioma, grade III), 4 ACG (diffuse astrocytoma, grade II), 6 AAC (anaplastic astrocytoma, grade III) and 18 GBM (glioblastoma multiforme, grade IV) diagnoses. Log-rank test was used to calculate difference between survival curves. $p < 0.05$ was considered as significant.

Moreover, by dividing the patients into two survival groups based on the median survival of the whole group (cut-off value = 24 months), patients with less than 24 months survival (~52%) expressed higher TDP1 levels (*p*-value = 0.0085, Likelihood ratio test) (**Figure 2.11**)**.** Thus again confirming the negative prognostic value of TDP1 in glioma.

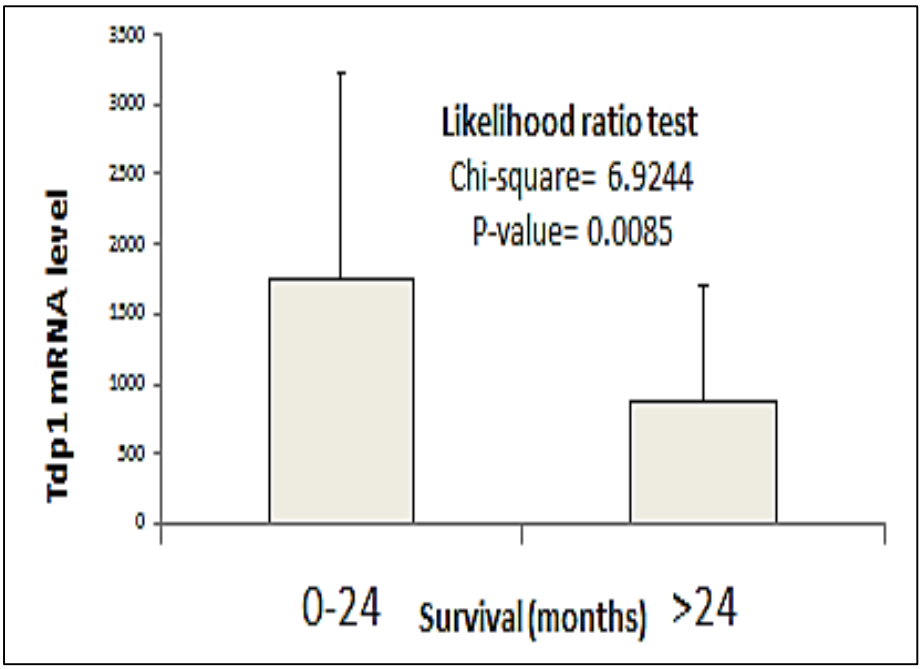


**Figure 2.11. The correlation between TDP1 level and the survival of patients.** Patients were divided into two groups based on their median survival (cut-off value = 24 months). Significant difference between the two groups was calculated using likelihood-ratio test. $p < 0.05$ was considered as significant.

### 2.3.9. Protein expression analysis of TDP1 in gliomas

To validate TDP1 as a diagnostic biomarker in glioma, we sought to identify the correlation between TDP1 protein expression level and glioma aggressiveness using western blot analysis. First of all, to ensure loading equal amounts of proteins, Bradford assay was used to measure protein concentration. BSA at concentrations 1, 2, 5, 10, and 20 µg/µl was used to create the standard curve and the best linear fit with an R2 value of >0.98 was used for further calculation of protein concentrations in glioma tissue extracts (**Figure 2.12**).

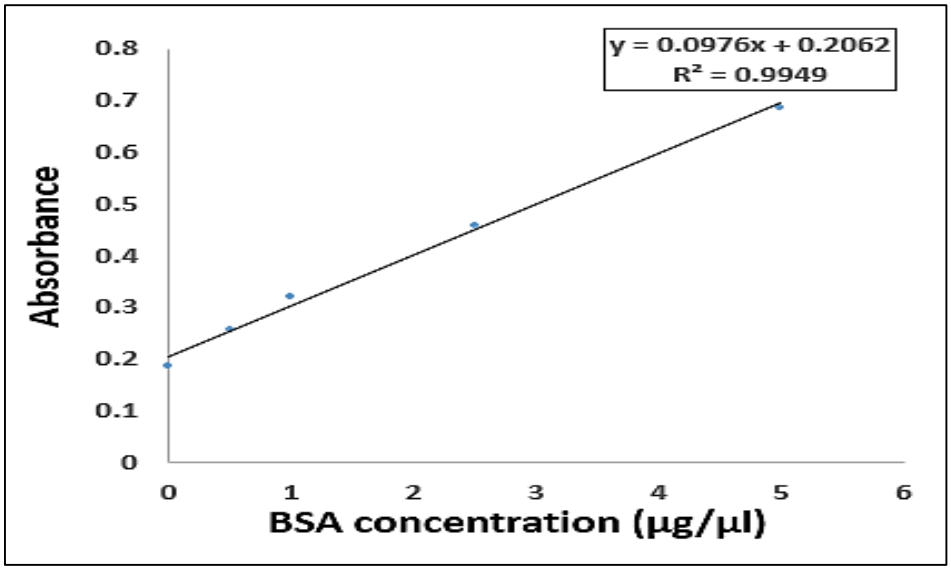


**Figure 2.12. Bradford assay calibration curve for Bovine Serum Albumin (BSA) standard solutions**. Absorbance values at 595 nm for five different concentrations of BSA were plotted and the best fit line was calculated in excel. The linear equation (x = protein concentration, y = absorbance value) was used to calculate the concentrations of the proteins in tissue homogenates.

25 µg of protein was loaded into SDS-PAGE gel for protein separation via western blot analysis. Protein bands localization and confirmation of protein transfer from gel to nitrocellulose membrane was performed using Ponceau red staining as shown in **Figure 2.13**. Western blot technique represents a rapid and accurate way for the exploration of differential protein expression among different samples. Using a specific antibody targeting TDP1 protein, TDP1 was visualized and its differential expression among glioma samples was identified and compared to that in non-neoplastic brain tissues.

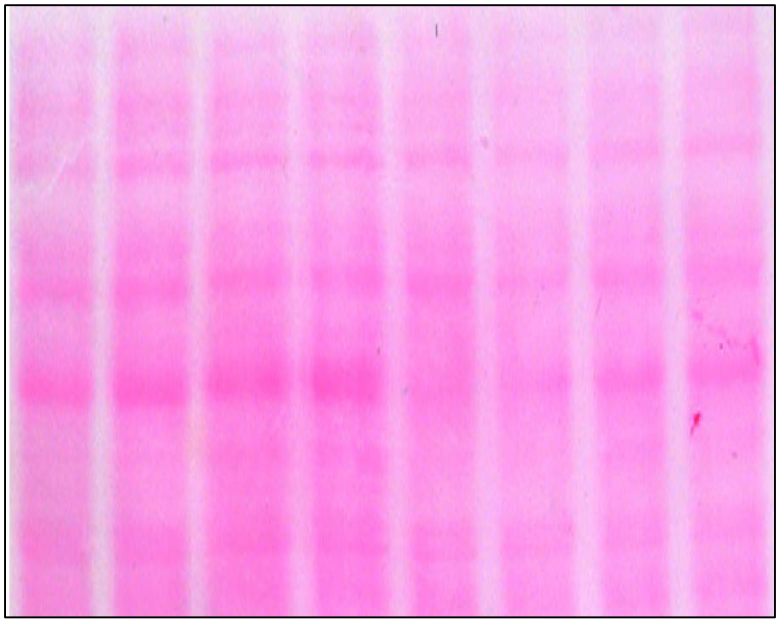

**Figure 2.13. Relative protein band intensity using reversible Ponceau red staining.**

Corresponding healthy brain tissue from the same patients could not be harvested, thus biopsies from patients with gliosis (brain inflammation) were obtained. Compared to the control brain tissues, TDP1 protein was overexpressed in high-grade gliomas consisting of AOD and GBM (**Figure 2.14.A**). Moreover, in astrocytoma lineage the increase in TDP1 level correlated with the tumor aggressiveness with an ascending increase in expression level from grade II (ACG) to grade IV (GBM) samples which revealed to possess the highest TDP1 protein levels (**Figure 2.14.B**).

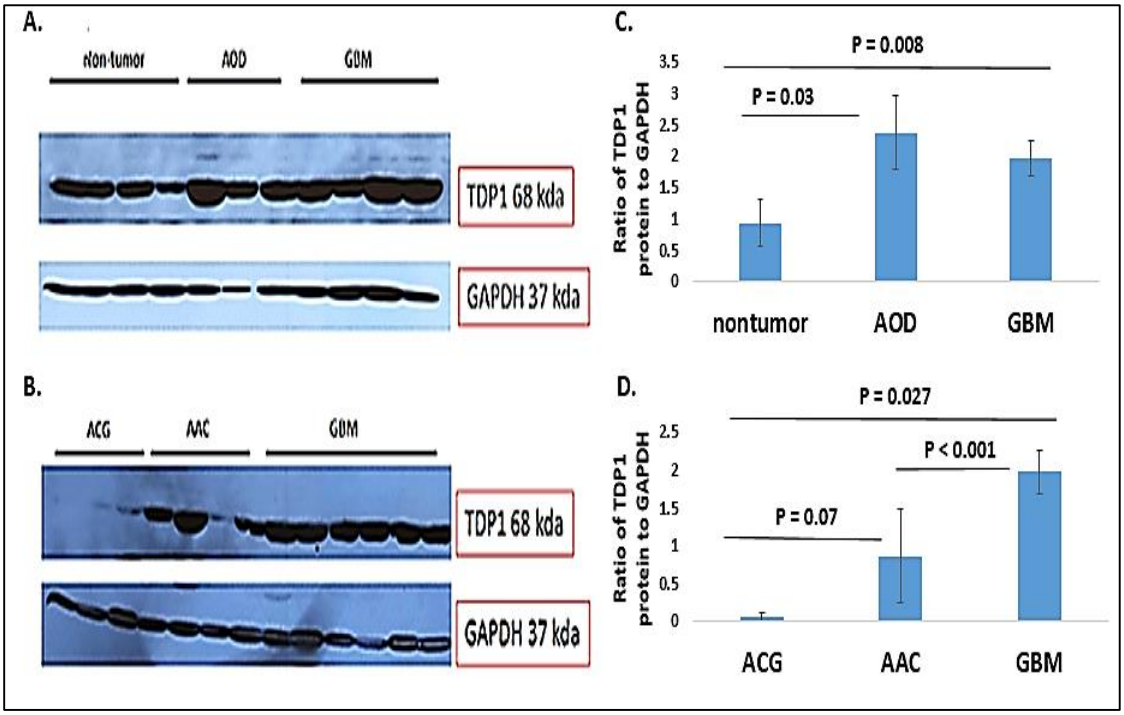


**Figure 2.14. Expression of TDP1 protein in 24 samples of human non-tumor brain and glioma tissues using western blot analysis.** (**A**), TDP1 protein expression in malignant glioma (3 AOD and 4 GBM) compared to non-tumor tissues. (**B**), TDP1 protein expression and correlation with the clinical grade of astrocytoma. (**C**) & (**D**), Graphical representation of the TDP1 protein expression profiles in (A) & (B) respectively. GAPDH was used as the loading control.

For validation of the results and to obtain quantitative data, the blots were analyzed using Image Studio Lite software. TDP1 signal measurements were normalized to corresponding GAPDH values. Then we compared the ratios between 4 non-tumor, 3 AOD and the 10 GBM (**Figure 2.14.C**) or between the astrocytoma samples (**Figure 2.14.D**). TDP1 expression in AOD and GBM differed significantly from that in non-tumor samples. Average TDP1 protein in AOD and GBM samples was about 2.5x and 2x higher than that in non-tumor samples respectively ($p = 0.03$ & $0.008$ respectively; student's t-test), indicating that TDP1 is upregulated in malignant glioma. Although not statistically significant due to small sample size, the average TDP1 protein level in AAC samples was about 14x higher than that in ACG samples ($p = 0.07$; student's t-test). Moreover, compared to ACG and AAC, GBM samples expressed the highest TDP1 levels (~ 31x higher than in ACG; $p = 0.027$ & ~ 2x higher than in AAC; $p < 0.001$ respectively, student's t-test).

## 2.4. Discussion

The inadequacy of the currently applied WHO classification system of glioma, which is based on the histopathological features of the tumor, to explain the wide variability in the patients' clinical outcome urged the need for better classification strategies. The high genetic heterogeneity of glioma directed researchers, neurologists, and pathologists to focus on molecular analysis as a tool for better categorization of glioma patients. Through the identification of novel prognostic biomarkers patients can be clustered thus providing more accurate diagnosis which is important for treatment selection.

Different cytogenetic techniques have been developed for genome-wide analysis purposes such as aCGH, FISH and the most recent microarray analysis. SNP array is one type of the latter that allows concurrent detection of CNAs, SNPs and LOH (138-141). Although expensive, SNP array is considered the most accurate and reliable compared to

aCGH and FISH. Using this technique we were able to identify different types of CNAs and allelic imbalances among glioma subtypes. Some of these genetic alterations showed significant correlation with glioma grade and clinical outcome of patients. Thus, revealing the importance of this technique in providing unbiased information that can aid in the diagnosis and prognosis of glioma.

Our data showed that some chromosomal abnormalities cluster with each other. For example, Chr. 7 gain coexisted with Chr. 10 and Chr. 9p losses while the presence of Chr. 1p and/or 19q losses was associated with no alteration in Chr. 7 or Chr. 9p. Moreover, some of the CNAs identified correlated with the patients' survival. For instance and consistent with previous studies, Chr. 1p/19q co-deletion associated with better prognosis. There was also a trend of poorer prognosis when Chr. 7 was amplified. These findings suggest the usefulness of molecular analysis for providing a better classification system of glioma that can cluster patients into diagnostic groups besides its applicability to provide prognostication information. Moreover, these molecular changes may represent novel therapeutic targets.

The huge number of chromosomal abnormalities identified in this study reveals that the DNA-repair pathways are significantly altered. Moreover, resistance to therapy plays an important role in the poor prognosis and focusing on DNA-repair mechanisms is a clinically relevant option since most of the chemotherapeutic agents used in the malignant glioma treatment have DNA-damaging properties. Furthermore, most of the studies have shown an inverse correlation between DNA-repair capacity and response to therapy in malignant gliomas (59). Of the major chromosomal imbalances that we identified, many of them involve genes that control the DNA-repair pathways either directly or indirectly. For example, Chr. 10 loss was associated with low or absent MGMT expression. MGMT as discussed in the previous chapter is important for the repair of the O6-meG DNA lesions produced by TMZ. Thus low or absent MGMT is expected to be associated with enhanced response to TMZ (142). Also Chr. 7 gain

resulted in the amplification of EGFR and EGFRvIII in some samples which as discussed in the previous chapter affect the response to IR through the activation of the NHEJ process thus mediating radioresistance (44).

With the focus on DNA-repair genes as potential prognostic or predictive biomarkers in glioma, various CNA losses and gains in several DNA-repair genes of different pathways including: BER, NER, MMR and DSB repair were recognized in this work. Compared to control samples, glioma biopsies revealed more frequency of CNAs in DNA-repair and repair-related genes evidencing their prognostic significance. Hence, studying gene expression profiles and genetic alterations associated with DNA-repair genes is an important tool for the identification of molecular biomarkers that can assist in glioma diagnosis, prognosis and treatment prediction.

In this work we were also able to identify chromosomal regions with LOH involving DDR and DNA-repair genes. LOH is a common phenomenon in cancer and it indicates the nonfunctionality of a tumor suppressor gene. In agreement with previous studies, and with the fact that inactivation of TP53 is the most prevalent mutation in cancer, LOH of TP53 locus was evidenced (143). The product of this gene (p53) is critical for the initiation of the DDR cascade through which the fate of the cell is determined and directed for apoptosis or cell-cycle arrest reliant on the damage severity. TP53 inactivation has been shown as a useful diagnostic tool in glioma since it is usually seen in LGGs and is hallmark of secondary GBMs (144,145), indicating that it is an early event in the tumor evolution (6). High p53 protein expression level resulting from mutations in the TP53 gene, correlated with poorer survival in pediatric malignant glioma patients (146). Moreover, *in vitro* experiments in U87MG GBM cell lines, showed that TP53 inactivation enhances the cells' sensitivity to BCNU and TMZ due to deficient DDR; suggesting a predictive value of this gene (147).

The loss of activity of MMR genes comes from the preclinical and the clinical studies that have shown an inverse effect on the response to TMZ. For example, acquired

loss of MSH6 was evident in recurrent GBM patients post TMZ+IR therapy and resulted in tumor progression due to resistance to TMZ (148-150). An interesting finding is the LOH of TDP1 (14q32.11) and POLB (8p11.21) loci, both of which are important interplayers in the BER pathway. A study by Tang *et al*. showed that Pol β mRNA expression was found to vary between GBM samples and normal brain tissue and its downregulation correlated with enhanced sensitivity to TMZ under BER inhibition via methoxyamine (52). On the other hand, the role of TDP1 in gliomagenesis or response of glioma to anticancer therapy has not yet been studied.

Previous studies have shown that polymorphisms in BER, NER and DSB repair genes correlated with glioma risk (susceptibility to develop glioma) and the survival of patients. A study by Liu *et al.* showed that three of the six SNPs that significantly associated with glioma risk in 373 Caucasian patients with glioma were nonsynonymous SNPs in XRCC1, PARP-1, APEX1 and ERCC1 (131). Other studies showed that certain SNPs in LIG4 (DSB repair) and ERCC6 (NER) were associated with poorer outcome (131,151). Thus, indicating the important prognostic value of DNA-repair genes in glioma. However, none of the TDP1 SNPs identified in this study correlated with glioma diagnosis, prognosis or with the clinical outcome of patients. Nevertheless, due to small sample size, a definitive answer regarding the applicability of TDP1 SNPs cannot be conferred from this study.

Being highly expressed in normal brain tissue and as an important interplayer in the BER, we focused on studying the molecular alterations associated with TDP1 which is carried on Chr. 14 and investigate its relevance in gliomagenesis. We found that TDP1 was amplified in 8% of biopsies all of which were AODs and GBMs indicating that TDP1 may correlate with glioma aggressiveness. Thus, in 20 glioma samples TDP1 protein level has been analyzed using western blot. TDP1 protein expression in 7 high-grade gliomas (4 GBM and 3 AOD) was found to be increased compared to that in 4 non-neoplastic brain tissues and in 13 astrocytoma biopsies there was a trend of increased

expression from WHO grade II to WHO grade IV. This indicates the translational activation of TDP1 in high-grade gliomas that may contribute to the gliomagenesis and malignant transformation. Since the glioma biopsies were taken before the patients had received radiation or chemotherapy, the TDP1 overexpression is unlikely to be the result of exogenous cytotoxic stress.

DNA-strand breaks are severe genomic lesions that can arise either endogenously by reactive oxygen species or exogenously by cytotoxic anticancer agents like ionizing radiation, alkylating agents, and TOP-poisons. If not repaired, DNA stability is breached which will eventually lead to cellular apoptosis. In normal cases efficient DNA-repair mechanisms are required to maintain the genetic integrity and thus the prevention of several abnormal conditions such as carcinogenesis. However, in the case of treatment with DNA-damaging anticancer drugs, DNA-repair is mainly considered a resistance mechanism that will aid in tumor growth and survival. In cancer tissues due to high mitotic rate of the cells and the wide genetic aberrations associated with their growth could lead to an enhanced expression of the DNA-repair enzyme to compensate for the increased demand to DNA repair including TDP1. In this study we provide the first evidence of the empirical role of TDP1 in glioma where we found an upregulation of TDP1 protein in glioma tissues.

In order to identify the prognostic significance of TDP1 in glioma, quantitative real-time PCR was performed to quantify TDP1 mRNA expression levels in glioma biopsies. They were correlated with patients' clinical outcome using Kaplan-Meier survival analysis. The analysis revealed that patients expressing high TDP1 levels had poorer outcome. This indicates that high TDP1 expression is a negative prognostic factor in patients with glioma. For validation of the correlation between TDP1 transcript level and survival, patients were divided into two groups based on their overall survival time ($\leq$24 vs. >24 months). Likelihood-ratio analysis showed a statistically significant difference in TDP1 expression level among the two groups with those who survived for

more than two years expressed lower TDP1. This is the first proof of the prognostic value of TDP1 in cancer. However, these results need to be further validated in a larger sample size.

As a summary, despite of the small sample size our findings showed that various chromosomal abnormalities correlated with glioma aggressiveness and prognosis. Thus microarray analysis is considered a useful tool for enhancing the classification of patients and selection of therapy through the identification of novel prognostic and predictive biomarkers. Moreover, our results showed that TDP1 was upregulated at protein level in human glioma tissues compared to non-neoplastic ones and also suggested that TDP1 is a potential prognostic biomarker for glioma patients' survival.

Our findings suggest that TDP1 may play a role in affecting the response of glioma to the DNA-damaging anticancer agents including TOP-poisons, IR and alkylating agents such as TMZ.

## 2.5. Conclusion

This chapter provides a convincing evidence of the potential role of TDP1 in glioma and that the increased TDP1 protein and gene expression levels correlate with advanced clinicopathological features and poorer survival respectively suggesting that TDP1 is a promising prognostic biomarker in glioma. Further validation for more reliable results is required by recruiting a larger sample size and using different methodologies.

This chapter directed us to the next step, which is the investigation of the underlying molecular mechanisms of how TDP1 may affect the response of gliomas to therapy. This would be achieved through the *in vitro* utilization of established malignant glioma cell lines and assess the effect of TDP1 on the response of the cells to the anticancer therapy. Thus, we can test the resistance to anticancer agents such as TMZ, topotecan, etoposide and doxorubicin. If proven to play a role in the resistance of the cells to DNA-damaging cytotoxic agents, this will move us from cell culture-based

studies to *in vivo* experiments by utilizing mice with glioma xenografts, this can be performed prior to clinical investigations. As a consequence, TDP1 may be indicated as a predictive biomarker that can aid in the prediction of response to therapy and thus treatment selection. Additionally, this may suggest the use of specific TDP1 inhibitors that can be incorporated to the therapeutic regimen to enhance the sensitivity of the cells and overcome the resistance to some DNA-damaging anticancer agents.

