CHARACTERIZING THERAPEUTICALLY RESISTANT HUMAN GLIOBLASTOMA CELLS

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A THESIS SUBMITTED

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF PHYSIOLOGY

NATIONAL UNIVERSITY OF SINGAPORE

2015

DECLARATION

I hereby declare that this thesis is my original work and has been written by me in its entirety. I have duly acknowledged all sources of information which has been used in the thesis.

This thesis has not been previously submitted for any degree in any university previously.

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01 APRIL 2015

ACKNOWLEDGEMENT

I thank many for the successful completion of this study. First and foremost, my supervisors A/P Celestial Yap and A/P Too Heng Phon for their advice and support during the PhD learning process. Next, I am also grateful to the past and present members from both their laboratories for the teaching of important benchwork techniques and experimental planning. I am also thankful to A/P Lim Kah Leong from my Thesis Advisory Committee for his assistance on the bioinformatics collaboration part of the project. In addition, I am grateful to colleagues and friends from the National Neuroscience Institute, Singapore; in particular those from the Department of Neurosurgery. Most significantly, I thank my family for their unwavering support and prayers during my candidature. They may not have understood, or even vaguely fathom what I was doing during my prolonged, albeit odd times in the laboratory. However, they never questioned the validity of my decision to complete the PhD. Last but not least, I thank the Lord for this PhD experience, and look forward to the day when science can translate into good medicine for the benefit of our patients.

'The capacity of man himself is only revealed when, under stress and responsibility, he breaks through his educational shell, and he may then be a splendid surprise to himself no less than to this teachers.'

Harvey W. Cushing : The Father of Neurosurgery (1869-1939)

SUMMARY

‘Glioblastoma multiforme’ retains its reputation as the most common and devastating primary brain tumour affecting adults. This disease’s therapeutic resistance fortify it as one of the most challenging cancers to manage. Despite the implementation of aggressive treatment which consists of maximal safe surgical resection, followed by concomitant chemotherapy and high-dose radiation, the overall prognosis remains abysmal. Tumour recurrence is an inevitable event. At this stage, the identification of surviving cells responsible for tumour recurrence has yet to be clinically determined. Based on these observations, we hypothesized that there are different cell populations within glioblastoma that possess innate resistance mechanisms against chemo-radiation induced cell death, allowing them to survive and initiate tumour recurrence.

For this project, we used an *in vitro* approach to explore key concepts underlying its notorious therapeutic resistance: tumour cell heterogeneity, the significance of MGMT (O6-methylguanine–DNA methyltransferase) in glioblastoma and the properties of a glioma stem cell population. . Here, the human glioblastoma cell line, LN229 was cultured to generate differentially-derived cell populations that are postulated to contribute to therapeutic resistance: ‘temozolomide-resistant’ lines that express MGMT and glioma ‘stem cell-like’ lines. These cells were characterized for their tumorigeneity and resistance to cell death, and found to be more resilient in comparison to the LN229 parent cells. In addition, they were also subjected to clinically

relevant doses of temozolomide (TMZ) and, or radiation therapy (RT); in order to simulate what is used in the clinical setting. Our results found that a subpopulation of LN229 ‘glioma stem cell-like’ cells was able to survive patient-relevant treatment. Hence, in order to explore these viable cells at a more in-depth level, their miRNA profiles were examined. We noted that there were miRNA clusters that demonstrated distinct changes in the presence of treatment. Subsequently, 3 selected miRNAs, miR-125a-3p, miR-629-p and miR-19b-3p were found to have significant downregulation in all 3 arms of treatment: TMZ only, RT only and combinatorial TMZ and RT. The miRNAs were functionally validated using miRNA mimics in LN229 ‘glioma stem cell-like’ cells. Independent overexpression of these miRNAs demnonstrated reduced cell viability when the transfected cells were subjected to treatment. Following this, these 2 miRNAs were mapped to potential mRNA targets using a prediction programme. The mRNA targets RFX1 and FLVCR2 were found to be correspondingly upregulated in the presence of these downregulated miRNAs. RFX1 and FLVCR2 were functionally validated using a siRNA knockdown experiment, where they showed reduced cell viability when the transfected cells were subjected to treatment. This was in concordance with our miRNA data. Most significantly, our validated mRNA targets were found to stratify survival in patient glioma databases, REMBRANDT and Gravendeel. Putting it all together, our findings suggest that miRNAs have strong potentiality to be used as tools to seek for therapeutic gene targets in glioblastoma.

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'I have made my peace with God. And I am ready to go'

A glioblastoma patient, Singapore

(Quote said at the time of third tumour recurrence)

Chapter 1

INTRODUCTION

1.1 Disease of interest: glioblastoma multiforme

Introduction

Gliomas belong to the group of primary brain tumours derived from the white matter cells of the central nervous system (CNS). These include glia cells, such as astrocytes and oligodendrocytes[1]. The gliomas are classified according to the criteria defined in the International Classification (IC) by the World Health Organization (WHO) in 2007[2,3]. This classification is currently based on histopathological features, including morphological evaluation of neoplastic cells by defined criteria such as cell density, nuclear polymorphism, mitoses, infiltrative pattern, vascular proliferation and, or necrosis. These criteria concur in identifying malignancy grades of glial-based tumours based on biological features and growth patterns[3,4]. Under this classification, glioblastoma multiforme (GBM) is the most common and unfortunately, the most malignant[5]. Globally, GBM accounts for approximately 1% of all adult malignancies[6], and is currently the most common primary CNS tumour affecting the adult population.