# CHAPTER III

# TYROSYL DNA-PHOSPHODIESTERASE I (TDP1) IS NOT SUFFICIENT TO PREDICT RESPONSE TO ANTICANCER THERAPY IN MALIGNANT GLIOMA

## 3.1. Introduction

Malignant glioma remains a treatment challenge in the world of tumor management. It is treated extensively with a maximal tumor resection followed by an aggressive adjuvant radiotherapy and temozolomide (TMZ), then succeeded by a single therapy of TMZ (**Figure 3.1**). Nevertheless, only 10% of the treated patients survive for 5 years after diagnosis and most deaths occur in the first 2 years (10,19).

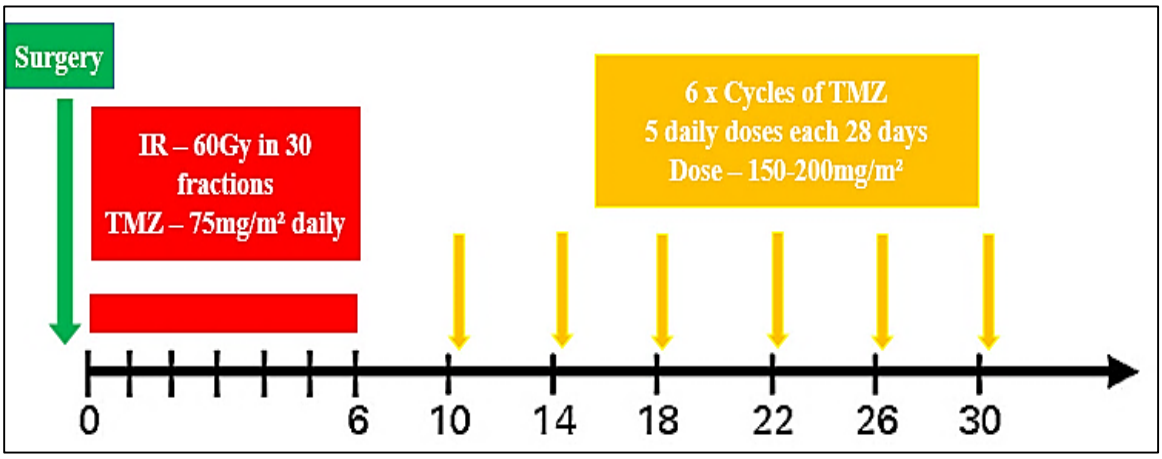


**Figure 3.1. Treatment of malignant glioma.** Schematic representation of glioma management started by surgical resection of tumor when possible followed by concurrent chemoradiotherapy, ionizing radiation (IR) and temozolomide (TMZ), followed by adjuvant TMZ.

Source: European Organization for Research on the Treatment of Cancer and the National Cancer Institute of Canada (EORTC-NCIC) (152).

Several factors account for the poor prognosis of malignant glioma. Genetic heterogeneity can be considered a major factor because it decreases the ability to provide an accurate diagnosis and hinders the ability to predict the clinical outcome of patients and to stratify them to treatment. A property that is also associated with malignant glioma is its inherent resistance to anticancer therapy which results in the recurrence of the majority of the cases. Consequently, untying the knots of glioma resistance and understanding the associated underlying molecular pathways can aid in the identification of novel therapeutic targets and in the individualization of therapy through defining patients who will benefit more to a specific anticancer regimen. In glioma, there are three main mechanisms of treatment resistance including: 1- poor penetration (due to efflux of the drugs through the ATP-binding cassette (ABC) transporters), 2- cancer stem cells, and 3- high DNA-repair capacity.

DNA-repair is a double-edged sword in the cancer world; defective DNA-repair processes result in carcinogenesis while efficient DNA-repair mechanisms are vital drivers of chemotherapy resistance. This can be evidenced by: 1- the results of our previous work that have shown a high frequency of genomic instability associated with glioma cases, thus predicting a deficiency in the DNA-repair mechanisms which resulted in the significant genetic aberrations identified, and 2- the findings of the previous studies that have shown an inverse correlation between the DNA-repair capacity and the therapeutic efficacy (59).

The first-line treatments of malignant glioma consisting of radiation (IR) and TMZ and the second-line agents mainly topoisomerase poisons (TOP-poisons) exert their cytotoxic function via damaging the DNA resulting eventually in single and double DNA strand breaks. If the DNA-damage response and repair processes were proficient, the produced DNA damage will be reversed resulting in cell survival and resistance to the anticancer therapy. The majority of the cytotoxic and mutagenic lesions produced by IR, TMZ and TOP I-poisons represent single-strand breaks which are recognized and

processed by the base excision repair pathway (BER) (43,153,154). The enzyme TDP1 plays a key role in the BER, by hydrolyzing blocked 3'-DNA termini and producing DNA products with 3'-phosphate ends (155). Thus facilitating the recruitment of PNKP to remove the 3'-phosphate and generate a free 3'-hydroxyl DNA termini that are ready for further processing via the remaining components of the BER complex (including: PARP-1, XRCC1, pol β, and Lig 3α) (155).

Previous preclinical studies have provided compelling evidence for the potential role of TDP1 in potentiating the resistance to several anticancer agents specifically to camptothecin and its derivatives. TDP1 was implicated in the repair of the irreversible topoisomerase I-DNA (TOP I-DNA) cleavage complexes induced by TOP I-poisons and the 3'-phosphoglycolate lesions associated with SSBs produced by IR (86,99). Moreover, it was suggested to play a role in the repair of the N3 and N7 methyladenine lesions produced by TMZ. Additionally, it was found that TDP1 is able to process the 5'-DNA lesions and repair irreversible topoisomerase II-DNA (TOP II-DNA) cleavage complexes induced by TOP II-poisons (103).

Due to the ability of TDP1 to repair various types of DNA lesions, the correlation between its level or its genetic status (wild-type vs. mutant) and the response to anticancer agents has been investigated in different kinds of cells. Cells expressing low TDP1 levels or harboring a mutant gene (associated with reduced enzymatic activity) were hypersensitive to camptothecin and topotecan (TOP I-poisons), etoposide (TOP II-poisons), and bleomycin (a source of 3'-phosphoglyclate SSBs) and were less able to resolve SSBs produced by IR (95,99,101,104,105). On the other hand, cells overexpressing TDP1 showed resistance to camptothecin and etoposide (103,107).

Consequently, several factors motivated the studying of the relevance of TDP1 in glioma including: 1- Our work that have shown a significant amplification of TDP1 in glioma specimens and its inverse correlation with the clinical outcome of patients. 2- The critical role of BER pathway in the fixation of the majority of the DNA lesions produced

by the anticancer agents used in the treatment of glioma. And 3- the BER is the most vital DNA-repair process responsible for the reversal of the SSBs induced by reactive-oxygen species in the brain tissue

The goals of this chapter are to investigate the effect of manipulating TDP1 level in malignant glioma cell lines on their sensitivity to TOP-poisons including topotecan, etoposide and doxorubicin beside the DNA-alkylating agent, TMZ. Moreover, we want to test the effect of small-molecule TDP1 inhibitors on potentiating the cytotoxicity of TOP-poisons. This work hypothesizes that malignant glioma cell lines overexpressing TDP1 are more resistant to treatment compared to parental cell lines while depleting TDP1 in these cells improves their response to therapy due to decreased DNA-repair capacity. Furthermore, this work hypothesizes that a combinational therapy of a TOP-poison and a TDP1 inhibitor is associated with enhanced cytotoxic effects compared to treatment with a TOP-poison alone.

## 3.2. Materials and methods

### 3.2.1. Drugs, cell lines and growth conditions

Topotecan, etoposide, and TMZ (Sigma-Aldrich, St. Louis, USA) were dissolved in dimethyl sulfoxide (DMSO) and diluted in culture medium. Doxorubicin (Novaplus) was diluted in culture medium. NSC128609 (T1) and NSC120686 (T2) were provided by the national cancer institute/developmental therapeutic program (NCI/DTP) as 10 mg powder that then were dissolved in DMSO and diluted in culture medium.

Our interest was to study the effect of TDP1 in malignant glioma, however the only commercially available cell lines are derived from glioblastoma multiforme patients. U87 (ATCC, Manassas, VA) and U251 (gift from Dr. William A Maltese)(156) were maintained in plastic flasks as adherent monolayers in Dulbecco's Modified Eagle Media (DMEM) (Invitrogen, Grand Island, NY) supplemented with 5% fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT), high glucose, L-glutamine, penicillin G

(10,000 U/ml), streptomycin (10,000 μg/ml) at 37 °C in a humidified 5% $CO_2$ incubator. The cells were passaged when confluent with 0.25% trypsin/EDTA (Life Technologies, Carlsbad, CA) to detach the cells from the plate.

### 3.2.2. Subcloning of TDP1 fragment from pCMV-sport6 into pIRES-hrGFP-II vector

A pCMV-sport 6 containing the full length cDNA of TDP1 (insert size = 1.1 kb) was obtained from open Biosystems (clone ID#: 3900062, Thermo Scientific, Huntsville, AL). The vector map is shown in **Figure 3.2.A.**

For stable transfection experiments, subcloning of TDP1 from pCMV-sport 6-TDP1 into pIRES-hrGFP-II vector was performed because the former lacks a selection marker which is needed for stable transfection in mammalian cells. Using the restriction enzymes *Not1* and *Sal1,* TDP1 was cut out from the pCMV-sport 6 plasmid. DNA fragments with 3'-overhangs consisting of the full length TDP1 and the remaining part of the plasmid were produced. Using the same restriction enzymes, pIRES-hrGFP-II vector (Stratagene, La Jolla, CA) was cut to produce DNA ends that are complementary to those of the TDP1 fragment. The vector map is shown in **Figure 3.2.B.** The cut pIRES plasmid and the TDP1 fragment were then ligated together in an ATP-dependent reaction using T4-DNA *ligase*. Bacteria were then transformed with the new recombinant plasmid and allowed to grow on ampicillin selective agar that allows only the growth of bacterial cells carrying the target plasmid. After selection, single colonies were picked and the plasmid DNA was extracted using miniprep (Qiagen, Germantown, MD). Briefly, the bacteria were harvested and lysed under alkaline conditions, then the plasmid DNA was adsorbed to a silica-gel membrane. This was followed by washing from salts and endonucleases followed by elution with DNase/RNase free water.

The presence of TDP1 insert in the pIRES-hrGFP-II vector was checked using *Not1* and *Sal1* restriction digesting. The two DNA fragments were visualized on 1%

agarose gel. Verification of TDP1 insert sequence in pIRES vector was performed using SP6 and T7 promoter primers. Midiprep (Qiagen, Germantown, MD) to purify clean and obtain sufficient amounts of the vector using alkaline lysis technique was performed. This allows the separation of plasmid DNA from the chromosomal DNA and protein. In an autoclaved 500 ml flask, 0.5 ml of the miniprep bacterial culture was diluted into 50 ml selective lysogenic broth medium containing ampicillin and then incubated and vigorously shaken overnight at 37$^{o}$C. The culture was then poured into 50 ml tube and centrifuged at 6250 rpm for 5 min at 4$^{o}$C. The supernatant was discarded and the bacterial pellet was resuspended in P1 buffer and lysed with P2 buffer. Then chilled P3 buffer was added for neutralization of the lysis. The mixture was centrifuged at 11500 rpm for 30 min at 4$^{o}$C. The supernatant containing the plasmid DNA was removed by filtration. QIAGEN-tips equilibrated with QBT buffer were used to filter the supernatant. The tip was then washed with QC buffer and the DNA attached to the resin membrane was eluted using QF buffer. The DNA was then precipitated using isopropanol and the pellet was washed with 70% ethanol and dissolved in EB buffer. The DNA yield was quantified using a spectrophotometer to measure the absorbance at 260 nm.

### 3.2.3. Transient and stable transfection of U87 and U251 cells and TDP1 protein overexpression

For transient overexpression of TDP1 in malignant glioma cell lines, the day before transfection U87 and U251 cells were seeded in 6-well plates (200,000 cells per well). On the day of transfection, 5 µg of the pCMV-sport6 vector containing the full length TDP1 or the empty vector were transfected into cells using the transfection reagent Xfect (BD Biosciences Clontech, Mountain View, CA). For stable overexpression of TDP1 experiments, the day before transfection U87 and U251 cells were seeded in 6-well plates (200,000 per well). On the day of transfection 5 µg of pIRES vector containing the full length TDP1gene or the empty vector were transfected into cells using Xfect

transfection reagent (BD Biosciences Clontech, Mountain View, CA). Cells transfected with the TDP1 containing vector (U87/TDP1 and U251/TDP1) or empty vector (U87/mock and U251/mock) were grown in the presence of 400 µg/ml G418 (GIBCO, Life Technologies, Carlsbad, CA). In both transient and stable transfection, the success of TDP1 overexpression was confirmed by real-time Q-PCR and western blot analyses.

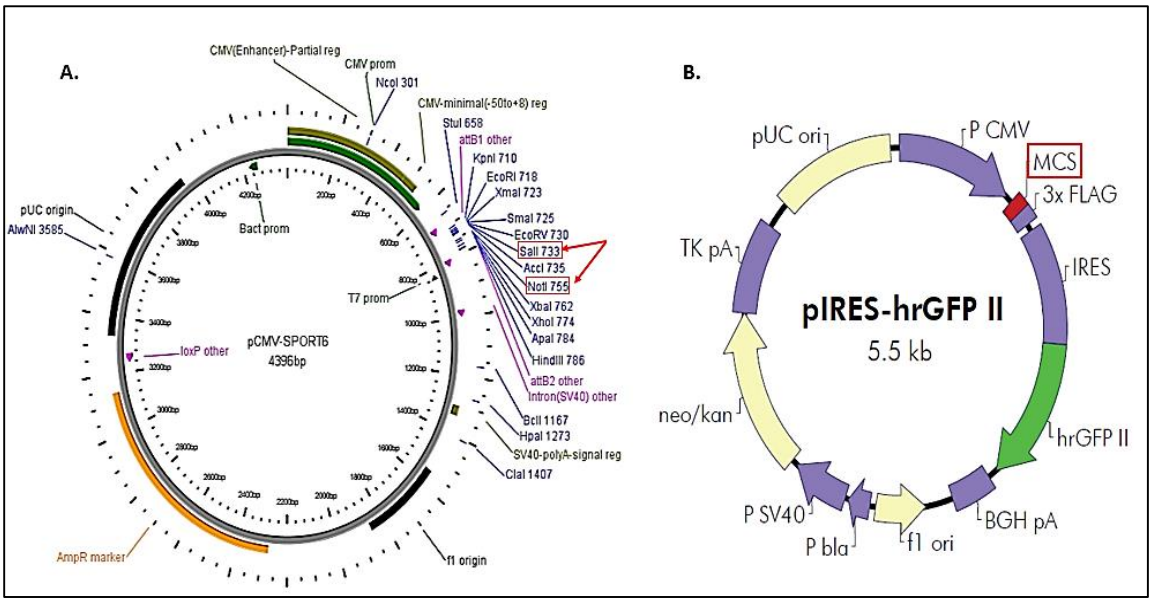


**Figure 3.2. Mammalian expression vectors maps**. (**A**), pCMV-sport 6 vector map. (**B**), pIRES-hrGFP-II map. The cDNA insert is expressed from a CMV promoter in both vectors but a neomycin selectable marker in pIRES-hrGFP II is present to allow for mammalian selection. MCS (multiple cloning site) represents the TDP1 insertion place and is flanked by Not1 and Sall restriction sites.

### 3.2.4. shRNA-lentiviral vectors, transduction and TDP1 protein knockdown in malignant glioma cell lines

Five small-hairpin RNA (shRNA) vectors (designated as 48-52) that are carried on pLKO.1 lentiviral vector (**Figure 3.3**) and that target different coding regions of TDP1 transcripts were obtained as bacterial stocks from Open Biosystems (Thermo Scientific, Huntsville, AL).

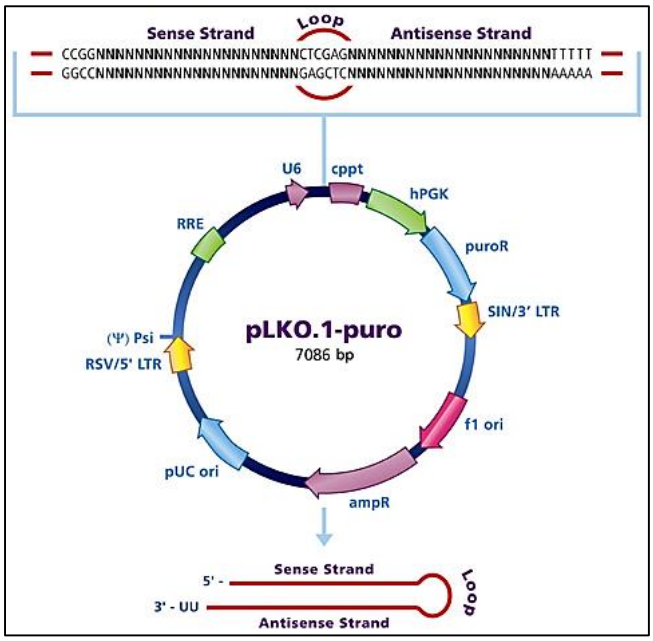


**Figure 3.3. pLKO.1 lentiviral vector map.** The length of the plasmid containing the shRNA insert is 7086 bp. This vector allows stable transduction with puromycin selectable marker (puroR). The shRNA insert consist of sense and antisense sequences connected by a short spacer of nucleotides to allow the formation of a loop structure which is important for shRNA processing inside the nucleus.

The sequences of the five shRNAs is shown in **Table 3.1**. All shRNA vectors target the two transcript variants of TDP1. The plasmids were extracted and purified from a 3-ml bacterial culture using Miniprep which is based on alkaline lysis technique (Qiagen, Germantown, MD). Plasmids were visualized on 1% agarose gel.

Lentiviral particles were generated as following: 1,000,000 TSA cells/well were seeded in a 6-well plate. The next day, the shRNA plasmids were co-transfected with packaging vectors (VsVg and PAX2) into cells (Polyfect Transfection Reagent, Qiagen, Germantown, MD) to generate recombinant lentiviruses. PLKO.1 empty vector was used as the negative control. Viral supernatants were harvested 24 and 48 h after transfection. The two samples were pooled and cleaned through a 0.45 µm filter then stored at -80ºC.

For the knockdown of TDP1 in U87/TDP1 cells, 200,000/well of U87/TDP1 cells were plated in a 6-well plate one day before transduction and infected with the lentiviral particles in the presence of 8 µg/ml polybrene. Then the cells were incubated for 24 hours, and the media was replaced with 2 ml fresh media. 48 hours later, U87/TDP1 cells with TDP1-knockdown were selected using 1 µg/ml puromycin. Cells were selected for 72 hours then transduction efficiency was validated using real-time Q-PCR and western blot analyses. Five shRNA clones were tested for the ability to knockdown TDP1 mRNA, and the best-performing clone was selected. For stable knockdown, cells were maintained in media supplemented with 1 µg/ml puromycin that was changed every three days.

### 3.2.5. Real-time q-PCR

RNA was isolated from malignant glioma cell lines using TRIzol (Life Technologies, Carlsbad, CA) and reverse transcribed into cDNA as described in the previous chapter. The primers used for the amplification and quantification of the transcript levels of TDP1, PARP-1, PNKP, XRCC1, TOP-I, TOP-II, MGMT, and GAPDH are listed in **Table 3.2.** Quantitative real-time PCR was performed on an ABI StepOne machine (Applied Biosystems, Foster City, CA).

**Table 3.1. List of The RNAi consortium (TRC) Lentiviral Human Tyrosyl DNA-Phosphodiesterase 1 (TDP1) small-hairpin RNA (shRNA)**

| TRC Lentiviral Human TDP1 shRNA | Sequence | Abbreviation | Target | Target site |
|---|---|---|---|---|
| TRCN0000048948 | 5'-ATAACCACTTTGATTCATCGG-3' | Sh48 | NM_018319 | 1516 |
| | | | NM_001008744 | 1293 |
| TRCN0000048949 | 5'-TATCCTTCTAAACTGGTCCGC-3' | Sh49 | NM_018319 | 1640 |
| | | | NM_001008744 | 1417 |
| TRCN0000048950 | 5'-TTTCATCACTACTAGATATGG-3' | Sh50 | NM_018319 | 331 |
| | | | NM_001008744 | 108 |
| TRCN0000048951 | 5'-TTTGTTTCAGAGAGATCGTGC-3' | Sh51 | NM_018319 | 1331 |
| | | | NM_001008744 | 1108 |
| TRCN0000048952 | 5'-ATCCGGTGCTTTGACATAAGG-3' | Sh52 | NM_018319 | 2076 |
| | | | NM_001008744 | 1853 |

### 3.2.6. Western blot analysis

For the lysis and homogenization of treated cells, appropriate volume of laemmli buffer was added directly to the wells. The western blot was performed as described in the previous chapter. Polyclonal mouse primary antibody against TDP1 (H-300, Santa Cruz Biotechnology) at 1/200 fold dilution was used to visualize the protein. 1:50,000-fold dilution of monoclonal mouse anti-GAPDH was used to confirm protein loading.

### 3.2.7. Glioma cell proliferation assay

MTT assay was used to evaluate cell proliferation. This is a colorimetric assay that measures the activity of mitochondrial reductase of viable cells. The yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) is reduced to purple formazan in the mitochondria and precipitates as violet crystals. Thus, this reaction proportionally correlates with the number of viable cells. Briefly, Cells were suspended with 200 µL media and plated on 96-well cell culture plates at $2\times10^3$ cells per well. After 24 h, the cells were treated for 72 hrs. with control media, or increasing concentration of 12.8 nM-200 µM topotecan, 12.8 nM-200 µM etoposide, 1.28 nM-20 µM doxorubicin, 64 nM-1000 µM TMZ or with 0.195-200 µM T1 and 0.097-100 µM T2 given alone or in combination with 0.05 µM or 0.5 µM topotecan, 0.1 µM or 1 µM etoposide, or 0.01 µM or 0.1 µM doxorubicin. Then the cells were incubated with MTT solution for 3 hours, the crystals were washed once with 100 µl cold HBSS and solubilized with 100 µl DMSO. The optical density was measured at 570 nm/690 nm using Spectramax Plus384 spectrophotometer (Molecular Devices, San Francisco, CA, USA). Normalization of data was performed in excel where the optical density of treated cells was divided by that for untreated cells (control; DMSO for topotecan, etoposide and temozolomide & media for doxorubicin). At least three independent experiments were performed for this assay.

### 3.2.8. Effect of treatment on genes expression levels

In order to assess the effect of treatment of U87/TDP1, U87/mock, U251/TDP1 and U251/mock [with topotecan, etoposide, doxorubicin or T1 and T2 given alone or in combination with one of the anticancer drugs] on the expression of TDP1, MGMT, TOP-I, TOP-IIβ, PARP-1, PNKP, and XRCC1. Cells were seeded in a 12 well plate at 50,000 cell/well density. After 24 hours the cells were treated for 72 hours with 20 µM T1, 20 µM T2, 0.5 µM topotecan, 1 µM etoposide or 0.1 µM doxorubicin given alone or in

combination with 20 µM of T1 or T2. Samples were then collected for real-time quantitative-PCR (Q-PCR) and western blot analyses.

**Table 3.2. List of primers used in real-time PCR.**

| Gene | Gene Symbol | Refseq accession number | Forward Primer | Reverse Primer | Amplicon size (bp) |
|---|---|---|---|---|---|
| Tyrosyl-DNA Phosphodiesterase 1 | TDP1 | NM_018319 & NM_001008744 | 5´-ACATCTCTGCTCCCAATGAC-3´ | 5´-CTCTCCAGATTTGTGGGTTC-3´ | 599 |
| O6-Methylguanine Methyltransferase | MGMT | NM_002412 | 5´-CCTGGCTGAATGCCTATTTC-3´ | 5´-GATGAGGATGGGGACAGGATT-3´ | 239 |
| Topoisomerase I | TOP-I | NM_003286 | 5´-TGGCAGAAATACGAGACTGCT-3´ | 5´-CCATCCAACTCTGGGTGTAGA-3´ | 239 |
| Topoisomerase IIβ | TOP-IIB | NM_001068 | 5'-AACTGGATGATGCTAATGATGCT-3' | 5'-TGGAAAAACTCCGTATCTGTCTC-3' | 137 |
| Poly ADP-ribose polymerase 1 | PARP-1 | NM_001618 | 5´-GAGGTGGATGGGTTCTCTGA-3´ | 5´-ACACCCCTTGCACGTACTTC-3´ | 177 |
| X-ray Repair Cross-Complementing protein 1 | XRCC1 | NM_006297 | 5´-GTGCTGAGTGGCTTCCAGAAC-3´ | 5´-TTGGCAAAGGCACAGATGAG-3´ | 122 |
| Polynucleotide Kinase 3'-Phosphatase | PNKP | NM_007254 | 5´-GTGTCCCAAGATGAGAAGAGAG-3´ | 5´-CTGGGTACAAGATCCTCCAGT-3´ | 220 |
| Glyceraldehyde 3-phosphate dehydrogenase | GAPDH | NM_002046 & NM_001256799 | 5´-ACCACAGTCCATGCCATCAC-3´ | 5´-TCCACCACCCTGTTGCTGTA-3´ | 452 |

### 3.2.9. Statistical analysis

All experiments have been performed at least three times on independent cell passages. Statistical analyses of differences between manipulated cells and the respective controls were performed using Student's t-test. The significance of data from real-time Q-PCR was determined using student's t-test. Statistical significance was considered as $p<0.05$.

## 3.3. Results

### 3.3.1. The effect of TDP1 overexpression on the susceptibility of malignant glioma cells to anticancer therapy.

Several reports have been published with the primary goal to assess the correlation between TDP1 level and response to different anticancer agents. The majority of these studies were performed in non-cancerous cell lines. In this work we sought to identify the significance of the effect of TDP1 level on the response of malignant glioma cell lines to anticancer therapy. TMZ is part of the mainstay therapy of newly-diagnosed GBM and the TOP-poisons are key second-line alternative agents for the treatment of recurrent glioma. Therefore, we assessed the effect of TDP1 overexpression on the sensitivity of malignant glioma cell lines to TMZ, topotecan, etoposide and doxorubicin.

U87 and U251 cells were stably transfected with the full length TDP1 cDNA. Since the pCMV-sport 6 vector lacks a mammalian selection marker, and in order to obtain stably transfected cells, pIRES vector containing the TDP1 insert was generated via subcloning from the pCMV-sport 6-TDP1 plasmid. The pIRES vector contains a neomycin expressing gene which allows mammalian selection using G418 drug. In order to have cell populations that persistently overexpress TDP1 gene, stable transfection of U87 and U251 cells with pIRES-hrGFP-II-TDP1 was performed. Consequently, the effect of high TDP1 level on the response of the cell lines was tested. Confirmation of stable TDP1 overexpression in U87 and U251 cells was performed using real-time PCR

which showed an upregulation of the gene by approximately 5000 and 3000 times in U87 and U251 respectively **Figure 3.4.A** and **3.5.A**, and by western blot analysis which showed 68 kda TDP1 protein bands **Figure 3.4.B** and **3.5.B**. Although U87 and U251 cells are expected to possess high TDP1 levels, but they express very low levels of TDP1 protein that sometimes are not detectable by western blot. They are established cell lines and after several passages they lose genes that are not important for their survival. Thus a positive control (U87 cells transiently transfected with pCMV-sport6-TDP1) was used for band localization.

To address our hypothesis that malignant glioma cell lines overexpressing TDP1 are more resistant to anticancer therapy compared to parental cells, we performed MTT proliferation assays. The cells were treated for 72 hours with an increasing concentration of topotecan (TOP I-poisons), etoposide or doxorubicin (TOP II-poison), or with TMZ (alkylating agent). By measuring the relative number of viable cells using MTT assay, we found that compared to malignant glioma cell lines transfected with the empty vector (U87/mock & U251/mock), those overexpressing TDP1 (U87/TDP1 & U251/TDP1) showed similar sensitivity levels to anticancer therapy **(Figures 3.6 and 3.7).**

The three TOP-poisons used resulted in a concentration dependent cytotoxicity toward U87 and U251 cell lines. On the other hand, both U87 and U251 cells showed less sensitivity to TMZ. At the lowest concentration used (12.8 nM), topotecan killed the U87 and U251 cells by 31% and 86% respectively. At the highest concentration of topotecan (200 µM) the cytotoxic levels reached about 86% and 95% toward U87 and U251 cells respectively.

The concentration of topotecan that killed 50% of U87 and U251 cells ($IC_{50}$) was about 320 nM and 9 nM respectively. Etoposide $IC_{50}$ was 8 µM toward U87 cells and 320 nM toward U251 cells. Doxorubicin showed similar cytotoxic effects toward U87 and U251 cells with an $IC_{50}$ of 6.4 nM. The cytotoxic curves were less steep when U87 and U251 cells were treated with TMZ. The highest U87 cell death was only about 35%

achieved at 8 µM, while for U251 cells the highest death was about 55% achieved at 1000 µM. Among the entire anticancer agents, the cytotoxic effects did not correlate with TDP1 level. In other words, TDP1 overexpression in malignant glioma cell lines did not increase their resistance to anticancer therapy.

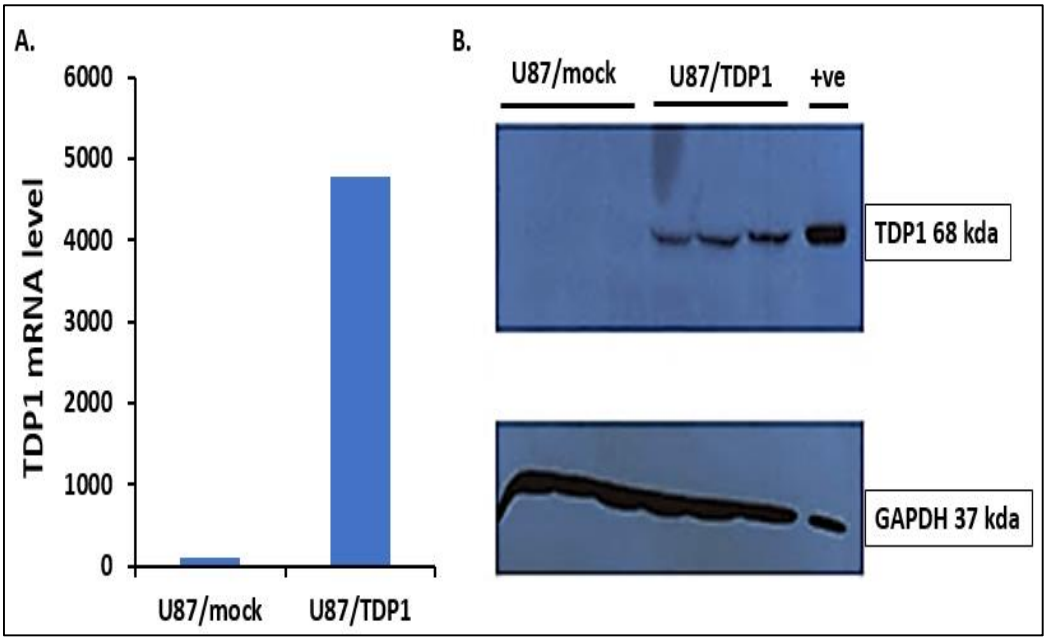


**Figure 3.4. TDP1 overexpression in U87 cell lines.** Analyses of TDP1 levels in U87 cells transfected with pIRES containing TDP1 full length cDNA (U87/TDP1) or pIRES empty vector (U87/mock). (**A**) Real-time PCR, TDP1 transcript levels were normalized to GAPDH and referred to the untransfected cells. (**B**) Western blot, control loading is shown by GAPDH.

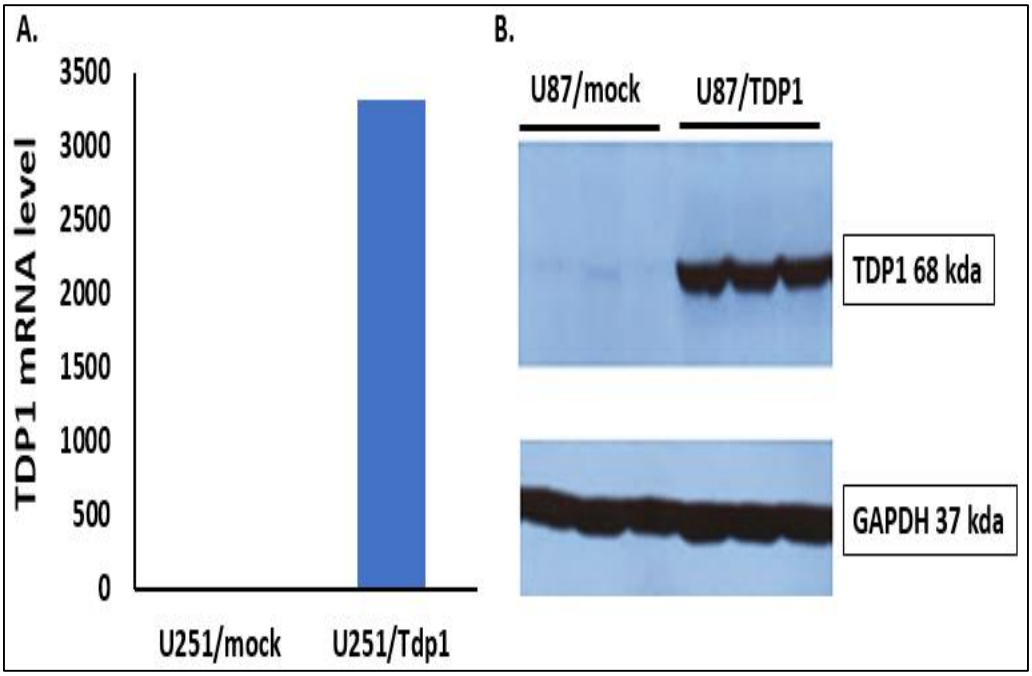


**Figure 3.5. TDP1 overexpression in U251 cell lines.** Analyses of TDP1 levels in U251 cells transfected with pIRES containing TDP1 full length cDNA (U251/TDP1) or pIRES empty vector (U251/mock). (**A**) Real-time PCR, TDP1 transcript levels were normalized to GAPDH and referred to the untransfected cells. (**B**) Western blot, control loading is shown by GAPDH.

### 3.3.2. The effect of TDP1 depletion on the susceptibility of U87/TDP1 cells to anticancer therapy.

To identify the effect of knocking-down TDP1 in U87/TDP1 cell lines on their response to anticancer agents, five shRNA clones targeting specific regions in TDP1 were used for the transduction experiment to produce U87/TDP1 cells with stable knockdown of TDP1. U87/TDP1 cells transduced with lentiviral particles carrying the five shRNA vectors and one control were allowed to grow with the presence of selection (1 µg/ml puromycin). Confirmation of TDP1 knockdown was determined by real-time PCR (**Figure 3.8**) and western blot analyses (**Figure 3.9**) showing that the level of TDP1

was significantly reduced in shTDP1 expressing cancer cells. The stable clones expressing shTDP1/48 (U87/TDP1/sh48) and shTDP1/50 (U87/TDP1/sh50) were chosen for subsequent experiments based on the degree of TDP1 knockdown which was about 80% reduction in TDP1 transcript levels from the control cells. U87/TDP1 cells expressing the empty vector (U87/TDP1/sh-ve) were used as the negative control.

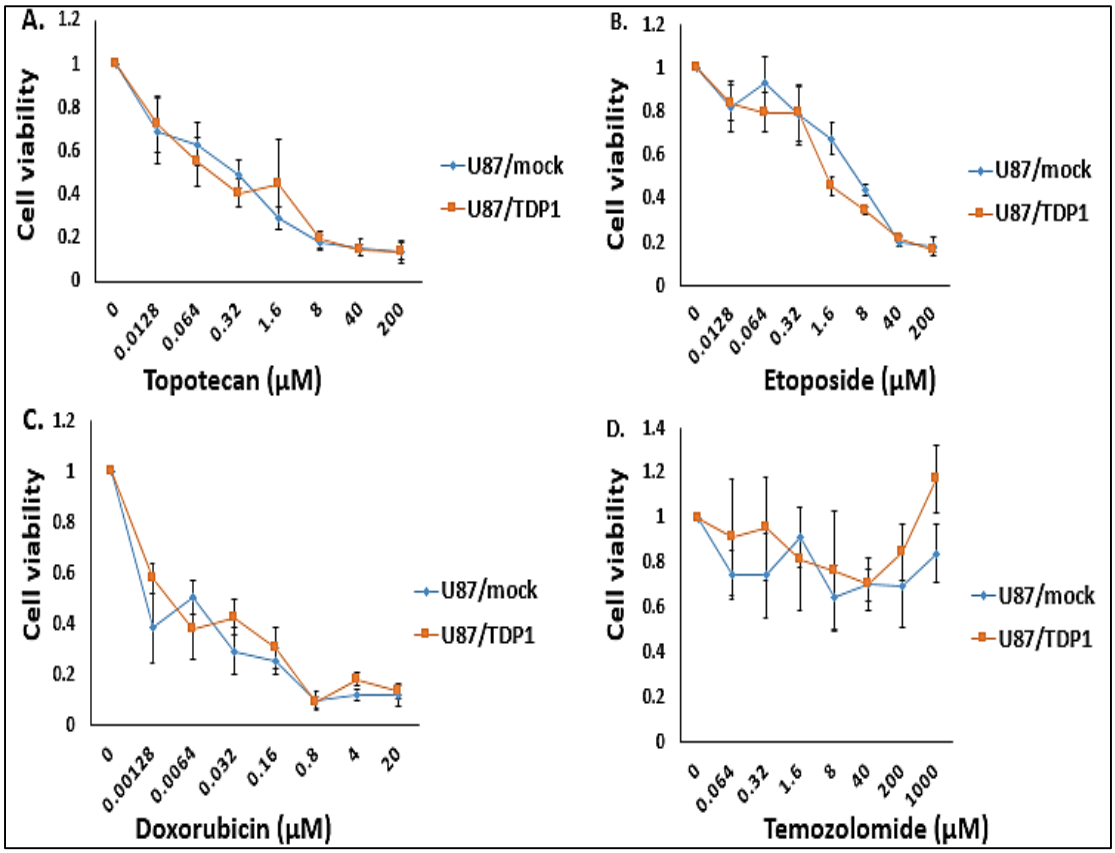


**Figure 3.6. MTT cytotoxicity assay to measure U87 cells sensitivity to anticancer agents and correlation with TDP1 level.** 2000 cells/well were treated for 72 h with an increasing concentration of topotecan (0.0128-200 µM), etoposide (0.0128-200 µM), doxorubicin (0.00128-20 µM), or temozolomide (0.064-1000 µM). Cell sensitivity of U87/TDP1 to anticancer agents was compared to that of U87/mock cells. Error bars represent standard deviation from triplicates. Data normalization to cells treated with DMSO or media was performed in excel. Student's t-test was used to calculate significant difference between viability curves.

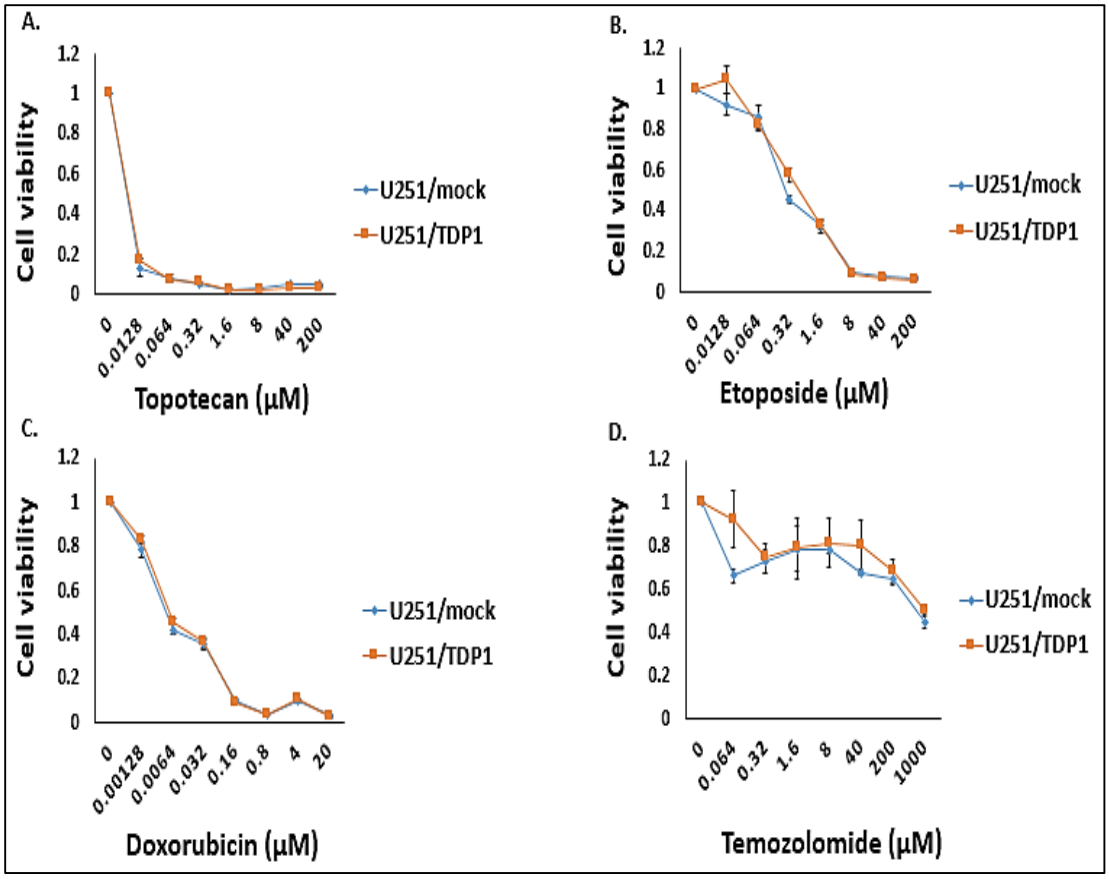


**Figure 3.7. MTT cytotoxicity assay to measure U251 cells sensitivity to anticancer agents and correlation with TDP1 level.** 2000 cells/well were treated for 72 h with an increasing concentration of topotecan (0.0128-200 µM), etoposide (0.0128-200 µM), doxorubicin (0.00128-20 µM), or temozolomide (0.064-1000 µM). Cell sensitivity of U251/TDP1 to anticancer agents was compared to that of U251/mock cells. Error bars represent standard deviation from triplicates. Data normalization to cells treated with DMSO or media was performed in excel. Student's t-test was used to calculate significant difference between viability curves.

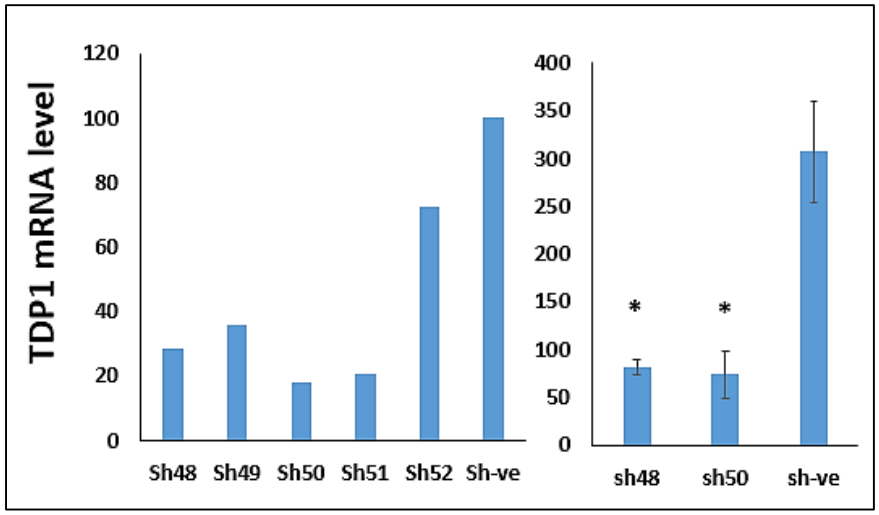


**Figure 3.8. Real-time PCR analysis of shRNA-Lentiviral knockdown of TDP1 in U87 cells overexpressing TDP1**. U87/TDP1 cells were transduced with recombinant lentiviruses carrying five different shRNA directed against TDP1 (sh48-52) and one PLKO.1 empty vector as the negative control (sh-ve).