General overview

I. Risk Factors

At present, the aetiology of GBM is not fully ascertained. From a clinical perspective, it is difficult to tell individual patients the exact cause of

their tumours, especially because there are no common risk factors[7]. However, general consensus amongst experts in the field is that GBM is likely multifactorial, with genetic and environmental agents proposed as probable risk factors[7]. Unlike several other types of cancers, up to date, there remains no established pattern of the disease's risk factors and, or target population. Family history of GBM may be rare. but when present, members are associated with a two-fold increase in the risk of developing glioma[8]. However, these cases are often seen in inherited tumour syndromes such as Turcot and Li-Fraumeni syndromes, neurofibromatosis type 1 and multiple enchondromatosis[9-11]. In these patients, the risk of other types of neoplasms are also apparent, indicating their genetic susceptibility is not exclusive to GBM alone. Furthermore, although genome-wide association studies did identify some susceptibility variants such as 20q13.33 (*RTEL*), 5p15.33 (*TERT*), 9p21.3 (*CDKN2BAS*), 7p11.2 (*EGFR*) and so forth, these genes were only weakly associated with glioma, possibly reflecting molecular subsets[12,13].

As for influence of environmental risk factors, it is difficult to prove a direct causation effect at this stage. This is taking into consideration the lesser number of studies, variability of risk factors and overall low statistical power. Expectantly, many of these risk factors, although often common in other human cancers, are likely to be merely chance associations in the context of GBM[14]. Therefore, the effect of diet choices[15-17], tobacco smoking[18,19], frequent use of mobile phones[20,21] remain preliminary in GBM tumours at this stage[20].To add pessimism to the situation, preventative measures, such as lifestyle changes, are ineffective in averting gliomas. Also, early diagnosis and

treatment unfortunately do not improve outcomes, precluding the utility of screening for this disease[8].

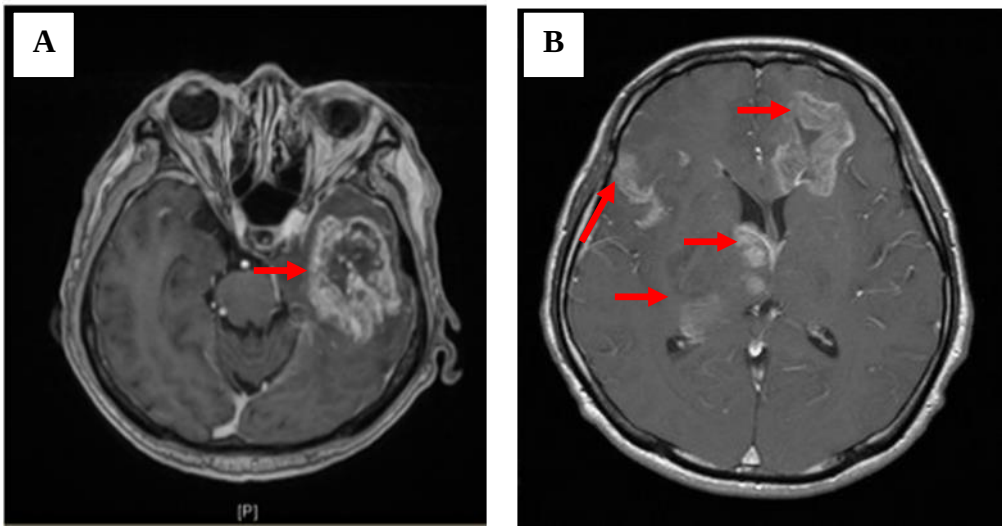
Currently, the only established risk factor for glioma development is previous exposure to ionizing radiation (IR)[8]. Historical data from atomic bomb survivors replicated glioma-specific risks paralleled with a linear dose-response at moderate doses[22]. In addition, 2 studies of childhood cancer survivors who had received relatively high dose radiation treatments for a primary cancer demonstrated a correlative increased risk of gliomas[23,24]. However, there are a few caveats to note in this context: firstly, in these epidemiological studies, the odds of an acquired neoplasm is not restricted to gliomas alone. Previous exposure to IR has also been found to be associated with other types of brain tumours, including meningiomas and schwannonas[25]. Next, although the link between IR and gliomas seem to be persuasive, this association is still not readily accepted in radiation science. One of the reasons is the longstanding concept of the brain being a highly differentiated organ with low mitotic activity, hence rendering it radioresistant[26]. Overall, we still need the input of high-quality, multi-centre studies, in order for better understanding of patient-environment interactions in the development of GBM.

II. Presenting symptoms and imaging modalities

A key point of clinical frustration is that GBM tumours are generally diagnosed at an advanced stage[27]. They can become very large before

producing symptoms of raised intracranial pressure via cerebral oedema and mass effect[28]. In the acute setting, corticosteroids may be able to reduce the oedema through rearrangement of the blood-brain-barrier, lowering mass effect and intracranial pressure[28]. However, such measures are only temporary for relief of symptoms—the tumour still needs to be addressed. When a brain tumour is suspected, magnetic resonance imaging (MRI) with and without gadonilium contrast is the diagnostic investigation of choice[29]. In the T1-weighted sequence, malignant gliomas typically enhance with gadonilium, and may have central areas of necrosis. These space-occupying lesions in the brain parenchyma are characteristically surrounded by white matter oedema. Malignant gliomas tumours are often unifocal, but infrequently, can be multifocal[8].