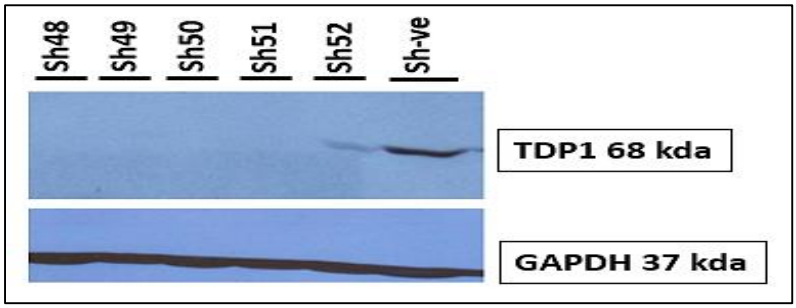


**Figure 3.9. Western blot analysis of shRNA-lentiviral knockdown of TDP1 in U87 cells overexpressing TDP1.** U87/TDP1 cells were transduced with recombinant lentiviruses carrying five different shRNA directed against TDP1 (sh48-52) and one PLKO.1 empty vector as the negative control (Sh-ve). Control loading is shown by GAPDH. Two TDP1 depleted cell lines were selected for further experiments (sh48 and sh50) and the control shRNA (sh-ve).

To address our hypothesis that low TDP1 levels enhance the sensitivity of malignant glioma cells to anticancer therapy, TDP1-knocked-down U87 cells were treated with a gradient concentration of topotecan, etoposide, doxorubicin or TMZ for 72 hours. Then the viability of cells was measured using MTT assay. As shown in **Figure 3.10.**, knockdown of TDP1 via transduction with shTDP1/48 or shTDP1/50 showed a trend of increased cytotoxicity of the anticancer agents toward U87/TDP1 cells. However, this sensitization effect was not statistically significant.

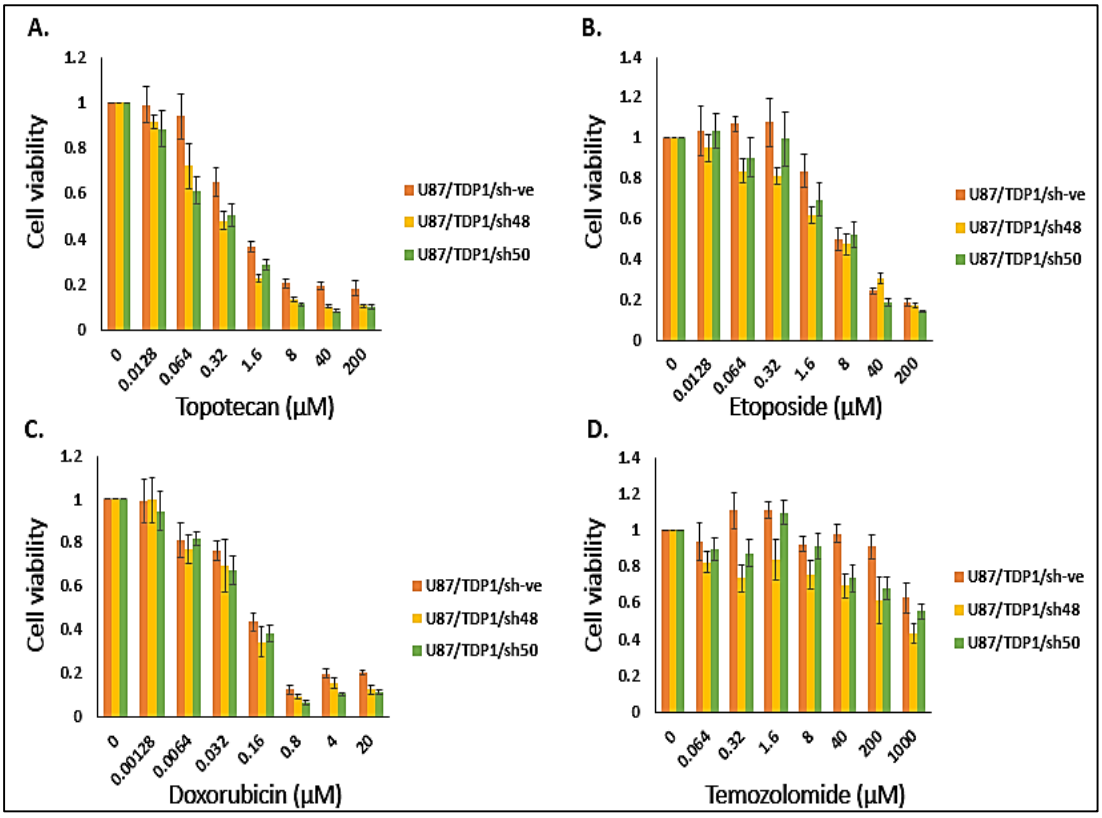


**Figure 3.10. Effect of TDP1 depletion on the susceptibility of U87/TDP1 cells to anticancer therapy.** Cell sensitivity of U87/TDP1 cells (2000 cell/well) stably transduced with TDP1 shRNAs or control shRNA to (**A**), topotecan; (**B**), etoposide; (**C**), doxorubicin; and (**D**), temozolomide. The antiproliferative effect of the drugs (72 h exposure) was assessed using the MTT assay.

### 3.3.3. The effect of small-molecule TDP1 inhibitors to potentiate the cytotoxicity of TOP-poisons toward malignant glioma cell lines

Manipulating TDP1 level in both U87 and U251 cell lines failed to prove our hypotheses of an enhanced sensitivity of cells to anticancer therapy upon reduced TDP1 expression via knockdown or increased resistance upon overexpression of the protein. Thus, to identify the effect of adding a TDP1 inhibitor to TOP-poisons on the sensitivity of cells to anticancer therapy, U87 cells were treated with an increasing concentration of a TDP1 inhibitor in the presence or absence of topotecan, etoposide, or doxorubicin. For this study we utilized small-molecule TDP1 inhibitor NSC128609 (T1) and NSC120686 (T2) that act as ligands and bind to the active site of the enzyme and prevent its binding to other substrates. Thus, they act as false substrates to the TDP1 enzyme. Hence, the available TDP1 enzyme and the whole SSB repair machinery responsible for the hydrolysis of the phosphodiesterase bond between TOP-enzyme and the DNA will be engaged with the false substrates, while the DNA breaks produced by the TOP-poisons; topotecan, etoposide and doxorubicin, will not be repaired and will accumulate in the cell leading eventually to cellular apoptosis.

To address our hypothesis that a combinational therapy of a TDP1-inhibitor and a TOP-poison is associated with better tumor cell-killing effect compared to treatment with a TOP-poison alone, we treated the cells for 72 hours with a gradient concentration of T1 or T2 alone or in combination with two different concentrations of the TOP-poisons.

Based on the MTT assay, the TDP1 inhibitor T1 had minimal cytotoxicity toward U87 cell lines with highest killing was only about 30% (**Figure 3.11.A**). On the other hand, T2 showed significant concentration dependent killing effect with an $IC_{50}$ was about 50 µM (**Figure 3.11.B**). The TDP1 level did not affect the response of the cells to these molecules (U87/TDP1 versus U87/mock). Moreover, our results showed that the combinational therapy of a TDP1 inhibitor and a TOP-poison resulted in enhanced cell killing effects compared to a monotherapy with a TDP1-inhibitor alone. Adding 0.05 µM

topotecan, 0.1 µM etoposide, or 0.01 µM doxorubicin to T1 resulted in about 15% to 60% reduction in cell viability in both U87/TDP1 and U87/mock cells (**Figure 3.12**). Using a 10x higher concentration of the TOP-poisons was associated with about 2x to 4x lower cell viability. On the other hand, adding TOP-poisons to T2 resulted in less significant reduction effects ranging from 12% to 40% (**Figure 3.13**). Thus, the potentiation of the cytotoxic effect was more pronounced when T1 was used. On the other hand, there was no difference in response between U87/TDP1 and U87/mock cells. Since U87 cells express negligible levels of TDP1 protein and since U87/TDP1 cells did not show differential response to the TDP1 inhibitors given alone or in combination with a TOP-poison, this may unfortunately suggest the non-specificity of these TDP1-inhibitors.

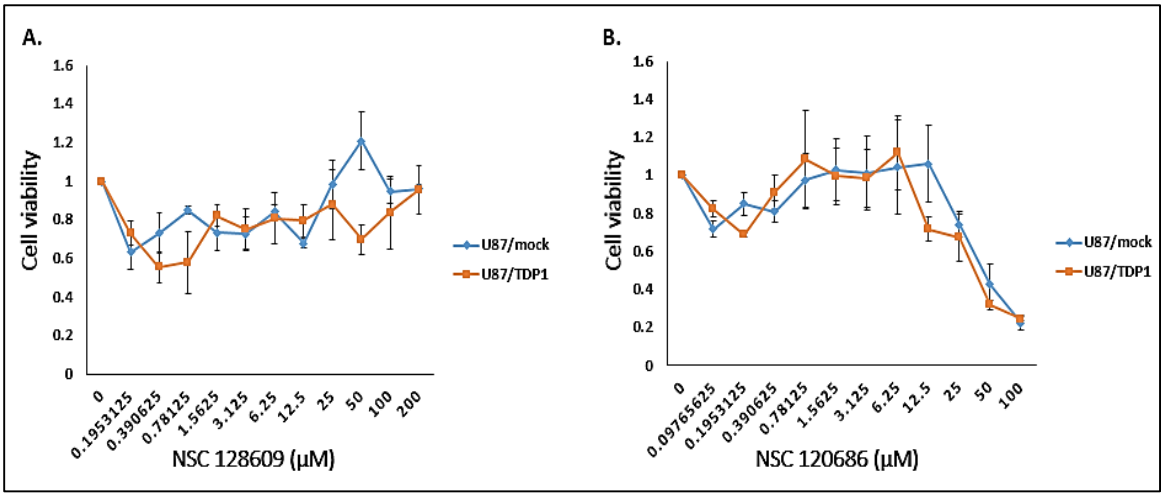


**Figure 3.11. Cell sensitivity to small-molecule TDP1 inhibitors in TDP1 overexpressing cells.** (**A**), NSC128609 (T1) and (**B**), NSC120686 (T2) show minimal cytotoxic effects in U87 cell lines. Viability after treatment of U87 cells transfected with the full length TDP1 (U87/TDP1) (2000 cell/well) was compared to that of cells transfected with the empty vector (U87/mock). Cell sensitivity was determined using MTT cytotoxicity assay 72 h after treatment with 0-200 µM of T1 and 0-100 µM of T2. Error bars represent standard deviation from triplicates.

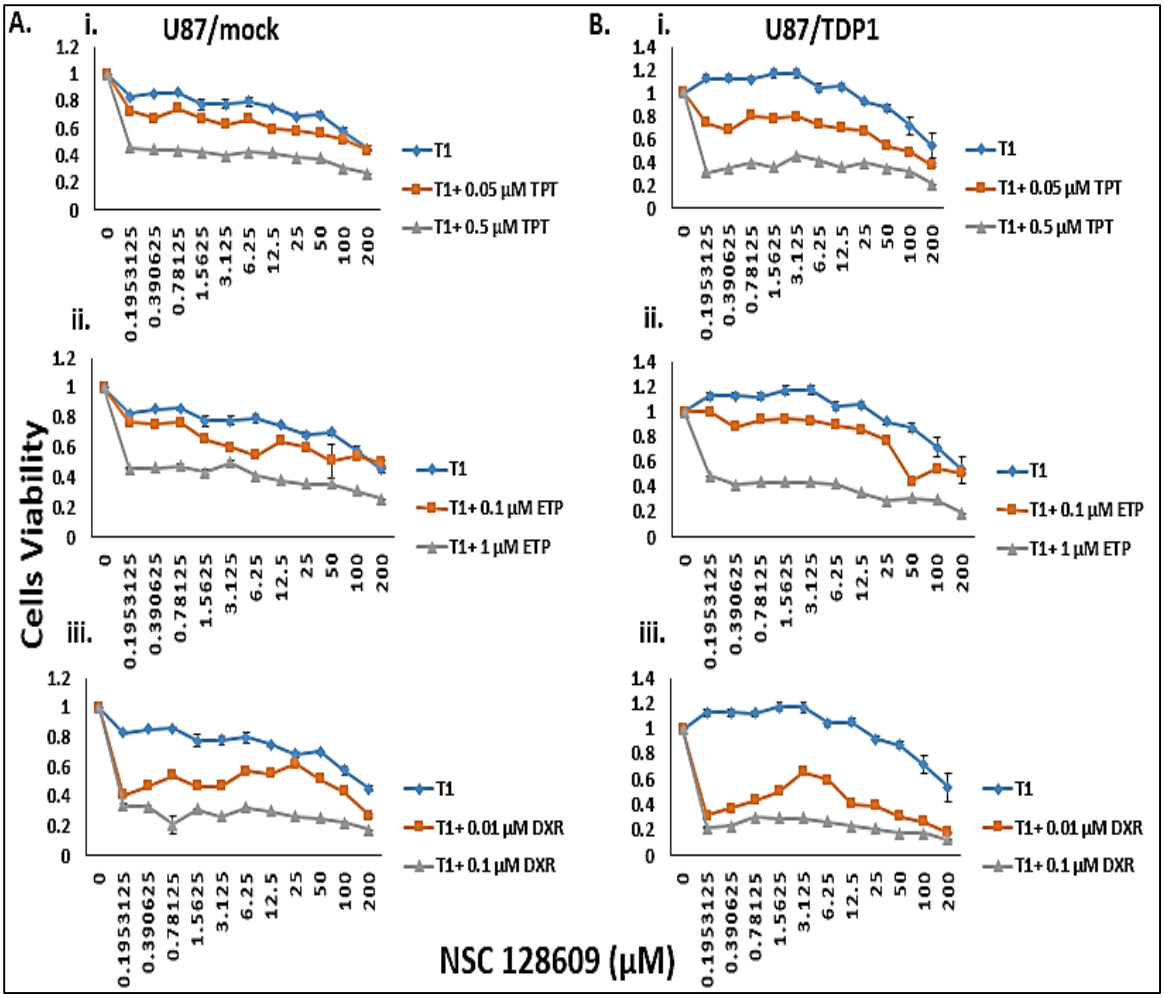


**Figure 3.12. MTT cytotoxicity assay. The addition of an anticancer agent potentiates the sensitivity of U87 cells to the TDP1 inhibitor NSC128609 (T1)**. Cells (2000/well) were treated with T1 for 72 h in the absence or presence of (i), topotecan (TPT) (0.05 or 0.5 µM); (**ii**), etoposide (ETP) (0.1 or 1 µM); or (**iii**), doxorubicin (DXR) (0.01 or 0.1 µM). The response to treatment in (**A**), U87/mock was comparable to that in (**B**), U87/TDP1. Data normalization to cells treated with DMSO or media was performed on excel. Error bars represent standard error from triplicates.

The previous findings suggest that there is a possible potentiation of the cytotoxic effect when utilizing a combinational therapy of a TDP1 inhibitor and a TOP-poison. However, in order to confirm that the observed differential effect is not due to the

anticancer agent itself; the cells were treated with the TOP-poison alone or in combination with 10 µM (reported $IC_{50}$ from a previous study) or 100 µM (10x higher than the $IC_{50}$) of T1. The TOP-poisons concentrations used were as mentioned before, 0.5 and 0.05 µM of topotecan, 0.1 and 1 µM of etoposide, or 0.01 and 0.1 µM of doxorubicin. After 72 hours of treatment, the cell viability was assessed via MTT cytotoxicity assay. In **Figure 3.14**, topotecan, etoposide and doxorubicin showed dose-dependent significant cytotoxic effects toward U87 cell lines.

As revealed in **Figure 3.14.A**, treatment with T1 alone at 100 µM resulted in about 38% reduction in cell viability. Compared to treatment with 100 µM T1 alone, the combinational therapy consisting of 100 µM T1 and 0.05 µM topotecan increased the cell killing by 1.5x. However, treatment with 0.05 µM topotecan alone or the combinational therapy resulted in comparable cytotoxic levels. Using a 10x higher topotecan concentration (0.5 µM) resulted in increased cytotoxic stress which was associated with higher cell death. However, in the combinational therapy, both {0.5 µM topotecan plus 100 µM T1} and {0.05 µM topotecan plus 100 µM T1} resulted in similar cytotoxic levels.

Utilizing 1 µM etoposide induced about 60% more cell death compared to the 0.1 µM concentration (**Figure 3.14.B**). A trend of enhanced sensitivity was observed when 0.1 µM etoposide was combined with 10 µM T1 rather than using 0.1 µM etoposide alone. This potentiation effect of etoposide cytotoxicity was statistically significant when 100 µM T1 was used. However, the benefit of adding T1 to etoposide was not evidenced when 1 µM of etoposide was used.

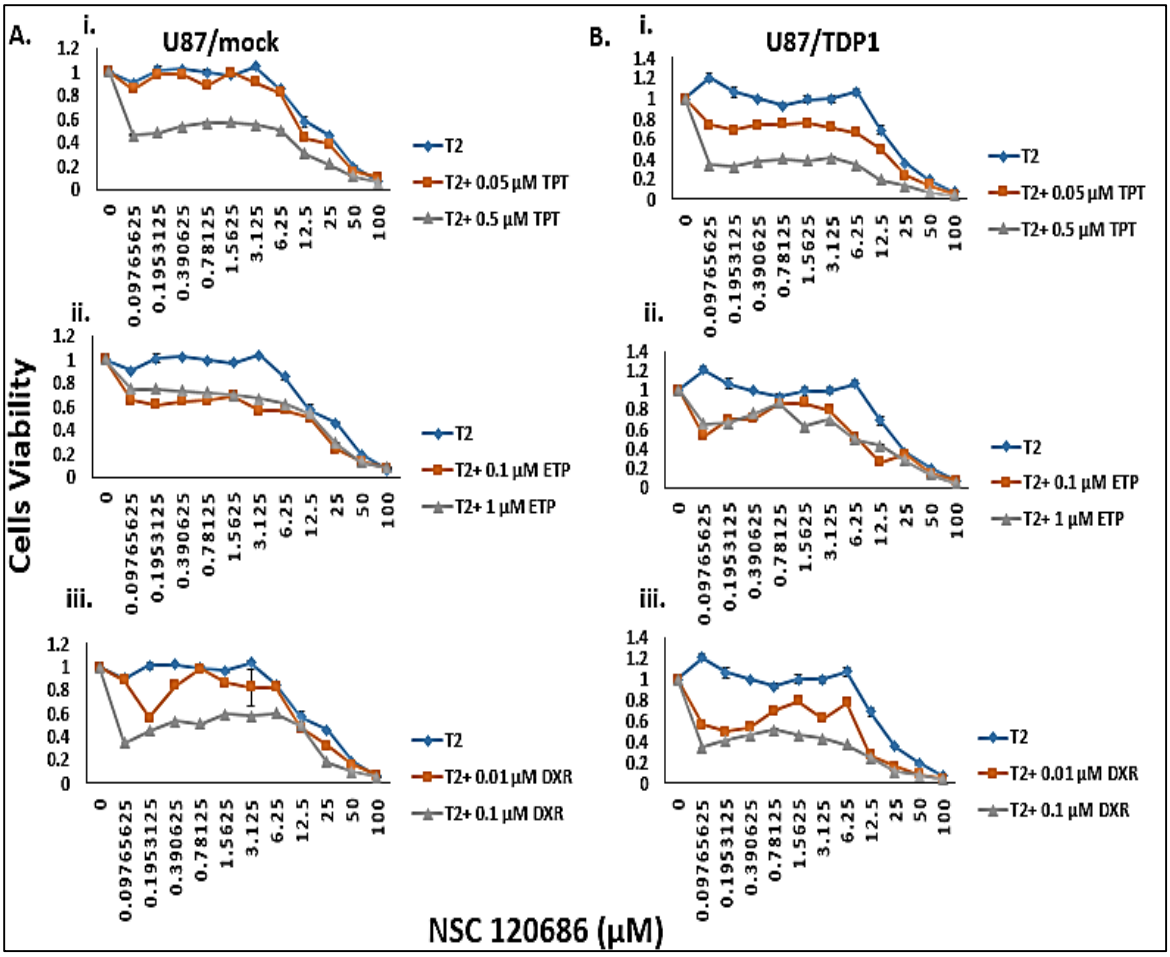


**Figure 3.13. MTT cytotoxicity assay. The addition of an anticancer agent potentiates the sensitivity of U87 cells to the TDP1 inhibitor NSC120686 (T2).** Cells (2000/well) were treated with T2 for 72 h in the absence or presence of (**i**), topotecan (TPT) (0.05 or 0.5 µM); (**ii**), etoposide (ETP) (0.1 or 1 µM); or (**iii**), doxorubicin (DXR) (0.01 or 0.1 µM). The response to treatment in (**A**), U87/mock was comparable to that in (**B**), U87/TDP1. Data normalization to cells treated with DMSO or media was performed on excel. Error bars represent standard error from triplicates.

Furthermore, doxorubicin resulted in cytotoxic effects that are dose dependent when used as a monotherapy (**Figure 3.14.C**). In the combinational therapy of doxorubicin and T1, there was a trend of enhanced cytotoxicity of doxorubicin but was not statistically significant. As monotherapies, increasing the concentration of topotecan,

etoposide, or doxorubicin by 10x resulted in a significant increase in U87 cell death. When combined with 10 μM T1, similar trend of dose-dependent TOP-poison cytotoxicity was observed. Nevertheless, when combined with 100 μM of T1, increasing the concentration of the TOP-poison was not associated with an increase in cell death (100 μM T1 + 0.05 μM topotecan vs. 100 μM T1 + 0.5 μM topotecan, 100 μM T1 + 0.1 μM etoposide vs. 100 μM T1 + 1 μM etoposide, and 100 μM T1 + 0.01 μM DXR vs. 100 μM T1 + 0.1 μM doxorubicin).

### 3.3.4 The effect of TDP1 overexpression or knockdown on the transcript levels of PARP-1, XRCC1, PNKP, TOP-I, TOP-IIβ, and MGMT

To explain our previous findings which revealed that modulating TDP1 level in malignant glioma cell lines did not affect their response to topotecan, etoposide, doxorubicin and temozolomide, we wanted to examine other DNA-repair genes that are known to be important for the final DNA-repair process of the DNA damage produced by these drugs. This is also supported by the fact that TDP1 does not work as a single enzyme and redundant alternative pathways are available for the DNA-repair.

BER has been found to be responsible for the repair of the TOP I-DNA cleavage complexes stalled by TOP I-poisons. Besides, it is part of the DNA-repair process for the N7-methylguanine produced by temozolomide which accounts for about 70% of the DNA damage produced by this drug. Since PARP-1, XRCC1, and PNKP are important components of the BER complex, and in order to obtain a complete and finalized BER process, this necessitates that all the genes involved in the machinery to be active. Also, in order to identify the effect of modulating the level of one of these genes, the level of all other genes must be fixed. Thus, we wanted to identify the effect of modulating TDP1 level on the three genes' expression levels.

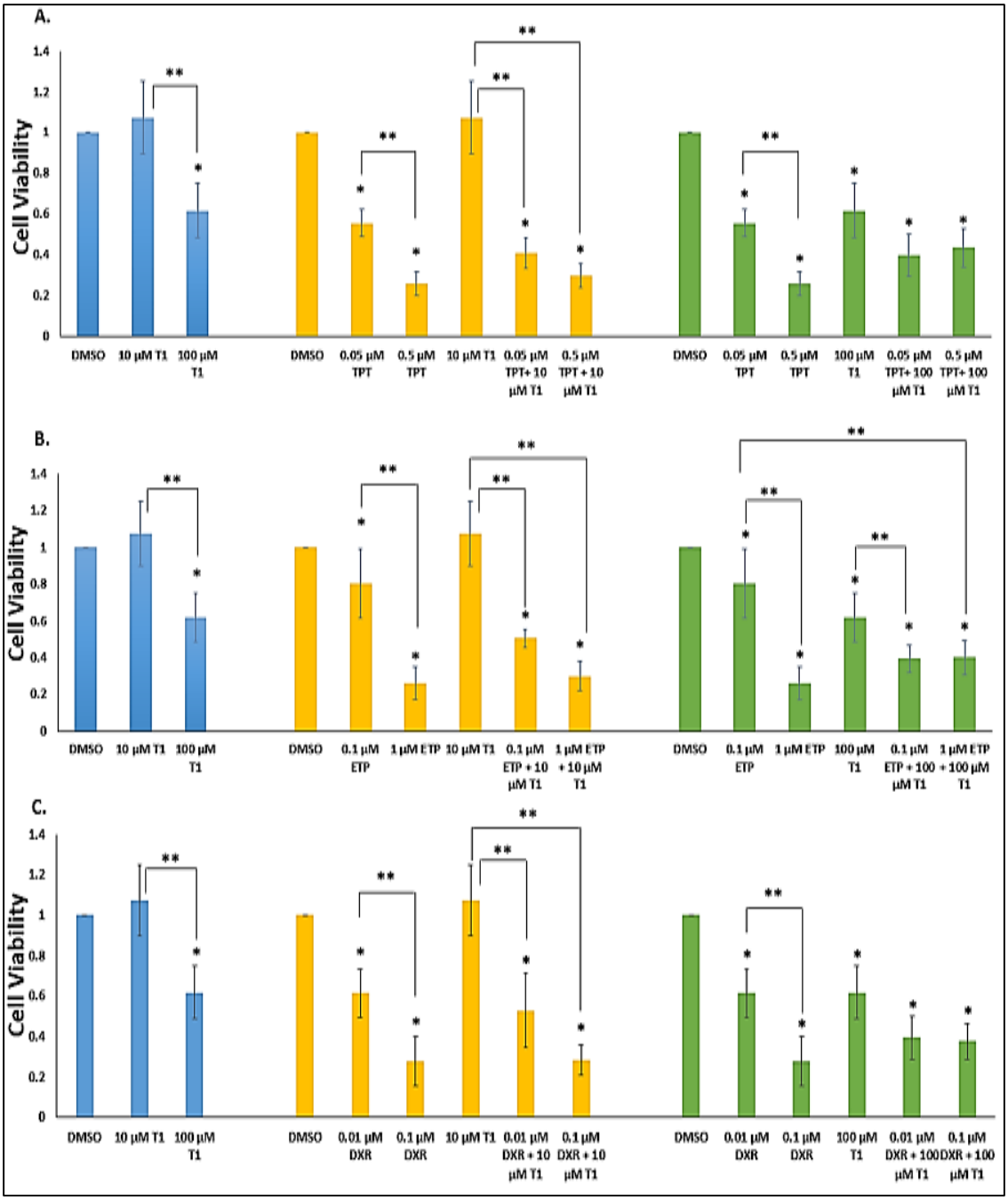


**Figure 3.14. MTT cytotoxicity assay. Treatment of U87 cells with a combinational therapy of NSC128609 and a TOP-poison.** Cells (2000/well) were treated with 10 µM or 100 µM NSC128609 (T1) for 72 h in the absence or presence of (**A**), Topotecan (TPT) (0.05 or 0.5 µM). (**B**), Etoposide (ETP) (0.1 or 1.0 µM). (**C**), Doxorubicin (DXR) (0.01 or 0.1 µM). (*****) represent statistically significant difference ($p < 0.05$) from control (cells treated with DMSO). (******) represent statistically significant differences ($p < 0.05$; student's t-test) between selected data lines. Error bars represent standard deviation from triplicates.

U87 and U251 cell lines transiently transfected with the full length TDP1 cDNA samples were analyzed for differential expression of various DNA-repair genes. As shown in **Figure 3.15.A & C,** TDP1 overexpression has resulted in a significant reduction of the mRNA expression levels of XRCC1 and PNKP in both U87/TDP1 and U251/TDP1 cell lines when compared to mock cells ($p < 0.001$ & $p = 0.002$ respectively; student's t-test). PARP-1 was expressed in very low levels in all types of cells but a significant upregulation was evidenced in U87/TDP1 cells compared to U87/mock cells ($p = 0.0003$, student's t-test). The reduction of the gene level of these SSB repair interplayers may contradict the effect of TDP1 overexpression, and the expected increased resistance of malignant glioma cells transfected with TDP1 to anticancer therapy may not be detected. On the other hand, TDP1 knockdown by TDP1/sh48 and TDP1/sh50 in U87/TDP1 was also associated with downregulation of XRCC1 compared to U87/TDP1 cells transduced with the control vector (TDP1/sh-ve) by percentages of 64% and 37% respectively (**Figure 3.15.B**, $p < 0.05$; student's t-test). PNKP was only significantly downregulated when TDP1 was knocked-down with TDP1/sh48 (**Figure 3.15.B**, $p = 0.0019$; student's t-test).

Since TOP-I and TOP-II are the cellular targets of topotecan, etoposide and doxorubicin; it is likely that the sensitivity to these drugs increases as the level of their target enzymes increases. Thus we wanted to identify the effect of modulating the TDP1 level in malignant glioma cell lines on the mRNA expression level of these two genes. **Figure 3.16.A** shows that TOP-I level has significantly increased by about three times after TDP1 overexpression in both U87 and U251 cell lines ($p < 0.005$, student's t-test). However, TOP-IIβ was downregulated by 1.34 times and 2 times in U87/TDP1 and U251/TDP1 respectively ($p < 0.05$, student's t-test) (**Figure 3.16.B**).

Although only about 5% of the DNA alkyl products generated by TMZ are O6-meG, it is considered to be the most severe type and is the main DNA-damage responsible for the cytotoxicity of this drug. MGMT is the sole DNA-repair enzyme that

fixes these adducts and hence induces resistance. Thus, we wanted to assess the effect of TDP1 level modulation on the expression level of MGMT in malignant glioma cell lines. We found that TDP1 overexpression has significantly increased MGMT mRNA expression level in both U87/TDP1 and U251/TDP1 compared to cells transfected with the empty vector by 1.3 times and 3.5 times respectively ($p < 0.05$, student's t-test) (**Figure 3.17.A**). A surprising finding is the extreme upregulation by about 124x of MGMT in U87/TDP1 cells transduced with TDP1/sh50 (**Figure 3.17.B**, $p < 0.0001$; student's t-test).

### 3.3.5 The effect of treatment of malignant glioma cell lines with anticancer agents and TDP1 inhibitors on the TDP1 level

In order to assess the effect of treating malignant glioma cells with anticancer agents or TDP1 inhibitors on the expression level of TDP1, we treated U87 cell lines stably overexpressing TDP1 with 20 µM of either T1 or T2, 0.05 µM topotecan, 0.1 µM etoposide, or 0.01 µM doxorubicin. The used concentrations cause minimal cell death but apply significant genotoxic stress that is required to assess their effect on the expression level of TDP1.

Treatment of U87/TDP1 with the anticancer agents at the mentioned concentrations did not affect TDP1 protein level, however treatment with TDP1 inhibitors, both T1 and T2 resulted in an upregulation of TDP1 protein level **Figure 3.18**. This may explain the inability of these inhibitors to potentiate the cytotoxicity of TOP-poisons because of the increased levels of TDP1 that are associated with enhanced repair of the DNA damage produced by these agents.

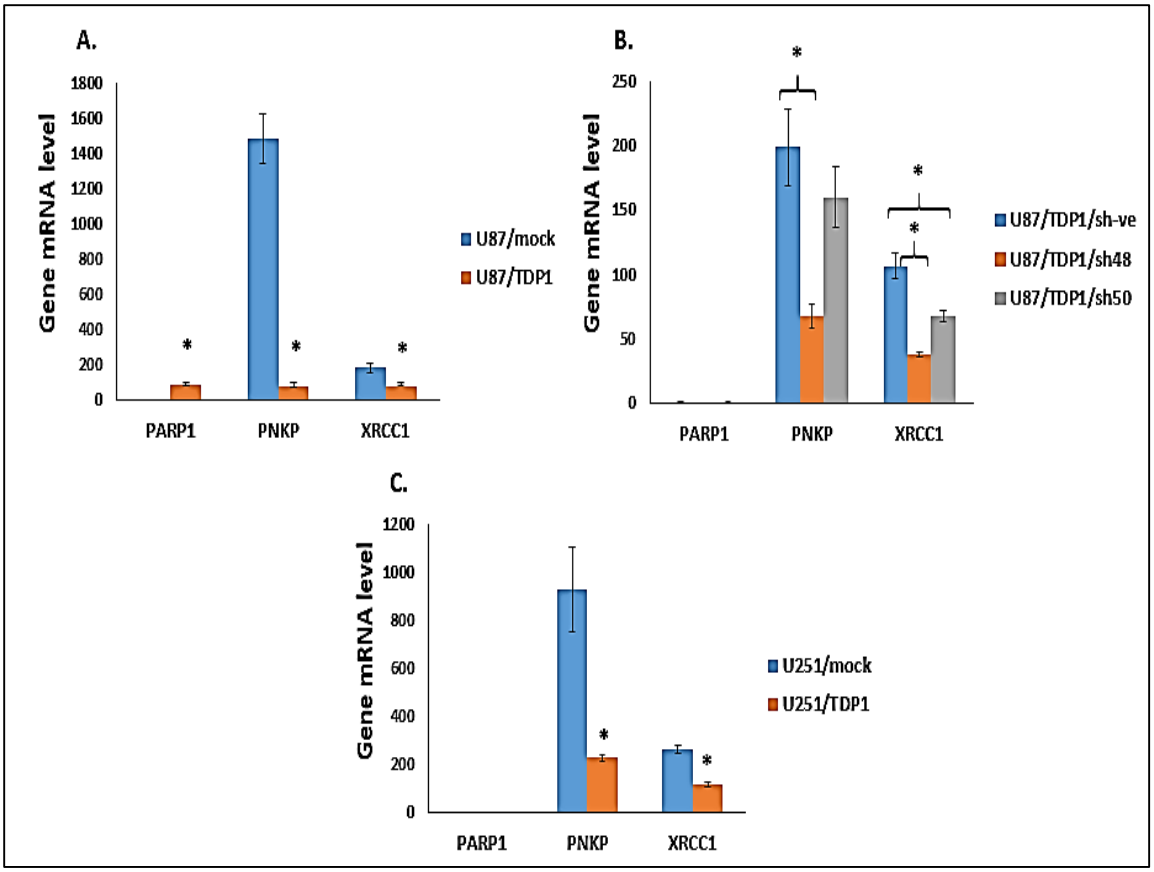


**Figure 3.15. Effect of manipulating TDP1 level in malignant glioma cell lines on the transcription level of base excision repair (BER) genes.** Real-time PCR was performed to measure PARP-1, PNKP and XRCC1 gene transcript levels in (**A**), U87 cell lines overexpressing TDP1 (U87/TDP1) compared to those transfected with empty vector (U87/mock), (**B**), U87/TDP1 cells knocked-down of TDP1 (U87/TDP1/sh48 & U87/TDP1/sh50) vs. control U87/TDP1 cells transduced with empty vector (U87/TDP1/sh-ve), and (**C**), U251 cell lines overexpressing TDP1 (U251/TDP1) compared to those transfected with empty vector (U251/mock). Gene mRNA levels were normalized to GAPDH and referred to the untransfected cells. Error bars represent standard deviation from triplicates. Data normalization was done in excel and student's t-test was used for significance testing.

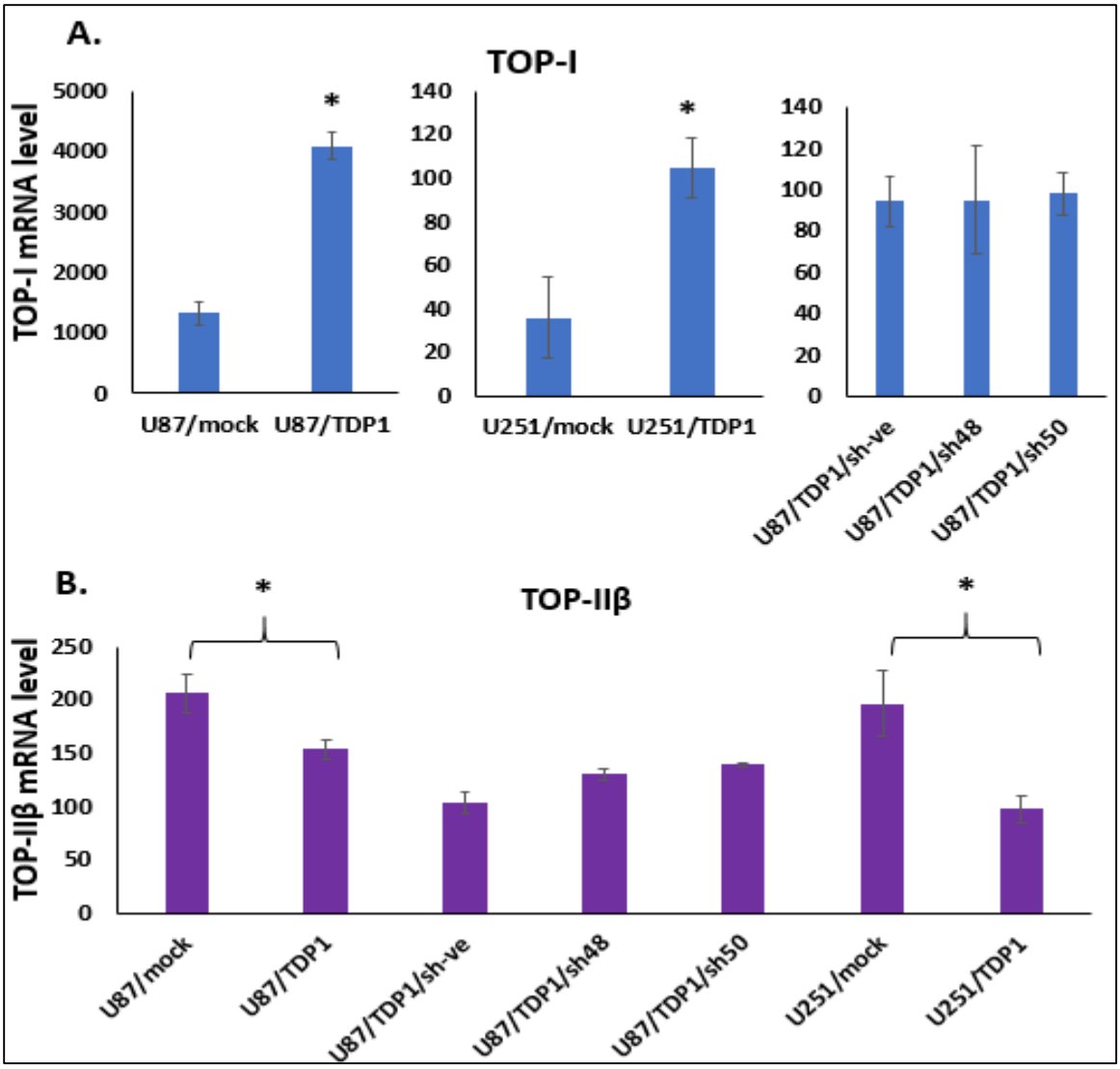


**Figure 3.16. Effect of manipulating TDP1 level in malignant glioma cell lines on transcription of topoisomerases.** Real-time PCR was performed to measure gene transcript levels of (**A**), topoisomerase I (TOP-I) and (**B**), topoisomerase IIβ (TOP-IIβ) in malignant glioma cell lines overexpressing TDP1 (U87/TDP1 and U251/TDP1) compared to those transfected with empty vector (U87/mock and U251/mock). Also in the same chart differential TOP-I and TOP-IIβ levels in U87/TDP1 cells knocked-down of TDP1 (U87/TDP1/sh48 & U87/TDP1/sh50) vs. control U87/TDP1 cells transduced with empty vector (U87/TDP1/sh-ve). Gene mRNA levels were normalized to GAPDH and referred to the untransfected cells. Error bars represent standard deviation from triplicates. Data normalization was done in excel and student's t-test was used for significance testing.

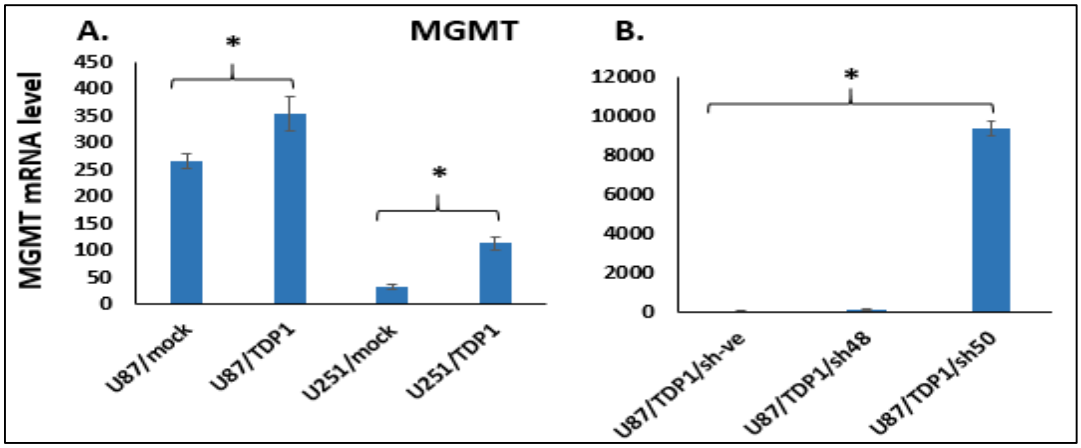


**Figure 3.17. Effect of manipulating TDP1 level in malignant glioma cell lines on transcription of O6-methylguanine methyltransferase (MGMT)**. Real-time PCR was performed to measure gene transcript levels of MGMT in (**A**), malignant glioma cell lines overexpressing TDP1 (U87/TDP1 and U251/TDP1) compared to those transfected with empty vector (U87/mock and U251/mock). (**B**), U87/TDP1 cells knocked-down of TDP1 (U87/TDP1/sh48 & U87/TDP1/sh50) vs. control U87/TDP1 cells transduced with empty vector (U87/TDP1/sh-ve). Gene mRNA levels were normalized to GAPDH and referred to the untransfected cells. Error bars represent standard deviation from triplicates. Data normalization was done in excel and student's t-test was used for significance testing.

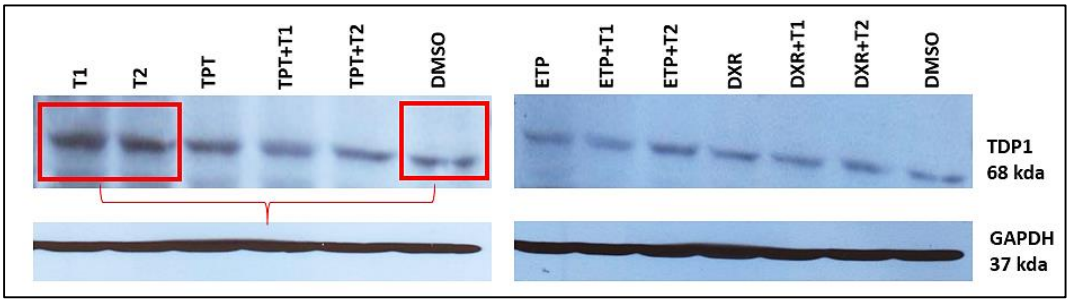


**Figure 3.18. Western blot Analysis of TDP1 level in U87/TDP1 cell lines treated with small-molecule TDP1 inhibitors and topoisomerase poisons given alone or in combinations**. 50,000 cell/well in a 12-well plate were treated for 72 h with 20 µM of either T1 (NSC128609), T2 (NSC120686), 0.05 µM topotecan (TPT), 0.1 µM etoposide (ETP), or 0.01 µM doxorubicin (DXR) or with a combination of T1 or T2 and a topoisomerase poison. Control loading is shown by GAPDH.

## 3.4. Discussion

Cellular response to DNA-damaging anticancer agents is a complex phenomenon and involves multiple and redundant pathways. For an efficient anti-neoplastic effect, the DNA damage produced by these agents needs to persist for a consequent cellular apoptosis to occur. Thus, DNA-repair pathways hinder the persistence of the DNA damages produced by the DNA-damaging anticancer agents and are considered critical determinants of the cytotoxicity of these agents.

In our analysis of TDP1 expression level in the previous chapter, we revealed that TDP1 protein is upregulated in glioma and this was correlated with tumor aggressiveness in terms of histological grade. Moreover, we showed that TDP1 acts as a negative prognostic factor where patients expressing high TDP1 transcript levels had poorer overall survival compared to those expressing low TDP1. Furthermore, previous studies have shown an overexpression of TDP1 in NSCLC and in colorectal carcinoma (110-112). Hence, indicating that cancer tissues may have acquired amplifications in TDP1 as a resistance mechanism, indicating that TDP1 could be a potential therapeutic target in glioma.