FIGURE 1: Representative MRI brain axial sections of T1-weighted post-gadonilium showing: A. unifocal GBM in the left temporal lobe, and B. scattered, multifocal GBM lesions in brain parenchyma (Red arrows point to the lesion(s) of interest)



(Courtesy of Department of Neurosurgery, NNI)

III. Current treatment options

Despite notable advances in understanding glioma biology over the last few decades, the overall prognosis of GBM patients remains abysmal. At present, the 'Stupp Protocol' is the accepted standard of care for the management of GBM; it consists of maximally safe surgical resection, followed by concomitant radiation therapy and chemotherapy in the form of temozolomide (TMZ)[30]. However since its implementation in 2005[30], the median survival period for malignant gliomas remain between 12-18 months. After first-line treatment, virtually all GBM patients experience disease progression after a median Progression Free Survival (PFS) of 7-10 months[31]. Long-term survival is less than 30% at 1 year, 5% at 3 years and 3% at 5 years. This is largely due to high rates of local recurrence: tumours typically recur within 2 cm of the operative site, but occasionally in some cases recurring further away from the primary site[32].

Temozolomide (Temodal®)

Given that complete surgical resection is not realistic in GBM, adjuvant therapies play an important role in the overall treatment. Temozolamide (TMZ), an alkylating agent, has proved to be an effective chemotherapy drug with relatively good side effect profile. It has 100% bioavailability as an oral agent[33]. Under physiological conditions, the blood-brain-barrier (BBB) protects the CNS, preventing systematically administered chemotherapeutic strategies to enter the brain parenchyma[34]. Paradoxically, under such circumstances, the BBB becomes a key limiting factor for GBM to

be adequately treated. In patients with GBMs, there is increased capillary permeability from the breakdown of the BBB, causing a known clinical phenomenon called vasogenic oedema. This is a common event principally seen in brain tumours[35]. The concern in GBMs is the overall lack of penetration into the otherwise unaffected brain parenchyma where the BBB is intact. This implies that firstly, a higher dose of drugs is required to reach the tumour of interest, and next, the drugs may not be able to effectively reach microscopic tumour cells that have infiltrated the normal brain tissues. However, owing to its small size and lipophilic properties, TMZ is able to cross the BBB more effectively compared to other chemotherapy drugs. Its concentrations in the central nervous system (CNS) are about 30% of plasma concentration. Once it has entered the CNS, TMZ is converted to the active metabolite, methyltriazene-1-yl-imidazole-4-carboxamide (MTIC). These pharmacological properties make it an ideal agent for treating CNS malignancies.

However, while concomitant adjuvant radiotherapy and TMZ offer a survival advantage over older chemotherapies, the improvement in median survival remains marginal, increasing only by 2 to 2.5 months[30,36]. Furthermore, despite survival benefit of TMZ, Stupp *et al* 2005's landmark study did not stipulate the relative contribution of drug administration during radiotherapy from the contribution of adjuvant therapy. Instead, the authors hypothesized that concurrent administration of both play important roles in enhancing the overall outcome[37]. Presently, the exact mechanism of how both combined therapies work together as an entity is still uncertain. However,

owing to TMZ's systemic tolerability, and lack of a better option for such a devastating disease, the use of the Stupp Protocol is current standard management for GBM today.

Radiation therapy

Despite IR exposure being identified as a significant risk factor (as previously mentioned), the paradoxical reality is that radiation therapy (RT) plays a central role in the treatment of brain tumours, and is considered the most efficient non-surgical intervention for malignant gliomas[38,39].

In GBM, the clinical practice of involved field RT, which is delivery of RT only to affected regions of the brain, is the standard approach for adjuvant RT. This rationale for limiting the RT field is based upon the observation that, following whole brain RT, recurrent GBM typically develop within 2 cm of the original tumour site in 80 to 90% of the patients[32,40,41]. Earlier studies also [sl1]demonstrated that a higher RT dose delivered to a smaller volume resulted longer patient survival time[42].In order to encompass infiltrating tumour cells, a total RT dose of 60 Gy is focused into the tumour cavity, plus a margin of radiographically normal tissue. Clinical data showed that RT doses beyond 60 Gy caused substantial toxicity to the brain parenchyma, triggering necrosis and, or leukencephalopathy[43]. Hence, the choice of 60 Gy as a cumulative dose has been clinically correlated with safety and patient survival[44,45]. In present times, we have the advantage of much improved and safer RT technology due to rapid progress in informatics, imaging and the delivery of

high-precision beams[46]. These improved technologies of planning and delivery of RT have increased the target precision in the brain parenchyma while limiting either the dose given to the normal tissues in the brain or the volume of normal tissues exposed to the high doses[47].

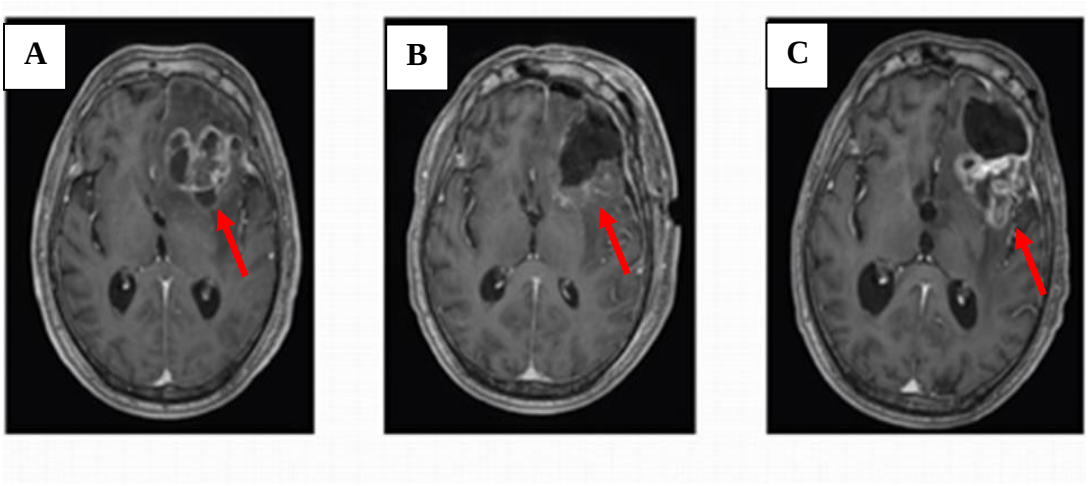
IV. Tumour Recurrence

One of the historical figures of neurosurgery, Walter Dandy, was the first to report GBM recurrence in the contralateral hemisphere after prophylactic radical hemispherectomy[48]. In spite of advancements in understanding the disease, the inevitability of tumour recurrence is accepted as the most notorious feature of GBM behaviour. As previously mentioned, studies that had analysed GBM recurrence patterns conclude that the vast majority of neoplastic cells are likely found centrally within the original tumour bed and, or up to 2cm beyond its radiographically-enhancing borders[49]. However, migrating cells are centimetres away from the tumour and occasionally, even in the contralateral hemisphere, provide the basis for the universal recurrence of these tumours[7].

Typically, patients succumb to tumour recurrence within 12 to 18 months. Several studies have demonstrated that the addition of TMZ to radiotherapy do not alter the fact that the most of the first relapses occur close to the original enhancing disease[49-52]. At the time of relapse, prognosis is particularly poor, with reports of near-100% mortality within 18 months. At

present, there is no standard treatment for patients with GBM recurrence after prior chemo-radiation therapy[53].

FIGURE 2: Time-sequential MRI brain axial sections of T1-weighted post-gadonilium showing tumour recurrence: A. Pre-operative scan with GBM in left fronto-temporal lobe, B. Immediate post-operative scan showing good tumour resection, and C. Scan image from MRI 6 months post-surgery, depicting tumour recurrence in surgical cavity (Red arrows point to tumour cavity)



The goals of re-surgery after tumour recurrence are firstly, to relieve the raised intracranial pressure caused by the space-occupying lesion for life-saving purposes; and next, for cytoreduction to reduce tumour burden within each patient. Up to this day, the practice of tumour debulking with extent of resection (EOR) $\geqslant$98% is currently the only factor known to improve patient survival[54].Nonetheless, in these cases, the effects of the re-operation are only temporary and impacts on the patient are at best, palliative. The following 2 chemotherapeutics to be discussed have been used in for a select group in these patients. However, they are costly, have potentially life-threatening side effects and their benefits, if present, are unexceptional.

Carmustine (Gliadel®)

Carmustine (BCNU (1,3-*bis* (2-chloroethyl)-1-nitrosourea)) has a long-standing history in GBM. It was one of the first drugs to be approved by the U.S. Food and Drug Administration (FDA) to treat malignant gliomas. BCNU is an alkylation agent that interferes in tumour development by inhibiting DNA synthesis and repair[55]. However, BCNU proved to have pharmacokinetic drawbacks: it has a short half-life and low oral bioavailability[56]. To overcome these shortcomings, Gliadel® wafers were developed to deliver local chemotherapy through carmustine-impregnated wafers to concentrate chemotherapy in the pre-cancerous peritumoral surgical cavity[57,58]. These wafers consist of 200 mg biodegradable polymer discs (1 mm width and 1 cm in diameter) that contain 3.85% BCNU by weight[59].

Initially, BCNU wafers implanted intra-operatively were shown to be effective in improving outcome in some studies[60]. However, subsequent studies showed that the addition of Gliadel® did not appear to significantly improve clinical outcome and was associated with increased cytotoxicity[61]. Even after tumour removal and addition of these wafers into the tumour cavity, the overall survival benefit remains modest. At present, in our local institution, the use of Gliadel® wafers are mostly reserved for patients who present with recurrent GBMs who have failed 1st-line therapy (i.e. Stupp Protocol).

Bevacizumab (Avastin®)

Over the years, a number of angiogenic agents have failed to demonstrate measurable efficacy in randomized trials[53]. However, bevacizumab, a monoclonal antibody to vascular endothelial growth factor (VEGF), managed to receive substantial attention in the past few years. However, there is recent consensus that despite the undeniable benefit, concerns regarding utility, indication, efficacy and side effects have been raised for its use in malignant gliomas[62]. We are now aware that not all GBMs are sensitive to anti-VEGF treatment[63]. In addition, some tumours that were initially sensitive to sensitive to the bevacizumb recurred with a more aggressive phenotype[64]. In fact, MRI scans done in this group of GBM patients treated with bevacizumab showed the development of multifocal recurrence that strongly indicated the presence of an infiltrative-invasive pattern[65]. Similar to BCNU, the use of bevacizumab is offered to patients who present with recurrent GBMs that are refractory to standard treatment protocol in our local institution.