This study was designed with the aim to identify the effect of manipulating TDP1 level in malignant glioma cell lines on their sensitivity to DNA-damaging anticancer therapy. We postulated that high TDP1 levels will induce resistance of malignant glioma cell lines to topotecan, etoposide, doxorubicin and TMZ while TDP1 knockdown will enhance their sensitivity. In U87 and U251 overexpressing TDP1 generated *in vitro* by stable transfection of the full length TDP1 gene, we found that high TDP1 levels did not induce resistance of these cells to topotecan, etoposide, doxorubicin or TMZ. Although TDP1 is expected to be mandatory for the initiation of the DNA-repair process especially for TOPI-DNA cleavage complexes since it acts to remove the protein that is blocking the 3'-end of the DNA, we could not prove that its overexpression in malignant glioma cell lines induces resistance and thus increases tumor cell survival. Moreover, we

demonstrated that TDP1 depletion using short hairpin RNAs targeting specific regions in the TDP1 gene in U87 cells stably overexpressing TDP1 was associated with a trend of enhanced sensitivity to topotecan, etoposide, doxorubicin and TMZ. However, this sensitization effect was not statistically significant and did not result in major differences in the cell viability.

Overexpression of TDP1in U2-OS cell lines did not increase their resistance to camptothecin, topotecan, gimatecan (new TOP I-poison), etoposide or doxorubicin (157). Moreover, silencing of TDP1 in the same type of cell lines did not enhance their sensitivity to gimatecan (157). This indicates the complexity of the DNA-damage repair pathways which can involve various interplayers. In the same study by Perego *et al.*, cosilencing of TDP1 and XRCC1 was associated with significant reduction in cell survival compared to control (157). Another study by Zhang *et al.* (2011) showed that the PARP-1 inhibitor (ABT-888) lost the ability to enhance the cytotoxicity of camptothecin in mouse embryonic fibroblast (MEF) cells when TDP1 was knocked-down (158). Indeed, recent studies revealed that TDP1 exists in a protein complex with XRCC1, PARP-1, PNKP, pol β and Lig 3α, pointing at the importance of the interaction between all the proteins for a final and complete DNA-repair process.

In our search for an explanation of these results, we wanted to analyze the effect of modulating TDP1 level on the expression of other DNA-repair genes especially those that are known to be important interplayers in the repair of the DNA damage produced by the tested agents. In U87 and U251 transiently transfected with the full length TDP1 gene, we extracted the RNA and performed a quantitative real-time PCR analyses for PNKP, XRCC1, PARP-1, TOP-I, TOP-IIβ and MGMT. We found that TDP1 overexpression was associated with significant reduction in the gene levels of XRCC1 and PNKP which as explained earlier are important for the complete and finalized BER process. Their downregulation could have contradicted the effect of TDP1 overexpression and thus the expected increased resistance to probably all the drugs was

not evidenced. On the other hand, TDP1 knockdown resulted in downregulation of XRCC1 and PNKP. However, the downregulation of the three genes was not associated with enhanced sensitivity to anticancer therapy, mostly because of the presence of alternative and redundant repair pathways to fix the DNA damages produced by these agents as have been discussed in the first chapter.

Topoisomerases are the target enzymes for topotecan, etoposide and doxorubicin, thus it is conceivable that the sensitivity of cells to these drugs will increase as the levels of TOP-I and TOP-II increases. Our results showed that TOP-I transcript levels were significantly increased in both U87 and U251 after TDP1 overexpression. This increase in TOP-I and the decrease in PNKP and XRCC1 could have enhanced the sensitivity of the cells to topotecan and thus masked the increased resistance effect expected after increased TDP1 levels. On the other hand, TOP-IIβ levels were significantly reduced in both U87 and U251. Although TOP-IIβ downregulation may be expected to be associated with lower sensitivity to etoposide and doxorubicin, other DNA-repair processes are involved for the fixation of TOP II-poisons induced DNA damage. Those include the tyrosyl DNA phosphodiesterase 2 (TDP2) that hydrolyzes the 5'-phosphotyrosyl linkage in the TOP II-DNA cleavage complexes and the DNA DSB repair pathways including HR and NHEJ which act to fix the DSBs produced by these agents (88,159,160).

MGMT is the sole enzyme responsible for the repair of O6-methylguanine, the most cytotoxic DNA lesion produced by TMZ (161). MGMT low expression level and promoter hypermethylation were associated with increased overall survival of patients and enhanced sensitivity to TMZ compared to those with unmethylated MGMT (57,162). However, still about 50% of patients with MGMT promoter methylation are resistant to TMZ suggesting the presence of other DNA-repair enzymes that play a role in the repair of the DNA-damage generated by this alkylating agent (163). Our results showed that MGMT mRNA level significantly increases after TDP1 overexpression in both U87 and U251 cell lines. Nevertheless, this was not associated with increased resistance of cells to

TMZ. More than 70% of the DNA adducts produced by TMZ are repaired via the BER process. DNA methylpurine-*N*-glycosylase (MPG) is the enzyme responsible for the removal of the alkyl group from the N3 and N7 resulting in an AP-site that as mentioned previously can be further repaired by TDP1 and the BER consisting of PARP-1, XRCC1, PNKP, pol β and Lig 3α (52). Since TDP1 overexpression was associated with downregulation of PNKP and XRCC1, as explained earlier these changes in transcript levels of other important components of the BER process may have opposed the expected increased resistance when overexpressing TDP1 in malignant glioma cell lines and thus a sensitization effect to TMZ was not noticed.

Our attempts to achieve our aims in proving the speculated role of TDP1 in malignant glioma cell lines on response to DNA-damaging anticancer therapy were not accomplished by manipulating TDP1 expression level in these cell lines. Thus we wanted to proceed and investigate the suggested potentiation effect of small-molecule TDP1 inhibitors to TOP-poisons. In our study we utilized two TDP1 inhibitors T1 and T2. They are small-molecule ligands that bind to the active site of the TDP1 and thus act as pseudosubstrates for the enzyme. Treatment of U87 cells with gradient concentrations of T1 or T2 alone or in combination with two different concentrations of topotecan, etoposide or doxorubicin resulted in a reduction in the cellular viability in a dose dependent manner. However, this potentiation effect did not correlate with the TDP1 level because U87/mock cells showed sensitivity levels that are comparable to those shown by U87/TDP1. This may indicate that these inhibitors are not specific to TDP1. Moreover, to confirm these results, we treated the parental U87 cell lines with topotecan, etoposide, or doxorubicin at two different concentrations in the presence or absence of T1. Our findings showed that combining T1 with TOP-poisons resulted in a trend of increased cell death compared to treatment with the TOP-poisons alone. However, this effect did not reach significance. An interesting finding is that when treating the cells with a 10x higher concentration of a TOP-poison the viability significantly reduced

compared to the lower concentration. This was applicable when a 10 µM of T1 was added, but was not seen when 100 µM of T1 was used. This could be due to the upregulation of TDP1 when the cells were treated with T1 which may result in the repair of the DNA damage produced by TOP-poisons. Thus, contradicting the sensitization effect expected from combining a TDP1 inhibitor and a TOP-poison.

The above findings suggest that T1 and T2 may not be efficient TDP1-inhibitors to potentiate the cytotoxicity of TOP-poisons. A problem that we confronted to study the potential benefit from incorporating TDP1 inhibitors to the treatment regimen, was that all of the up to date developed TDP1 inhibitors are not specific for the enzyme and they inhibit several phosphodiesterase processes in the cell. Thus, the identification of novel selective and specific TDP1 inhibitors and the investigation of their role to enhance the sensitivity of cancer cells to anti-neoplastic therapy is required.

In summary, this study provides some evidence that redundant pathways are acting to limit the cytotoxicity of topotecan, etoposide, doxorubicin and TMZ. Modulation of TDP1 expression level in malignant glioma cell lines U87 and U251 was not sufficient to induce resistance to the previous DNA-damaging anticancer drugs with no difference in the viability between cells expressing low or high. Moreover, manipulating TDP1 level was associated with changes in the transcript levels of different DNA-repair enzymes that are known to play a role in affecting the sensitivity of the cells to the tested anticancer drugs including PARP-1, XRCC1 and PNKP (BER process), TOP-I and TOP-II, and MGMT which emphasizes the importance of studying several DNA-repair genes instead of focusing on a single gene when attempting to identify novel therapeutic targets.

## 3.5. Conclusion

This chapter emphasizes the fact that the response to the DNA-damaging anticancer agents is complex and several DNA-repair pathways which are redundant and

intertwined are vital determinants of the response of cancerous cells to the anti-neoplastic agents. Targeting TDP1 alone was not sufficient to modulate the response of malignant glioma cell lines to TOP-poisons and to TMZ. Thus, insisting the importance of targeting several cellular pathways in glioma instead of focusing on a single molecular signature. As a consequence, future targeting of several DNA-repair genes is required in order to be able to identify novel therapeutic strategies. Moreover, once identified and characterized preclinically for their synergistic effect, potential TDP1-inhibitors should be incorporated in the anticancer treatment regimen of glioma especially those including TOP I-poisons. If proven efficacious, these inhibitors could represent promising adjuvant anticancer agents that can be tested clinically for their efficiency to improve the sensitivity of glioma to therapy and the clinical outcome of patients.

# CHAPTER IV

# CONCLUSION, CHALLENGES AND FUTURE WORK

## 4.1. Summary and conclusions

Gliomas are the most common type of intracranial tumors and despite the advanced diagnostic tools and the aggressive treatment strategies; the prognosis is still poor with a median survival of 14.6 months for patients with glioblastoma. Although the WHO classification is considered the only clinically applied strategy for classifying patients into diagnostic and prognostic groups and for the stratification to therapy, it is inadequate to explain the variability in the clinical outcome between patients in the same pathological group. Thus, there is an urgent need for the identification of new, novel and more reliable prognostic factors that can classify gliomas into subgroups based on the genetic and molecular profiles of the patient and the tumor itself. Moreover, resistance to therapy represents a major problem in glioma that accounts for tumor progression and recurrence. Thus, comprehensive molecular analysis of glioma represents an important strategy for the identification of prognostic and predictive biomarkers and new therapeutic targets.

With the focus on the DNA-repair genes, comprehensive genetic analysis of the representative glioma population in this study revealed significant different gene expression profiles and numerous genetic alterations in genes of several DNA-repair pathways compared to control samples. A newly discovered DNA-repair gene that has shown significant amplification in glioma biopsies is tyrosyl-DNA phosphodiesterase 1 (TDP1). The product of this gene has been shown to hydrolyze various types of 3’-DNA lesions especially the phosphodiester bond in the irreversible topoisomerase I-DNA (TOP I-DNA) cleavage complexes produced mainly by topoisomerase I poisons (TOP I-poisons). Previous explorations have identified correlations between TDP1 and response to anticancer agents in different types of cell lines. Mutant and low levels of TDP1

resulted in enhanced sensitivity to camptothecin, topotecan and bleomycin, while TDP1 overexpression was associated with resistance to camptothecin and etoposide.

Further analysis of TDP1 transcript and protein levels in a subset of glioma samples revealed a correlation with glioma aggressiveness and clinical outcome. High TDP1 levels were found to be associated with poorer overall survival. Furthermore, glioma samples showed higher TDP1 protein levels compared to non-neoplastic tissues. Additionally, in astrocytic lineage, TDP1 protein level was found to be upregulated in higher grade samples and GBM tissues showed the highest expression levels. These findings suggest the negative prognostic value of TDP1, besides its possible utility to provide information regarding glioma subclassification. TDP1 as a molecular biomarker in glioma would be more ideal if we could prove its ability to predict response to therapy. As a consequence, and in order to understand the underlying molecular mechanism of how TDP1 may modulate the response of glioma to anticancer therapy, we moved forward to our *in vitro* investigations.

The findings of our *in vitro* analyses of the effect of TDP1 level on the response to the DNA-damaging anticancer agents revealed that targeting TDP1 alone was not enough to modulate the sensitivity of GBM cell lines to TOP-poisons and temozolomide. Overexpression of TDP1 in the GBM cell lines U87 and U251 did not increase their resistance to topotecan, etoposide, doxorubicin or temozolomide. Moreover, TDP1 knockdown in U87 cells overexpressing TDP1 resulted in only minimal enhancement of response to therapy. Thus, emphasizing the importance of the comprehensive role of several proteins in the DNA-repair process. This was evident by the differential expression levels of several DNA-repair genes when TDP1 level was manipulated in GBM cell lines. Moreover, although adding small-molecule TDP1 inhibitors was associated with a trend of enhanced cytotoxicity of TOP-poisons toward U87 cell lines, the effect was not significant and did not correlate with the TDP1 expression level probably due to the nonspecificity of these molecules.

In summary, our findings provide an unprecedented evidence of the role of TDP1 as a prognostic biomarker and as a potential tool for the subcategorization of patient with glioma. The enrollment of a larger sample size is required for further confirmation of TDP1 utility in glioma. On the other hand, TDP1 predictive value needs further validation where the role of other DNA-repair genes need to be investigated in combination with TDP1. A potentially vital area of study in glioma is the role of the base excision repair (BER) pathway in modulating the tumor response to anticancer therapy. Almost all of the up to date studies with limited practicality are focusing on the role of a single gene in the BER process. However the involvement of other genes especially TDP1 in these studies may represent a useful tool for better understanding of glioma response to therapy and to develop novel strategies to enhance the cytotoxicity of the currently implied anti-neoplastic agents.

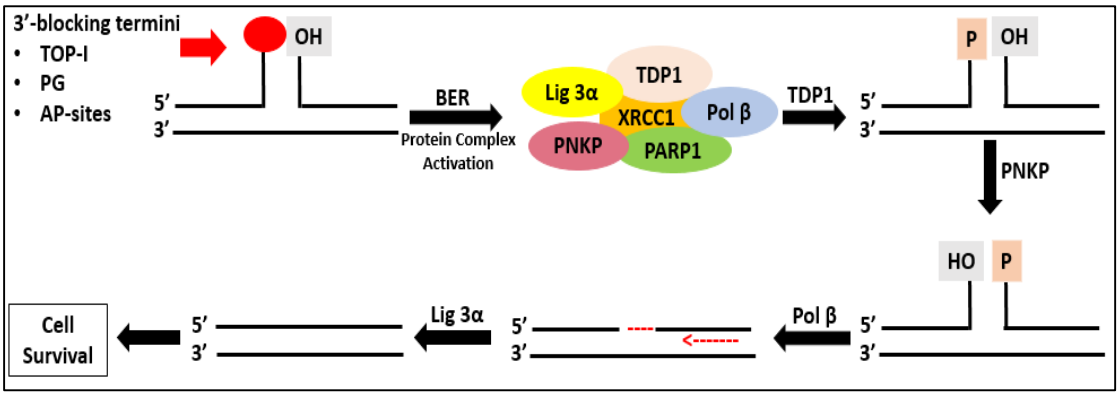


**Figure 4.1. A model for the involvement of Tyrosyl DNA-phosphodiesterase I (TDP1) in the repair of 3'-DNA lesions associated with DNA single strand breaks (SSBs) via the base excision repair (BER) process**. The SSBs are formed from several sources including irreversible topoisomerase I-DNA cleavage complexes, apurinic/apyrimidinic-sites (AP-sites) which are generated spontaneously due to base loss or induced or through enzymatic excision, and 3'-phosphoglycolate (3'-PG) which arises from reactive oxygen species produced endogenously or exogenously via anticancer agents.

## 4.2. Challenges

To achieve our attempts to exploring the effect of TDP1 level on the sensitivity of GBM cell lines to DNA-damaging anticancer agents, we required to manipulate TDP1 level exogenously via overexpression and knockdown experiments. This resulted in changes in the transcription level of other DNA repair genes. Thus, the identification of glioma cell lines that express endogenous high TDP1 levels is needed. However, trials to manipulate TDP1 level in these cell lines may also affect other genes' levels. Thus, a possible better way to assess the importance of TDP1 in glioma, is to introduce the cells to both a wild-type and a mutated TDP1 (that is associated with low or negligible enzymatic activity). This may represent a direct approach of investigating the importance of TDP1 in glioma cell line survival after treatment with anticancer therapy.

## 4.3. Future work

The role of TDP1 to predict the survival of patients and its utility to provide more accurate information regarding glioma diagnosis need to be verified in a larger population of glioma patients. This population need to include patients of both lineages (astrocytoma and oligodendroglioma) and should include all the histological grades in order to be able to provide a comprehensive view of the role of TDP1 in glioma. Moreover, high-throughput screening techniques to analyze TDP1 protein expression levels need to be implied. Regarding *in vitro* investigation of the role of TDP1 to predict the response to the DNA-damaging anticancer agents, it would be interesting to obtain glioma cell lines that express high endogenous TDP1 levels. The tendency of enhanced sensitivity to anticancer therapy when TDP1 was knocked-down in U87 cell lines did not reach significance because of the negligible TDP1 expression levels in the parental cell lines. Thus, knockdown of TDP1 in a glioma cell line that intrinsically express high TDP1 levels may yield promising results. Moreover, if new specific and selective TDP1

inhibitors were to be found, it will be beneficial to test their ability to potentiate the cytotoxicity of anticancer therapy in glioma.

If the above investigations produced significant results, *in vivo* analysis of the role of TDP1 would be the next step. Orthotopic xenografts mice models to resemble the glioma microenvironment will be utilized. The xenografts would be representative to low, medium and high TDP1 levels (in terms of protein level or enzymatic activity). Mice will be treated with various types of DNA-damaging anticancer agents such as topotecan, etoposide, doxorubicin and temozolomide. Then, the differential tumor response to therapy should be measured in terms of the time required for the mice to develop symptoms due to tumor burden and by measuring the tumor size. Also measurements of mice survival should be obtained and correlated with TDP1 level.

Based on the findings of our research besides the reports of the previous studies, a strong suggestion is to study the role of BER in glioma. TDP1 should be included in the investigational experiments and the role of several BER genes in affecting the response to DNA-damaging anticancer agents should be analyzed. The source of the importance of studying the comprehensive role of BER emerges from the previous studies that have shown a minimal change in the sensitivity to anticancer agents when only modulating a single gene.

If significant results are obtained from the preclinical investigation of the role of TDP1 in affecting the response of glioma to TOP-poisons and TMZ, the results can be translated into clinical studies. TOP-poisons in combination with small-molecule TDP1 inhibitors instead of TMZ can be incorporated in glioma therapy as an alternative regimen. This would be applicable in patients expressing unmethylated MGMT, thus resistance to TMZ would be expected. Moreover, patients expressing high TDP1 levels would be expected to show resistance to TOP-poisons, hence these agents will not represent potential second-line agents for the treatment of recurrent glioma. All of these

approaches will aid in paving the way of personalized medicine and individualization of therapy.

Despite the challenges that will be associated with investigating the relevance of TDP1 in glioma, if proven to play a role in predicting response to therapy, TDP1 may represent an important building block in the enhancement of glioma diagnosis, prognosis and response to therapy.

# APPENDIX A

## LIST OF CNAs AND LOH EVENTS IN GLIOMA STUDY POPULATION

**Table A.1. Summary of the most significant ($p < 0.0001$) loci/chromosomal regions showing CNAs (gain or loss) or LOH events in the glioma study population**

| Pathway | Chromosome | Gene | Cytoband | Genetic Alteration | Frequency (%) |
|---|---|---|---|---|---|
| DDR | 3 | ATR | 3p14.1 - 3q26.32 | CNA gain | 2 |
| | 3 | ATR | 3q22.3 - 3q25.1 | CNA loss | 4 |
| | 3 | ATRIP | 3p21.31 | CNA loss | 8 |
| | 3 | ATRIP | 3p25.1 - 3p21.1 | CNA gain | 4 |
| | 11 | ATM | 11q22.3 | LOH | 28 |
| | 17 | TP53 | 17p13.1 | LOH | 17 |
| | 17 | TP53 | 17p13.1 | LOH | 18 |
| | 17 | TP53 | 17p13.1 | LOH | 19 |
| BER | 14 | APEX1 | 14q11.2 - 14q32.31 | CNA loss | 6 |
| | 1 | PARP1 | 1q42.12 - 1q42.13 | CNA gain | 2 |
| | 14 | PARP2 | 14q11.2 - 14q32.31 | CNA loss | 6 |
| | 3 | PARP3 | 3p25.1 - 3p21.1 | CNA gain | 4 |
| | 19 | PNKP | 19q12 - 19q13.43 | CNA loss | 14 |
| | 19 | PNKP | 19q13.32 - 19q13.33 | CNA gain | 10 |
| | 19 | XRCC1 | 19q12 - 19q13.43 | CNA loss | 16 |
| | 19 | XRCC1 | 19q13.31 | CNA gain | 10 |
| NER | 19 | ERCC1 | 19q12 - 19q13.43 | CNA loss | 40 |
| | 19 | ERCC1 | 19q13.31 - 19q13.32 | CNA gain | 20 |
| | 19 | ERCC2 | 19q12 - 19q13.43 | CNA loss | 20 |
| | 19 | ERCC2 | 19q13.31 - 19q13.32 | CNA gain | 10 |
| | 13 | ERCC5 | 13q11 - 13q34 | CNA loss | 8 |
| | 10 | ERCC6 | 10p11.23 - 10q26.3 | CNA loss | 20 |
| | 5 | ERCC8 | 5q11.2 - 5q12.2 | CNA loss | 6 |
| | 5 | ERCC8 | 5q11.2 - 5q12.1 | CNA gain | 18 |
| | 2 | ERCC3 | 2q14.3 | LOH | 26 |
| MMR | 3 | MLH1 | 3p25.1 - 3p21.1 | CNA gain | 2 |