1.2. Therapeutic resistance in human glioblastoma: current concepts

Since gliomas were first recognized in the mid-19th century, we have accrued a tremendous amount of data on this disease but have enjoyed little improvement in its survivability[66].Like all cancers, a major obstacle in GBM treatment is its resistance to anticancer modalities[67]. As notably observed in modern brain imaging, true responses with partial or complete regression of tumour are rare in GBMs. This is an indication that current treatments, if

successful, arrest growth rather than actually kill[68]. To add on to GBM's grim profile, we are also aware that none of the available salvage treatments has shown real evidence of improved survival[8]. Therefore, delaying onset of recurrence or the extent of tumour dispersal will represent a significant advance in managing this disease[27].

There are several mechanisms and molecular abnormalities known to be involved in GBM therapeutic resistance, including genetic changes and variability, increased expression of target proteins, alteration of drug target, failure of the therapy to reach or enter target cell, increased DNA damage repair, reduced apoptosis and so forth. In addition, as previously mentioned, the presence of the BBB makes it challenging for therapies to penetrate the brain. All these cumulative factors add to this particular tumour being able to persistently evade both current and novel therapies. Although there are several factors involved in GBM therapeutic resistance, the following are commonly implicated:

I. Tumour heterogeneity

GBM is characterized by a variety of genetic abnormalities. This phenomenon of *inter-* and *intra-tumoral* heterogeneity raises therapeutic challenges, because cells bearing different abnormalities may respond differently to therapy[34]. Studies have shown that there is a considerable evidence of heterogeneity within GBM tumours in a variety of biological, physiological and biochemical properties[69,70] among subclones of the same

tumour in their response to adjuvant treatment[71-73]. Therefore, the unfortunate reality implies that attempts at treating subpopulations within a single lesion is likely futile in completely eliminating all tumour cells. [74,75]. However, at this stage, the identification of surviving cells responsible for tumour recurrence has yet to be clinically confirmed.

II. MGMT (O^6-methylguanine DNA methyltransferase)

The enzyme O^6-methylguanine-DNA transferase (MGMT) functions as a DNA damage repair entity. MGMT encodes a DNA repair protein that removes alkyl adducts at the O^6 position of guanine, and less frequently, at the O^4 position of thymine[76]. In normal cells, endogenous MGMT expression protects them from carcinogens; however, on the contrary, it can also protect cancer cells from chemotherapeutic alkylating agents, such as TMZ[77,78]. The flurry of interest in MGMT was a consequence of a publication following Stupp *et al* 2005's landmark paper that resulted in the establishment of the now famous 'Stupp Protocol'[30] by Heigi *et al*[79]. This study attempted to identify patients who would benefit from the addition of TMZ to radiotherapy. Here, the authors found that methylation of MGMT promoter turned off its own gene transcription. This in turn, caused reduction in intracellular MGMT, and thereby inhibiting the DNA repair mechanism. According to their results, although the benefit was small, patients with a methylated promoter demonstrate a longer progression-free survival with chemotherapy plus radiotherapy[37]. The TCGA effort also found that in their cohort of GBM tumours, the MGMT promoter methylation status correlated with mismatch

repair deficiency and a hypermutator phenotype—all of which are known to be associated with resistance in GBMs[80].

However, owing to the immense complexity of GBMs, we acknowledge the contribution of this particular gene in therapeutic failure is not singular. Despite this, the methylation status of MGMT remains to be the most well-studied molecular feature in GBM at this point in time[81], with both large-scale molecular[80] and clinical[79,82] data to substantiate its significance. It is hence imperative, that it maintains its bearing in GBM, until proven otherwise.

III. Glioma stem cells

The cancer stem cell model proposes that firstly, a tumour is organized according to the cell hierarchy of the organ in which it arises. Next, it is driven and maintained by a minority of transformed population of tissue-specific stem and, or progenitor cells[83,84]. At present, the debate regarding whether if it is the tumour stem cell, progenitor counterpart or both that is the actual contender for the disease remains unresolved[39]. Incidentally, this concept is not novel in GBMs. In 1926, the father of neurosurgery, Cushing, together with Bailey had already proposed the idea of brain tumours arising from progenitor cells[85]. In addition, GBM is also one of those tumours in which cancer stem cells have been identified. These so-called 'glioma stem cells' (GSCs) are thought to be responsible for maintaining tumours post-therapy and re-populating them after gross total surgical resection. Moreover, it has

also been shown that the most aggressive and, or refractory cancers, usually contain the highest number of cancer stem cells[86,87]. The natural history of GBM is in concordance with this, thus reinstating the therapeutic importance of this particular cancer cell subpopulation.

1.3. Molecular classification of glioblastoma

Current WHO classification for gliomas

At present, glioma patients are diagnosed according to histopathological findings. This relies on a morphology-based classification from the World Health Organization (WHO) criteria[1]. Essentially, the WHO system divides diffuse gliomas into tumour subsets: astrocytomas, oligodendrogliomas and oligoastrocytomas. These glial-based tumours are then graded into increasing histological degrees of malignancy (WHO I, II, III, IV). Malignancy features include the presence of nuclear atypia, mitotic activity, microvascular proliferation and necrosis. Based on the current WHO criteria[1], malignant gliomas refer to those that fall under the WHO III and WHO IV tumours. As previously mentioned, the overall prognosis is especially dismal for the WHO IV group.