Table A.1 Continued

| | | | | | |
|---|---|---|---|---|---|
| | 14 | MLH3 | 14q11.2 - 14q32.31 | CNA loss | 10 |
| | 2 | MSH2 | 2p21 | CNA gain | 2 |
| | 5 | MSH3 | 5q12.3 - 5q14.3 | CNA gain | 6 |
| | 1 | MSH4 | 1p31.1 - 1p13.2 | CNA loss | 16 |
| | 2 | PMS1 | 2q32.1 - 2q33.1 | CNA loss | 2 |
| | 7 | PMS2 | 7p22.3 - 7q21.11 | CNA gain | 12 |
| | 7 | PMS2 | 7p22.1 | CNA loss | 4 |
| | 2 | MSH2 | 2p21 | LOH | 33 |
| | 6 | MSH5 | 6p21.33 | LOH | 15 |
| | 2 | MSH6 | 2p16.3 | LOH | 14 |
| | 2 | MSH6 | 2p16.3 | LOH | 15 |
| DSBR | 13 | BRCA2 | 13q12.12 - 13q21.1 | CNA loss | 8 |
| | 13 | LIG4 | 13q11 - 13q34 | CNA loss | 6 |
| | 13 | LIG4 | 13q33.2 - 13q33.3 | CNA gain | 2 |
| | 11 | MRE11A | 11q14.1 - 11q22.3 | CNA gain | 2 |
| | 5 | RAD50 | 5q15 - 5q33.2 | CNA gain | 4 |
| | 7 | XRCC2 | 7q34 - 7q36.3 | CNA gain | 30 |
| | 14 | XRCC3 | 14q32.33 | CNA loss | 12 |
| | 5 | XRCC4 | 5q14.2 - 5q14.3 | CNA gain | 18 |
| | 22 | XRCC6 | 22q13.1 - 22q13.2 | CNA loss | 14 |
| | 17 | BRCA1 | 17q21.31 | LOH | 38 |
| | 2 | XRCC5 | 2q35 | LOH | 33 |
| | 16 | ERCC4 | 16p13.3 - 16p12.3 | CNA gain | 2 |
| *miscellaneous | 19 | LIG1 | 19q12 - 19q13.43 | CNA loss | 20 |
| | 19 | LIG1 | 19q13.32 - 19q13.33 | CNA gain | 10 |
| | 20 | PCNA | 20p12.3 | CNA gain | 12 |
| | 20 | PCNA | 20p13 - 20p12.3 | CNA loss | 6 |
| | 12 | POLE | 12q24.33 | CNA gain | 6 |
| | 14 | POLE2 | 14q11.2- 14q32.31 | CNA loss | 6 |

Table A.1 Continued

| | | | | | |
|---|---|---|---|---|---|
| | 6 | POLH | 6p21.1 | CNA gain | 4 |
| | 6 | POLH | 6p21.1 | CNA loss | 4 |
| | 18 | POLI | 18q12.3- 18q22.1 | CNA loss | 2 |
| | 18 | POLI | 18p11.32- 18q21.32 | CNA gain | 2 |
| | 5 | POLK | 5q12.3 - 5q14.3 | CNA gain | 6 |
| | 10 | POLL | 10p11.23-10q26.3 | CNA loss | 26 |
| | 7 | POLM | 7p22.3 - 7q21.11 | CNA gain | 20 |
| | 3 | POLQ | 3q13.33 | CNA loss | 8 |
| | 3 | POLQ | 3p14.1 - 3q26.32 | CNA gain | 2 |
| | 5 | POLS | 5p15.33 - 5p14.2 | CNA loss | 4 |
| | 5 | POLS | 5p15.33 - 5p15.2 | CNA gain | 6 |
| | 14 | TDP1 | 14q31.3 - 14q32.13 | CNA gain | 8 |
| | 20 | TOP1 | 20p13 - 20q13.12 | CNA gain | 14 |
| | 8 | TOP1MT | 8q24.3 | CNA gain | 16 |
| | 3 | TOP2B | 3p25.1 - 3p21.1 | CNA gain | 4 |
| | 17 | TOP3A | 17p11.2 | CNA gain | 4 |
| | 22 | TOP3B | 22q11.21 - 22q11.22 | CNA gain | 10 |
| | 17 | LIG3 | 17q12 | LOH | 28 |
| | 8 | POLB | 8p11.21 | LOH | 36 |
| | 14 | TDP1 | 14q32.11 | LOH | 42 |
| | 14 | TDP1 | 14q32.11 | LOH | 42 |
| | 17 | TOP2A | 17q21.2 | LOH | 13 |

# APPENDIX B

# PUBLICATIONS AND ABSTRACTS

## Publications

**Al-Keilani MS,** Agarwal S, Alqudah MA, Sibenaller Z, Ryken TC, and Assem M. Role of tyrosyl DNA-phosphodiesterase as a prognostic and predictive factor in malignant glioma. In preparation for pubishing.

Alqudah MA, **Al-Keilani MS**, Agarwal S, Sibenaller Z, Ryken TC, and Assem M. NOTCH3 is a Prognostic Factor that Promotes Glioma Cell Proliferation, Migration and Invasion via Activation of CCND1 and EGFR. PlosOne, 2013, in press.

Agarwal S, **Al-Keilani MS**, Alqudah MA, Sibenaller Z, Ryken TC, and Assem M. Tumor derived mutations of Protein Tyrosine Phosphatase Receptor Type K affect its function and alter sensitivity to chemotherapeutics in glioma. PlosOne, May 2013, 8(5): e62852.

Assem M, **Al-Keilani MS**, Agarwal S, Alqudah MA, Sibenaller Z, and Ryken TC. Enhancing diagnosis, prognosis and therapeutic outcome prediction of gliomas using genomics. OMICS, March 2012, 16(3): 113-122.

## Abstracts

**Al-Keilani MS**, Agarwal S, Alqudah MA, Sibenaller Z, Ryken TC, and Assem M. Tyrosyl DNA Phosphodiesterase I (TDP1): A Promising Predictive and Prognostic Biomarker in Malignant Glioma. Pharmaceutics Graduate Student Research Meeting. University of Nebraska Medical Center, College of Pharmacy, Omaha state. June 2012.

**Al-Keilani MS**, Agarwal S, Alqudah MA, Sibenaller Z, Ryken TC, and Assem M. Tyrosyl DNA Phosphodiesterase I (TDP1) is a Prognostic Factor and its Inhibition Synergizes Response to Topoisomerase Poisons in Malignant Glioma. American Association for Cancer Research 2013 Annual Meeting. The Walter E. Washington Convention Center, Washington, D.C. April 2013.

# REFERENCES

(1) Borgo MC, Pereira JL, Lima FB, Brandao RA, Carvalho GT, Costa BS. Glioblastoma multiforme in childhood: a case report. Clinics (Sao Paulo) 2010;65(9):923-925.

(2) Zalatimo O, Zoccoli CM, Patel A, Weston CL, Glantz M. Impact of genetic targets on primary brain tumor therapy: what's ready for prime time? Adv Exp Med Biol 2013;779:267-289.

(3) Schwartzbaum JA, Fisher JL, Aldape KD, Wrensch M. Epidemiology and molecular pathology of glioma. Nat Clin Pract Neurol 2006 Sep;2(9):494-503; quiz 1 p following 516.

(4) Maher EA, Furnari FB, Bachoo RM, Rowitch DH, Louis DN, Cavenee WK, et al. Malignant glioma: genetics and biology of a grave matter. Genes Dev 2001 Jun 1;15(11):1311-1333.

(5) van Thuijl HF, Ylstra B, Wurdinger T, van Nieuwenhuizen D, Heimans JJ, Wesseling P, et al. Genetics and pharmacogenomics of diffuse gliomas. Pharmacol Ther 2013 Jan;137(1):78-88.

(6) Ohgaki H, Kleihues P. Genetic pathways to primary and secondary glioblastoma. Am J Pathol 2007 May;170(5):1445-1453.

(7) Kim YH, Nobusawa S, Mittelbronn M, Paulus W, Brokinkel B, Keyvani K, et al. Molecular classification of low-grade diffuse gliomas. Am J Pathol 2010 Dec;177(6):2708-2714.

(8) Nakamura M, Watanabe T, Yonekawa Y, Kleihues P, Ohgaki H. Promoter methylation of the DNA repair gene MGMT in astrocytomas is frequently associated with G:C --> A:T mutations of the TP53 tumor suppressor gene. Carcinogenesis 2001 Oct;22(10):1715-1719.

(9) Hofer S, Lassman AB. Molecular markers in gliomas: impact for the clinician. Target Oncol 2010 Sep;5(3):201-210.

(10) Vredenburgh JJ, Desjardins A, Reardon DA, Friedman HS. Experience with irinotecan for the treatment of malignant glioma. Neuro Oncol 2009 Feb;11(1):80-91.

(11) Klautke G, Schutze M, Bombor I, Benecke R, Piek J, Fietkau R. Concurrent chemoradiotherapy and adjuvant chemotherapy with Topotecan for patients with glioblastoma multiforme. J Neurooncol 2006 Apr;77(2):199-205.

(12) Franceschi E, Cavallo G, Scopece L, Paioli A, Pession A, Magrini E, et al. Phase II trial of carboplatin and etoposide for patients with recurrent high-grade glioma. Br J Cancer 2004 Sep 13;91(6):1038-1044.

(13) Pope WB, Sayre J, Perlina A, Villablanca JP, Mischel PS, Cloughesy TF. MR imaging correlates of survival in patients with high-grade gliomas. AJNR Am J Neuroradiol 2005 Nov-Dec;26(10):2466-2474.

(14) Wood JR, Green SB, Shapiro WR. The prognostic importance of tumor size in malignant gliomas: a computed tomographic scan study by the Brain Tumor Cooperative Group. J Clin Oncol 1988 Feb;6(2):338-343.

(15) Jiang H, Ren X, Zhang W, Ma J, Sui D, Jiang Z, et al. A new prognostic scoring scale for patients with primary WHO grade III gliomas based on molecular predictors. J Neurooncol 2013 Feb;111(3):367-375.

(16) Gottesman MM. Mechanisms of cancer drug resistance. Annu Rev Med 2002;53:615-627.

(17) Bredel M. Anticancer drug resistance in primary human brain tumors. Brain Res Brain Res Rev 2001 Apr;35(2):161-204.

(18) Stupp R, Tonn JC, Brada M, Pentheroudakis G, ESMO Guidelines Working Group. High-grade malignant glioma: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol 2010 May;21 Suppl 5:v190-3.

(19) Stupp R, Hegi ME, Mason WP, van den Bent MJ, Taphoorn MJ, Janzer RC, et al. Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial. Lancet Oncol 2009 May;10(5):459-466.

(20) Wen PY, Kesari S. Malignant gliomas in adults. N Engl J Med 2008 Jul 31;359(5):492-507.

(21) Xu T, Chen J, Lu Y, Wolff JE. Effects of bevacizumab plus irinotecan on response and survival in patients with recurrent malignant glioma: a systematic review and survival-gain analysis. BMC Cancer 2010 Jun 2;10:252-2407-10-252.

(22) Wang Y, Jiang T. Understanding high grade glioma: molecular mechanism, therapy and comprehensive management. Cancer Lett 2013 May 1;331(2):139-146.

(23) Tentori L, Graziani G. Recent approaches to improve the antitumor efficacy of temozolomide. Curr Med Chem 2009;16(2):245-257.

(24) Quinn JA, Jiang SX, Reardon DA, Desjardins A, Vredenburgh JJ, Friedman AH, et al. Phase II trial of temozolomide (TMZ) plus irinotecan (CPT-11) in adults with newly diagnosed glioblastoma multiforme before radiotherapy. J Neurooncol 2009 Dec;95(3):393-400.

(25) Cecchi M, Vaiani M, Ceroti M, Banfi R. A retrospective observational analysis to evaluate the off-label use of bevacizumab alone or with irinotecan in recurrent glioblastoma. Int J Clin Pharm 2013 Mar 28.

(26) Grabenbauer GG, Gerber KD, Ganslandt O, Richter A, Klautke G, Birkmann J, et al. Effects of concurrent topotecan and radiation on 6-month progression-free survival in the primary treatment of glioblastoma multiforme. Int J Radiat Oncol Biol Phys 2009 Sep 1;75(1):164-169.

(27) Lesimple T, Riffaud L, Frappaz D, Ben Hassel M, Gedouin D, Bay JO, et al. Topotecan in combination with radiotherapy in unresectable glioblastoma: a phase 2 study. J Neurooncol 2009 Jun;93(2):253-260.

(28) Massimino M, Spreafico F, Cefalo G, Riccardi R, Tesoro-Tess JD, Gandola L, et al. High response rate to cisplatin/etoposide regimen in childhood low-grade glioma. J Clin Oncol 2002 Oct 15;20(20):4209-4216.

(29) Parkinson JF, Wheeler HT, McDonald KL. Contribution of DNA repair mechanisms to determining chemotherapy response in high-grade glioma. J Clin Neurosci 2008 Jan;15(1):1-8.

(30) Smith J, Tho LM, Xu N, Gillespie DA. The ATM-Chk2 and ATR-Chk1 pathways in DNA damage signaling and cancer. Adv Cancer Res 2010;108:73-112.

(31) Kemp MG, Lindsey-Boltz LA, Sancar A. The DNA damage response kinases DNA-dependent protein kinase (DNA-PK) and ataxia telangiectasia mutated (ATM) Are stimulated by bulky adduct-containing DNA. J Biol Chem 2011 Jun 3;286(22):19237-19246.

(32) Pietenpol JA, Stewart ZA. Cell cycle checkpoint signaling: cell cycle arrest versus apoptosis. Toxicology 2002 Dec 27;181-182:475-481.

(33) Reinhardt HC, Schumacher B. The p53 network: cellular and systemic DNA damage responses in aging and cancer. Trends Genet 2012 Mar;28(3):128-136.

(34) Robertson AB, Klungland A, Rognes T, Leiros I. DNA repair in mammalian cells: Base excision repair: the long and short of it. Cell Mol Life Sci 2009 Mar;66(6):981-993.

(35) de Boer J, Hoeijmakers JH. Nucleotide excision repair and human syndromes. Carcinogenesis 2000 Mar;21(3):453-460.

(36) Friedberg EC. How nucleotide excision repair protects against cancer. Nat Rev Cancer 2001 Oct;1(1):22-33.

(37) Li GM. Mechanisms and functions of DNA mismatch repair. Cell Res 2008 Jan;18(1):85-98.

(38) Hsieh P, Yamane K. DNA mismatch repair: molecular mechanism, cancer, and ageing. Mech Ageing Dev 2008 Jul-Aug;129(7-8):391-407.

(39) Schonn I, Hennesen J, Dartsch DC. Cellular responses to etoposide: cell death despite cell cycle arrest and repair of DNA damage. Apoptosis 2010 Feb;15(2):162-172.

(40) Burma S, Chen BP, Chen DJ. Role of non-homologous end joining (NHEJ) in maintaining genomic integrity. DNA Repair (Amst) 2006 Sep 8;5(9-10):1042-1048.

(41) Helleday T, Lo J, van Gent DC, Engelward BP. DNA double-strand break repair: from mechanistic understanding to cancer treatment. DNA Repair (Amst) 2007 Jul 1;6(7):923-935.

(42) Lans H, Marteijn JA, Vermeulen W. ATP-dependent chromatin remodeling in the DNA-damage response. Epigenetics Chromatin 2012 Jan 30;5:4-8935-5-4.

(43) Loser DA, Shibata A, Shibata AK, Woodbine LJ, Jeggo PA, Chalmers AJ. Sensitization to radiation and alkylating agents by inhibitors of poly(ADP-ribose) polymerase is enhanced in cells deficient in DNA double-strand break repair. Mol Cancer Ther 2010 Jun;9(6):1775-1787.

(44) Mukherjee B, McEllin B, Camacho CV, Tomimatsu N, Sirasanagandala S, Nannepaga S, et al. EGFRvIII and DNA double-strand break repair: a molecular mechanism for radioresistance in glioblastoma. Cancer Res 2009 May 15;69(10):4252-4259.

(45) Kao GD, Jiang Z, Fernandes AM, Gupta AK, Maity A. Inhibition of phosphatidylinositol-3-OH kinase/Akt signaling impairs DNA repair in glioblastoma cells following ionizing radiation. J Biol Chem 2007 Jul 20;282(29):21206-21212.

(46) McCord AM, Jamal M, Williams ES, Camphausen K, Tofilon PJ. CD133+ glioblastoma stem-like cells are radiosensitive with a defective DNA damage response compared with established cell lines. Clin Cancer Res 2009 Aug 15;15(16):5145-5153.

(47) Bobola MS, Blank A, Berger MS, Stevens BA, Silber JR. Apurinic/apyrimidinic endonuclease activity is elevated in human adult gliomas. Clin Cancer Res 2001 Nov;7(11):3510-3518.

(48) Naidu MD, Mason JM, Pica RV, Fung H, Pena LA. Radiation resistance in glioma cells determined by DNA damage repair activity of Ape1/Ref-1. J Radiat Res 2010;51(4):393-404.

(49) Galia A, Calogero AE, Condorelli R, Fraggetta F, La Corte A, Ridolfo F, et al. PARP-1 protein expression in glioblastoma multiforme. Eur J Histochem 2012 Feb 27;56(1):e9.

(50) Russo AL, Kwon HC, Burgan WE, Carter D, Beam K, Weizheng X, et al. In vitro and in vivo radiosensitization of glioblastoma cells by the poly (ADP-ribose) polymerase inhibitor E7016. Clin Cancer Res 2009 Jan 15;15(2):607-612.

(51) Friedman HS, Kerby T, Calvert H. Temozolomide and treatment of malignant glioma. Clin Cancer Res 2000 Jul;6(7):2585-2597.

(52) Tang JB, Svilar D, Trivedi RN, Wang XH, Goellner EM, Moore B, et al. N-methylpurine DNA glycosylase and DNA polymerase beta modulate BER inhibitor potentiation of glioma cells to temozolomide. Neuro Oncol 2011 May;13(5):471-486.

(53) Liu L, Gerson SL. Targeted modulation of MGMT: clinical implications. Clin Cancer Res 2006 Jan 15;12(2):328-331.

(54) Christmann M, Verbeek B, Roos WP, Kaina B. O(6)-Methylguanine-DNA methyltransferase (MGMT) in normal tissues and tumors: enzyme activity, promoter methylation and immunohistochemistry. Biochim Biophys Acta 2011 Dec;1816(2):179-190.

(55) Mojas N, Lopes M, Jiricny J. Mismatch repair-dependent processing of methylation damage gives rise to persistent single-stranded gaps in newly replicated DNA. Genes Dev 2007 Dec 15;21(24):3342-3355.

(56) Esteller M, Garcia-Foncillas J, Andion E, Goodman SN, Hidalgo OF, Vanaclocha V, et al. Inactivation of the DNA-repair gene MGMT and the clinical response of gliomas to alkylating agents. N Engl J Med 2000 Nov 9;343(19):1350-1354.

(57) Hegi ME, Diserens AC, Gorlia T, Hamou MF, de Tribolet N, Weller M, et al. MGMT gene silencing and benefit from temozolomide in glioblastoma. N Engl J Med 2005 Mar 10;352(10):997-1003.

(58) Della Puppa A, Persano L, Masi G, Rampazzo E, Sinigaglia A, Pistollato F, et al. MGMT expression and promoter methylation status may depend on the site of surgical sample collection within glioblastoma: a possible pitfall in stratification of patients? J Neurooncol 2012 Jan;106(1):33-41.

(59) Frosina G. DNA repair and resistance of gliomas to chemotherapy and radiotherapy. Mol Cancer Res 2009 Jul;7(7):989-999.

(60) Agnihotri S, Gajadhar AS, Ternamian C, Gorlia T, Diefes KL, Mischel PS, et al. Alkylpurine-DNA-N-glycosylase confers resistance to temozolomide in xenograft models of glioblastoma multiforme and is associated with poor survival in patients. J Clin Invest 2012 Jan 3;122(1):253-266.

(61) Hunter C, Smith R, Cahill DP, Stephens P, Stevens C, Teague J, et al. A hypermutation phenotype and somatic MSH6 mutations in recurrent human malignant gliomas after alkylator chemotherapy. Cancer Res 2006 Apr 15;66(8):3987-3991.

(62) Felsberg J, Thon N, Eigenbrod S, Hentschel B, Sabel MC, Westphal M, et al. Promoter methylation and expression of MGMT and the DNA mismatch repair genes MLH1, MSH2, MSH6 and PMS2 in paired primary and recurrent glioblastomas. Int J Cancer 2011 Aug 1;129(3):659-670.

(63) Yip S, Miao J, Cahill DP, Iafrate AJ, Aldape K, Nutt CL, et al. MSH6 mutations arise in glioblastomas during temozolomide therapy and mediate temozolomide resistance. Clin Cancer Res 2009 Jul 15;15(14):4622-4629.

(64) Maxwell JA, Johnson SP, McLendon RE, Lister DW, Horne KS, Rasheed A, et al. Mismatch repair deficiency does not mediate clinical resistance to temozolomide in malignant glioma. Clin Cancer Res 2008 Aug 1;14(15):4859-4868.

(65) Kim NK, Ahn JY, Song J, Kim JK, Han JH, An HJ, et al. Expression of the DNA repair enzyme, N-methylpurine-DNA glycosylase (MPG) in astrocytic tumors. Anticancer Res 2003 Mar-Apr;23(2B):1417-1423.

(66) Silber JR, Bobola MS, Blank A, Schoeler KD, Haroldson PD, Huynh MB, et al. The apurinic/apyrimidinic endonuclease activity of Ape1/Ref-1 contributes to human glioma cell resistance to alkylating agents and is elevated by oxidative stress. Clin Cancer Res 2002 Sep;8(9):3008-3018.

(67) Montaldi AP, Sakamoto-Hojo ET. Methoxyamine sensitizes the resistant glioblastoma T98G cell line to the alkylating agent temozolomide. Clin Exp Med 2012 Jul 25.

(68) Cheng CL, Johnson SP, Keir ST, Quinn JA, Ali-Osman F, Szabo C, et al. Poly(ADP-ribose) polymerase-1 inhibition reverses temozolomide resistance in a DNA mismatch repair-deficient malignant glioma xenograft. Mol Cancer Ther 2005 Sep;4(9):1364-1368.

(69) Tentori L, Leonetti C, Scarsella M, D'Amati G, Vergati M, Portarena I, et al. Systemic administration of GPI 15427, a novel poly(ADP-ribose) polymerase-1 inhibitor, increases the antitumor activity of temozolomide against intracranial melanoma, glioma, lymphoma. Clin Cancer Res 2003 Nov 1;9(14):5370-5379.

(70) Tentori L, Portarena I, Torino F, Scerrati M, Navarra P, Graziani G. Poly(ADP-ribose) polymerase inhibitor increases growth inhibition and reduces G(2)/M cell accumulation induced by temozolomide in malignant glioma cells. Glia 2002 Oct;40(1):44-54.

(71) Davar D, Beumer JH, Hamieh L, Tawbi H. Role of PARP inhibitors in cancer biology and therapy. Curr Med Chem 2012;19(23):3907-3921.

(72) McEllin B, Camacho CV, Mukherjee B, Hahm B, Tomimatsu N, Bachoo RM, et al. PTEN loss compromises homologous recombination repair in astrocytes: implications for glioblastoma therapy with temozolomide or poly(ADP-ribose) polymerase inhibitors. Cancer Res 2010 Jul 1;70(13):5457-5464.

(73) Wang JC. DNA topoisomerases. Annu Rev Biochem 1996;65:635-692.