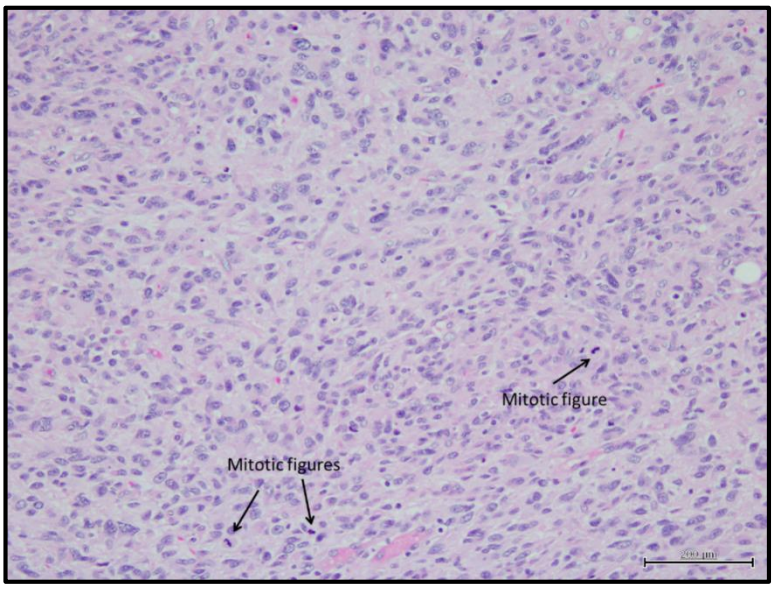
TABLE 1: WHO Classification of glial tumours (Adapted from Louis *et al* (2007)[1])

WHO Grading	Type of glioma	Morphological criteria
Grade I	Pilocytic astrocytoma	Low cellularity Rosenthal fibres May have microvascular proliferation
Grade II	Diffuse astrocytoma	Well-differentiated neoplastic astrocytic cells Moderately increased cellularity Absent mitotic activity No microvascular proliferation or necrosis
Grade III	Anaplastic astrocytoma	Distinct nuclei atypia Increased cellularity Marked mitotic activity No microvascular proliferation or necrosis
Grade IV	**Glioblastoma multiforme (GBM)**	**Pleomorphic tumour cells** **Distinct nuclei atypia** **Increased cellularity** **Brisk mitotic activity** **Presence of microvascular proliferation and, or necrosis**

(*Note: this table is not exhaustive. For the purposes of this project, the focus will be on adult gliomas of astrocytic origin*)

FIGURE 3: Microscopic haematoxylin-eosin slide pictures of a GBM tumour showing evidence of: A. Mitotic activity and, B. Microvascular proliferation. In general, both slides demonstrate areas of increased cellularity, nuclei atypia and tumour cell pleomorphism. (*Note: the surgery of malignant gliomas uses a cytoreduction approach whereby the tumour is removed as much as possible with preservation of normal brain margins. Hence, normal brain tissue is not usually removed, especially in eloquent areas that control neurological function.*)

A



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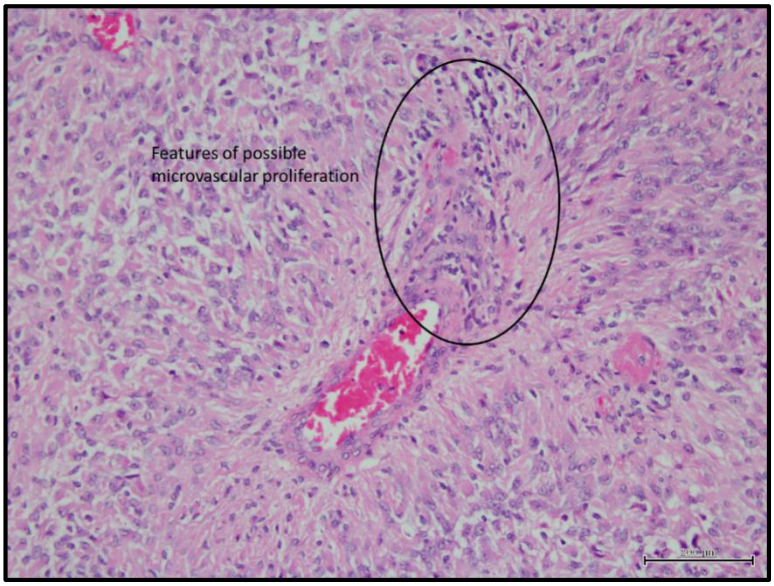


(Pictures and analysis courtesy of Dr Tang YL, TTSH Pathology, and Dr B Yan, KKWCH Pathology)

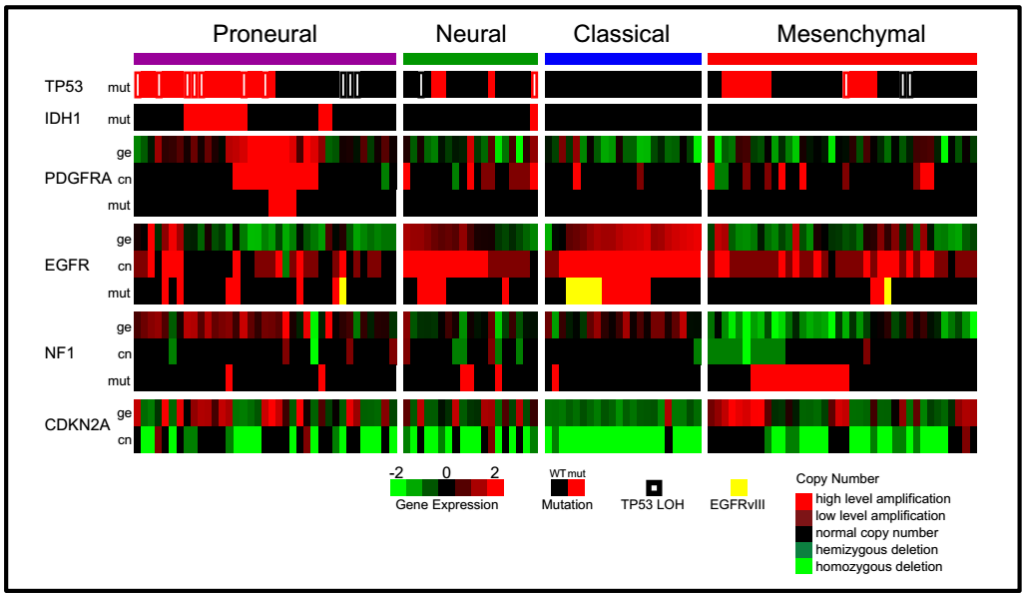
Although the WHO classification for gliomas is still used as the standard method of the diagnosis in clinical practice, we are aware that as a visually-based criteria, it is likely to have certain limitations, such as subjectivity and the risk of inter-observer variations[88,89]. What has become clear in recent years is that cancer cells, although may have morphological similarities, are functionally heterogeneous[90]. Furthermore, observation from a clinical standpoint is the variable response of the patients with the same histological diagnosis towards standard treatment. 'Glioblastoma multiforme' as aptly termed by Harvey Cushing has been a challenging disease to understand owing to its notorious heterogeneity.

Insights into glioblastoma subtyping

At present, the traditional view of human cancers, including GBM, as a homogeneous population is considered obsolete[90]. With the advent of genome-wide profiling studies, we are now aware of the existence of molecular subclasses within GBM[80,91-94]. This has been made possible with the development of various high-throughput genomic technologies to allow comprehensive surveys of human cancer genomes[80].In 2006, the National Cancer Institute (NCI, USA) initiated an effort known as The Cancer Genome Atlas (TCGA) Research Network to generate a comprehensive catalogue of genomic abnormalities driving tumorigenesis in several cancers[80], including GBM. The exercise was based on the observation that GBM, although histologically identical, will be heterogeneous at a molecular level. More importantly, the aim of deeper subtyping was to enable the use of identified

molecular traits in patient prognosis stratification, independent of currently used clinical indicators. In their landmark paper, TCGA provided a detailed view of the genomic changes in a GBM cohort containing 206 patient samples[80]. This initiative was followed by a series of studies that has since proven the existence of 4 molecular sub-groups (Proneural, Neural, Classical and Mesenchymal) based on gene expression, genetic aberrations and survival profiles. TCGA complied their findings with the input of gene expression, microarray-based comparative genomic hybridization (aCGH), methylation status and miRNA data. This cumulative data was subsequently correlated with patient demographics such as age, gender, diagnosis, treatment and survival patterns.

FIGURE 4: Diagram showing GBM molecular subtyping findings by TCGA (Adapted from Verhaak *et al*, 2010[95])