(74) Wang JC. Cellular roles of DNA topoisomerases: a molecular perspective. Nat Rev Mol Cell Biol 2002 Jun;3(6):430-440.

(75) Pommier Y. Topoisomerase I inhibitors: camptothecins and beyond. Nat Rev Cancer 2006 Oct;6(10):789-802.

(76) Pourquier P, Ueng LM, Kohlhagen G, Mazumder A, Gupta M, Kohn KW, et al. Effects of uracil incorporation, DNA mismatches, and abasic sites on cleavage and religation activities of mammalian topoisomerase I. J Biol Chem 1997 Mar 21;272(12):7792-7796.

(77) Pourquier P, Pilon AA, Kohlhagen G, Mazumder A, Sharma A, Pommier Y. Trapping of mammalian topoisomerase I and recombinations induced by damaged DNA containing nicks or gaps. Importance of DNA end phosphorylation and camptothecin effects. J Biol Chem 1997 Oct 17;272(42):26441-26447.

(78) Lesher DT, Pommier Y, Stewart L, Redinbo MR. 8-Oxoguanine rearranges the active site of human topoisomerase I. Proc Natl Acad Sci U S A 2002 Sep 17;99(19):12102-12107.

(79) Chrencik JE, Burgin AB, Pommier Y, Stewart L, Redinbo MR. Structural impact of the leukemia drug 1-beta-D-arabinofuranosylcytosine (Ara-C) on the covalent human topoisomerase I-DNA complex. J Biol Chem 2003 Apr 4;278(14):12461-12466.

(80) Meng LH, Liao ZY, Pommier Y. Non-camptothecin DNA topoisomerase I inhibitors in cancer therapy. Curr Top Med Chem 2003;3(3):305-320.

(81) Pommier Y, Redon C, Rao VA, Seiler JA, Sordet O, Takemura H, et al. Repair of and checkpoint response to topoisomerase I-mediated DNA damage. Mutat Res 2003 Nov 27;532(1-2):173-203.

(82) Das BB, Dexheimer TS, Maddali K, Pommier Y. Role of tyrosyl-DNA phosphodiesterase (TDP1) in mitochondria. Proc Natl Acad Sci U S A 2010 Nov 16;107(46):19790-19795.

(83) Pommier Y, Pourquier P, Fan Y, Strumberg D. Mechanism of action of eukaryotic DNA topoisomerase I and drugs targeted to the enzyme. Biochim Biophys Acta 1998 Oct 1;1400(1-3):83-105.

(84) Burden DA, Osheroff N. Mechanism of action of eukaryotic topoisomerase II and drugs targeted to the enzyme. Biochim Biophys Acta 1998 Oct 1;1400(1-3):139-154.

(85) Nitiss JL. Targeting DNA topoisomerase II in cancer chemotherapy. Nat Rev Cancer 2009 May;9(5):338-350.

(86) El-Khamisy SF, Caldecott KW. TDP1-dependent DNA single-strand break repair and neurodegeneration. Mutagenesis 2006 Jul;21(4):219-224.

(87) Li TK, Liu LF. Tumor cell death induced by topoisomerase-targeting drugs. Annu Rev Pharmacol Toxicol 2001;41:53-77.

(88) Cortes Ledesma F, El Khamisy SF, Zuma MC, Osborn K, Caldecott KW. A human 5'-tyrosyl DNA phosphodiesterase that repairs topoisomerase-mediated DNA damage. Nature 2009 Oct 1;461(7264):674-678.

(89) Yang SW, Burgin AB,Jr, Huizenga BN, Robertson CA, Yao KC, Nash HA. A eukaryotic enzyme that can disjoin dead-end covalent complexes between DNA and type I topoisomerases. Proc Natl Acad Sci U S A 1996 Oct 15;93(21):11534-11539.

(90) Interthal H, Pouliot JJ, Champoux JJ. The tyrosyl-DNA phosphodiesterase Tdp1 is a member of the phospholipase D superfamily. Proc Natl Acad Sci U S A 2001 Oct 9;98(21):12009-12014.

(91) Weidlich IE, Dexheimer T, Marchand C, Antony S, Pommier Y, Nicklaus MC. Inhibitors of human tyrosyl-DNA phospodiesterase (hTdp1) developed by virtual screening using ligand-based pharmacophores. Bioorg Med Chem 2010 Jan 1;18(1):182-189.

(92) Raymond AC, Rideout MC, Staker B, Hjerrild K, Burgin AB,Jr. Analysis of human tyrosyl-DNA phosphodiesterase I catalytic residues. J Mol Biol 2004 May 14;338(5):895-906.

(93) Interthal H, Chen HJ, Champoux JJ. Human Tdp1 cleaves a broad spectrum of substrates, including phosphoamide linkages. J Biol Chem 2005 Oct 28;280(43):36518-36528.

(94) Inamdar KV, Pouliot JJ, Zhou T, Lees-Miller SP, Rasouli-Nia A, Povirk LF. Conversion of phosphoglycolate to phosphate termini on 3' overhangs of DNA double strand breaks by the human tyrosyl-DNA phosphodiesterase hTdp1. J Biol Chem 2002 Jul 26;277(30):27162-27168.

(95) Zhou T, Lee JW, Tatavarthi H, Lupski JR, Valerie K, Povirk LF. Deficiency in 3'-phosphoglycolate processing in human cells with a hereditary mutation in tyrosyl-DNA phosphodiesterase (TDP1). Nucleic Acids Res 2005 Jan 12;33(1):289-297.

(96) Interthal H, Champoux JJ. Effects of DNA and protein size on substrate cleavage by human tyrosyl-DNA phosphodiesterase 1. Biochem J 2011 Jun 15;436(3):559-566.

(97) Hawkins AJ, Subler MA, Akopiants K, Wiley JL, Taylor SM, Rice AC, et al. In vitro complementation of Tdp1 deficiency indicates a stabilized enzyme-DNA adduct from tyrosyl but not glycolate lesions as a consequence of the SCAN1 mutation. DNA Repair (Amst) 2009 May 1;8(5):654-663.

(98) El-Khamisy SF, Saifi GM, Weinfeld M, Johansson F, Helleday T, Lupski JR, et al. Defective DNA single-strand break repair in spinocerebellar ataxia with axonal neuropathy-1. Nature 2005 Mar 3;434(7029):108-113.

(99) El-Khamisy SF, Hartsuiker E, Caldecott KW. TDP1 facilitates repair of ionizing radiation-induced DNA single-strand breaks. DNA Repair (Amst) 2007 Oct 1;6(10):1485-1495.

(100) Zhou T, Akopiants K, Mohapatra S, Lin PS, Valerie K, Ramsden DA, et al. Tyrosyl-DNA phosphodiesterase and the repair of 3'-phosphoglycolate-terminated DNA double-strand breaks. DNA Repair (Amst) 2009 Aug 6;8(8):901-911.

(101) Murai J, Huang SY, Das BB, Dexheimer TS, Takeda S, Pommier Y. Tyrosyl-DNA phosphodiesterase 1 (TDP1) repairs DNA damage induced by topoisomerases I and II and base alkylation in vertebrate cells. J Biol Chem 2012 Apr 13;287(16):12848-12857.

(102) Das BB, Antony S, Gupta S, Dexheimer TS, Redon CE, Garfield S, et al. Optimal function of the DNA repair enzyme TDP1 requires its phosphorylation by ATM and/or DNA-PK. EMBO J 2009 Dec 2;28(23):3667-3680.

(103) Barthelmes HU, Habermeyer M, Christensen MO, Mielke C, Interthal H, Pouliot JJ, et al. TDP1 overexpression in human cells counteracts DNA damage mediated by topoisomerases I and II. J Biol Chem 2004 Dec 31;279(53):55618-55625.

(104) Interthal H, Chen HJ, Kehl-Fie TE, Zotzmann J, Leppard JB, Champoux JJ. SCAN1 mutant Tdp1 accumulates the enzyme--DNA intermediate and causes camptothecin hypersensitivity. EMBO J 2005 Jun 15;24(12):2224-2233.

(105) Katyal S, el-Khamisy SF, Russell HR, Li Y, Ju L, Caldecott KW, et al. TDP1 facilitates chromosomal single-strand break repair in neurons and is neuroprotective in vivo. EMBO J 2007 Nov 14;26(22):4720-4731.

(106) Hirano R, Interthal H, Huang C, Nakamura T, Deguchi K, Choi K, et al. Spinocerebellar ataxia with axonal neuropathy: consequence of a Tdp1 recessive neomorphic mutation? EMBO J 2007 Nov 14;26(22):4732-4743.

(107) Nivens MC, Felder T, Galloway AH, Pena MM, Pouliot JJ, Spencer HT. Engineered resistance to camptothecin and antifolates by retroviral coexpression of tyrosyl DNA phosphodiesterase-I and thymidylate synthase. Cancer Chemother Pharmacol 2004 Feb;53(2):107-115.

(108) Conda-Sheridan M, Reddy PV, Morrell A, Cobb BT, Marchand C, Agama K, et al. Synthesis and biological evaluation of indenoisoquinolines that inhibit both tyrosyl-DNA phosphodiesterase I (Tdp1) and topoisomerase I (Top1). J Med Chem 2013 Jan 10;56(1):182-200.

(109) Nguyen TX, Morrell A, Conda-Sheridan M, Marchand C, Agama K, Bermingham A, et al. Synthesis and biological evaluation of the first dual tyrosyl-DNA phosphodiesterase I (Tdp1)-topoisomerase I (Top1) inhibitors. J Med Chem 2012 May 10;55(9):4457-4478.

(110) Liu C, Zhou S, Begum S, Sidransky D, Westra WH, Brock M, et al. Increased expression and activity of repair genes TDP1 and XPF in non-small cell lung cancer. Lung Cancer 2007 Mar;55(3):303-311.

(111) Alagoz M, Gilbert DC, El-Khamisy S, Chalmers AJ. DNA repair and resistance to topoisomerase I inhibitors: mechanisms, biomarkers and therapeutic targets. Curr Med Chem 2012;19(23):3874-3885.

(112) Gilbert DC, Chalmers AJ, El-Khamisy SF. Topoisomerase I inhibition in colorectal cancer: biomarkers and therapeutic targets. Br J Cancer 2012 Jan 3;106(1):18-24.

(113) Vitucci M, Hayes DN, Miller CR. Gene expression profiling of gliomas: merging genomic and histopathological classification for personalised therapy. Br J Cancer 2011 Feb 15;104(4):545-553.

(114) Huse JT, Holland E, DeAngelis LM. Glioblastoma: molecular analysis and clinical implications. Annu Rev Med 2013;64:59-70.

(115) Smith JS, Tachibana I, Passe SM, Huntley BK, Borell TJ, Iturria N, et al. PTEN mutation, EGFR amplification, and outcome in patients with anaplastic astrocytoma and glioblastoma multiforme. J Natl Cancer Inst 2001 Aug 15;93(16):1246-1256.

(116) Ohka F, Natsume A, Wakabayashi T. Current trends in targeted therapies for glioblastoma multiforme. Neurol Res Int 2012;2012:878425.

(117) Abrey LE, Louis DN, Paleologos N, Lassman AB, Raizer JJ, Mason W, et al. Survey of treatment recommendations for anaplastic oligodendroglioma. Neuro Oncol 2007 Jul;9(3):314-318.

(118) Knudson AG,Jr. Mutation and cancer: statistical study of retinoblastoma. Proc Natl Acad Sci U S A 1971 Apr;68(4):820-823.

(119) Liu HB, Peng YP, Dou CW, Su XL, Gao NK, Tian FM, et al. Comprehensive study on associations between nine SNPs and glioma risk. Asian Pac J Cancer Prev 2012;13(10):4905-4908.

(120) Simon M, Hosking FJ, Marie Y, Gousias K, Boisselier B, Carpentier C, et al. Genetic risk profiles identify different molecular etiologies for glioma. Clin Cancer Res 2010 Nov 1;16(21):5252-5259.

(121) Pan WR, Li G, Guan JH. Polymorphisms in DNA repair genes and susceptibility to glioma in a chinese population. Int J Mol Sci 2013 Feb 5;14(2):3314-3324.

(122) Freire P, Vilela M, Deus H, Kim YW, Koul D, Colman H, et al. Exploratory analysis of the copy number alterations in glioblastoma multiforme. PLoS One 2008;3(12):e4076.

(123) Assem M, Sibenaller Z, Agarwal S, Al-Keilani MS, Alqudah MA, Ryken TC. Enhancing diagnosis, prognosis, and therapeutic outcome prediction of gliomas using genomics. OMICS 2012 Mar;16(3):113-122.

(124) Cancer Genome Atlas Research Network. Comprehensive genomic characterization defines human glioblastoma genes and core pathways. Nature 2008 Oct 23;455(7216):1061-1068.

(125) Verhaak RG, Hoadley KA, Purdom E, Wang V, Qi Y, Wilkerson MD, et al. Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. Cancer Cell 2010 Jan 19;17(1):98-110.

(126) Theeler BJ, Yung WK, Fuller GN, De Groot JF. Moving toward molecular classification of diffuse gliomas in adults. Neurology 2012 Oct 30;79(18):1917-1926.

(127) Sasai K, Nodagashira M, Nishihara H, Aoyanagi E, Wang L, Katoh M, et al. Careful exclusion of non-neoplastic brain components is required for an appropriate evaluation of O6-methylguanine-DNA methyltransferase status in glioma: relationship between immunohistochemistry and methylation analysis. Am J Surg Pathol 2008 Aug;32(8):1220-1227.

(128) Rodriguez FJ, Thibodeau SN, Jenkins RB, Schowalter KV, Caron BL, O'neill BP, et al. MGMT immunohistochemical expression and promoter methylation in human glioblastoma. Appl Immunohistochem Mol Morphol 2008 Jan;16(1):59-65.

(129) Preusser M, Charles Janzer R, Felsberg J, Reifenberger G, Hamou MF, Diserens AC, et al. Anti-O6-methylguanine-methyltransferase (MGMT) immunohistochemistry in glioblastoma multiforme: observer variability and lack of association with patient survival impede its use as clinical biomarker. Brain Pathol 2008 Oct;18(4):520-532.

(130) Kim S, Dougherty ER, Shmulevich I, Hess KR, Hamilton SR, Trent JM, et al. Identification of combination gene sets for glioma classification. Mol Cancer Ther 2002 Nov;1(13):1229-1236.

(131) Liu Y, Scheurer ME, El-Zein R, Cao Y, Do KA, Gilbert M, et al. Association and interactions between DNA repair gene polymorphisms and adult glioma. Cancer Epidemiol Biomarkers Prev 2009 Jan;18(1):204-214.

(132) Liu Y, Zhang H, Zhou K, Chen L, Xu Z, Zhong Y, et al. Tagging SNPs in non-homologous end-joining pathway genes and risk of glioma. Carcinogenesis 2007 Sep;28(9):1906-1913.

(133) Jiang L, Fang X, Bao Y, Zhou JY, Shen XY, Ding MH, et al. Association between the XRCC1 polymorphisms and glioma risk: a meta-analysis of case-control studies. PLoS One 2013;8(1):e55597.

(134) Otomo T, Hishii M, Arai H, Sato K, Sasai K. Microarray analysis of temporal gene responses to ionizing radiation in two glioblastoma cell lines: up-regulation of DNA repair genes. J Radiat Res 2004 Mar;45(1):53-60.

(135) Morandi E, Zingaretti C, Chiozzotto D, Severini C, Semeria A, Horn W, et al. A cDNA-microarray analysis of camptothecin resistance in glioblastoma cell lines. Cancer Lett 2006 Jan 8;231(1):74-86.

(136) Hester SD, Reid L, Nowak N, Jones WD, Parker JS, Knudtson K, et al. Comparison of comparative genomic hybridization technologies across microarray platforms. J Biomol Tech 2009 Apr;20(2):135-151.

(137) Beroukhim R, Lin M, Park Y, Hao K, Zhao X, Garraway LA, et al. Inferring loss-of-heterozygosity from unpaired tumors using high-density oligonucleotide SNP arrays. PLoS Comput Biol 2006 May;2(5):e41.

(138) Nannya Y, Sanada M, Nakazaki K, Hosoya N, Wang L, Hangaishi A, et al. A robust algorithm for copy number detection using high-density oligonucleotide single nucleotide polymorphism genotyping arrays. Cancer Res 2005 Jul 15;65(14):6071-6079.

(139) Zhou X, Rao NP, Cole SW, Mok SC, Chen Z, Wong DT. Progress in concurrent analysis of loss of heterozygosity and comparative genomic hybridization utilizing high density single nucleotide polymorphism arrays. Cancer Genet Cytogenet 2005 May;159(1):53-57.

(140) Lyons-Weiler M, Hagenkord J, Sciulli C, Dhir R, Monzon FA. Optimization of the Affymetrix GeneChip Mapping 10K 2.0 Assay for routine clinical use on formalin-fixed paraffin-embedded tissues. Diagn Mol Pathol 2008 Mar;17(1):3-13.

(141) Monzon FA, Hagenkord JM, Lyons-Weiler MA, Balani JP, Parwani AV, Sciulli CM, et al. Whole genome SNP arrays as a potential diagnostic tool for the detection of characteristic chromosomal aberrations in renal epithelial tumors. Mod Pathol 2008 May;21(5):599-608.

(142) Wick W, Platten M, Weller M. New (alternative) temozolomide regimens for the treatment of glioma. Neuro Oncol 2009 Feb;11(1):69-79.

(143) Arslantas A, Artan S, Oner U, Muslumanoglu MH, Ozdemir M, Durmaz R, et al. Genomic alterations in low-grade, anaplastic astrocytomas and glioblastomas. Pathol Oncol Res 2007;13(1):39-46.

(144) Ohgaki H, Kleihues P. Genetic pathways to primary and secondary glioblastoma. Am J Pathol 2007 May;170(5):1445-1453.

(145) Zalatimo O, Zoccoli CM, Patel A, Weston CL, Glantz M. Impact of genetic targets on primary brain tumor therapy: what's ready for prime time? Adv Exp Med Biol 2013;779:267-289.

(146) Pollack IF, Finkelstein SD, Woods J, Burnham J, Holmes EJ, Hamilton RL, et al. Expression of p53 and prognosis in children with malignant gliomas. N Engl J Med 2002 Feb 7;346(6):420-427.

(147) Xu GW, Mymryk JS, Cairncross JG. Inactivation of p53 sensitizes astrocytic glioma cells to BCNU and temozolomide, but not cisplatin. J Neurooncol 2005 Sep;74(2):141-149.

(148) Cahill DP, Levine KK, Betensky RA, Codd PJ, Romany CA, Reavie LB, et al. Loss of the mismatch repair protein MSH6 in human glioblastomas is associated with tumor progression during temozolomide treatment. Clin Cancer Res 2007 Apr 1;13(7):2038-2045.

(149) Hunter C, Smith R, Cahill DP, Stephens P, Stevens C, Teague J, et al. A hypermutation phenotype and somatic MSH6 mutations in recurrent human malignant gliomas after alkylator chemotherapy. Cancer Res 2006 Apr 15;66(8):3987-3991.

(150) Yip S, Miao J, Cahill DP, Iafrate AJ, Aldape K, Nutt CL, et al. MSH6 mutations arise in glioblastomas during temozolomide therapy and mediate temozolomide resistance. Clin Cancer Res 2009 Jul 15;15(14):4622-4629.

(151) Berntsson SG, Wibom C, Sjostrom S, Henriksson R, Brannstrom T, Broholm H, et al. Analysis of DNA repair gene polymorphisms and survival in low-grade and anaplastic gliomas. J Neurooncol 2011 Dec;105(3):531-538.

(152) Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med 2005 Mar 10;352(10):987-996.

(153) Trivedi RN, Almeida KH, Fornsaglio JL, Schamus S, Sobol RW. The role of base excision repair in the sensitivity and resistance to temozolomide-mediated cell death. Cancer Res 2005 Jul 15;65(14):6394-6400.

(154) Caldecott KW. Single-strand break repair and genetic disease. Nat Rev Genet 2008 Aug;9(8):619-631.

(155) Lebedeva NA, Rechkunova NI, Lavrik OI. AP-site cleavage activity of tyrosyl-DNA phosphodiesterase 1. FEBS Lett 2011 Feb 18;585(4):683-686.

(156) Overmeyer JH, Young AM, Bhanot H, Maltese WA. A chalcone-related small molecule that induces methuosis, a novel form of non-apoptotic cell death, in glioblastoma cells. Mol Cancer 2011 Jun 6;10:69-4598-10-69.

(157) Perego P, Cossa G, Tinelli S, Corna E, Carenini N, Gatti L, et al. Role of tyrosyl-DNA phosphodiesterase 1 and inter-players in regulation of tumor cell sensitivity to topoisomerase I inhibition. Biochem Pharmacol 2012 Jan 1;83(1):27-36.

(158) Zhang YW, Regairaz M, Seiler JA, Agama KK, Doroshow JH, Pommier Y. Poly(ADP-ribose) polymerase and XPF-ERCC1 participate in distinct pathways for the repair of topoisomerase I-induced DNA damage in mammalian cells. Nucleic Acids Res 2011 May;39(9):3607-3620.

(159) Zeng Z, Cortes-Ledesma F, El Khamisy SF, Caldecott KW. TDP2/TTRAP is the major 5'-tyrosyl DNA phosphodiesterase activity in vertebrate cells and is critical for cellular resistance to topoisomerase II-induced DNA damage. J Biol Chem 2011 Jan 7;286(1):403-409.

(160) Heisig P. Type II topoisomerases--inhibitors, repair mechanisms and mutations. Mutagenesis 2009 Nov;24(6):465-469.

(161) Roos WP, Batista LF, Naumann SC, Wick W, Weller M, Menck CF, et al. Apoptosis in malignant glioma cells triggered by the temozolomide-induced DNA lesion O6-methylguanine. Oncogene 2007 Jan 11;26(2):186-197.

(162) Hegi ME, Diserens AC, Godard S, Dietrich PY, Regli L, Ostermann S, et al. Clinical trial substantiates the predictive value of O-6-methylguanine-DNA methyltransferase promoter methylation in glioblastoma patients treated with temozolomide. Clin Cancer Res 2004 Mar 15;10(6):1871-1874.

(163) Murat A, Migliavacca E, Gorlia T, Lambiv WL, Shay T, Hamou MF, et al. Stem cell-related "self-renewal" signature and high epidermal growth factor receptor expression associated with resistance to concomitant chemoradiotherapy in glioblastoma. J Clin Oncol 2008 Jun 20;26(18):3015-3024.