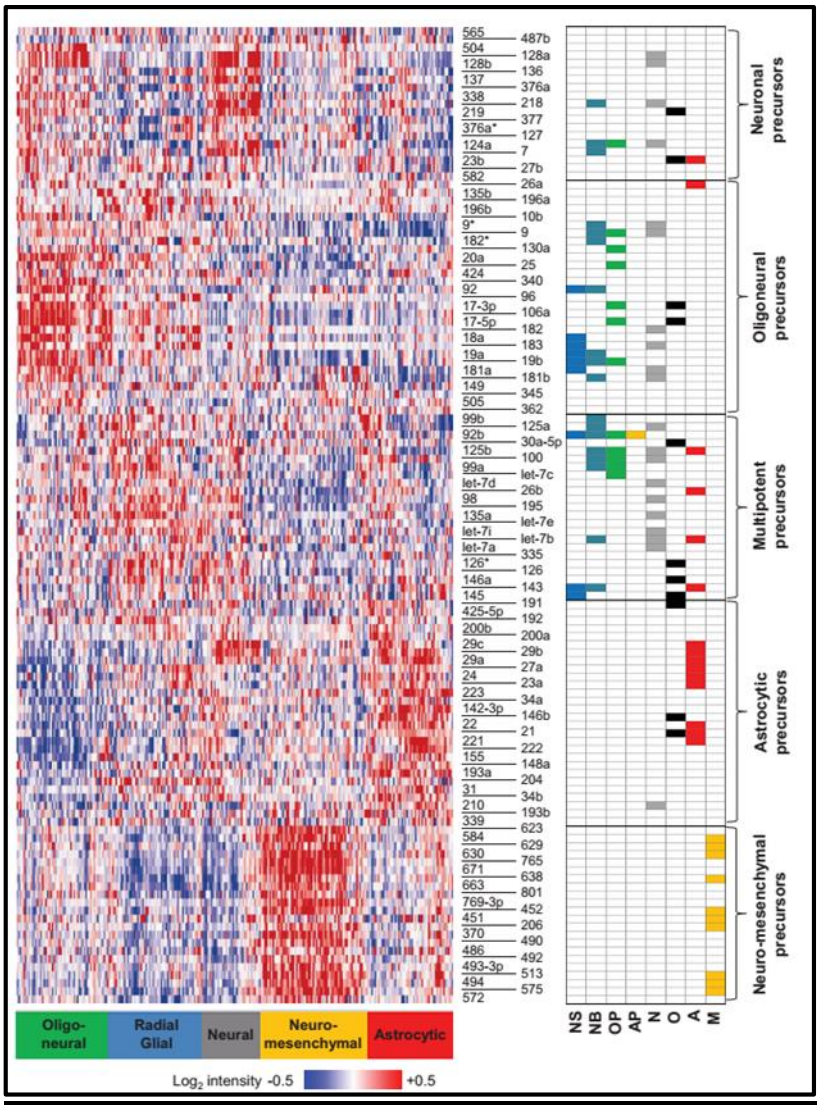


In essence, the introduction of gene expression profiling has provided researchers a useful method to classify tumours[96-98]. This is useful from a clinical perspective: a more insightful classification will be able to help the managing physician better delineate a general diagnosis. This is helpful in firstly, guiding clinical decision-making and next, in allowing effective disease prognostication. More importantly, molecular details in the classification can help us to identify areas of knowledge deficit for research. Examples of malignant primary brain tumours that have been exponentially better understood from molecular subtyping include medulloblastomas[98] and ependymomas[99]. However, in the context of GBM, in the current TCGA subclasses 'Proneural, Neural, Classical and Mesenchymal', exact mechanisms underlying the relationships of some subtypes to neural differentiation and patient survival remain unknown at this stage[93,95]. Nonetheless, as a result of the TCGA initiative, other efforts were attempted to assess whether GBMs could be further subdivided into protein-based subtypes for better prognositication[100,101]. Despite their promising results, direct genetic links to protein subtyping remain incomplete at this stage.

In addition to mRNA data, the TCGA database also included an extensive molecular network for GBM involving miRNAs[102]. Together with the recent insights of experimentally-validated miRNAs[103]and mRNA-miRNA networks[102], miRNA-mediated mechanisms are now cited as key contributors to GBM's notorious heterogeneity. A method attempting to elucidate these potential relationships was described by Kim *et al* 2011[104]. Here, the authors used a high-throughput microRNA approach for expression-based clustering

to identify clinically and genetically distinct glioblastomas subclasses. Their findings showed that in contrast to mRNA-based GBM subclasses, miRNA-defined GBM subclasses demonstrated strong differences in genetic alterations, patient demographics, treatment response and patient survival[104].

FIGURE 5: Diagram showing the developmental taxonomy of 5 miRNA clusters in relation to 5 GBM subclasses. (Adapted from Kim *et al*, 2011[104])



1.4. MicroRNAs in human glioblastoma

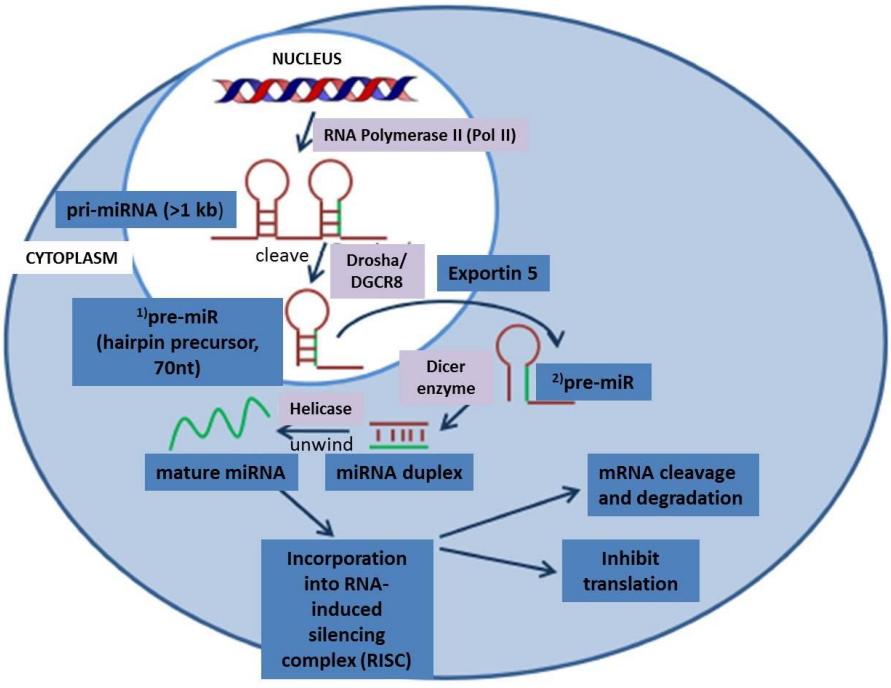
Introduction: overview of miRNAs

MicroRNAs (miRNAs) are a relatively novel class of small, non-coding molecules typically 22 nucleotides in length[105]. They are highly conserved and account for approximately 1% of the human genome[106]. Of significance is that many miRNAs are tissue-specifically expressed[107]. Thus on this basis, it is expected that miRNA expression profiles will help to guide functional studies in various developmental pathways[108]. The exact number of miRNAs genes in the human genome is still unknown and previously estimated to be range from 500 to 1000[108,109]. However, the recent miRBase report showed the number of human mature miRNAs to exceed 2,500 (release 21, June 2014). The established roles of miRNAs include the regulation of gene expression, organogenesis, and not surprisingly, tumorigenesis[110-112].

MicroRNA biogenesis is a multi-step process. (See **Figure 6**). They begin as long primary stem-loop transcripts (more than 1 kb) encoded by a cell's own genome: these being termed as 'pri-miRNA'. Pri-miRNAs are wrapped in hairpin-stem-loop secondary structures that undergo a 2-step maturation process, to eventually produce the functional miRNA. Essentially, miRNAs are transcribed by RNA polymerase II (Pol II) into long primary miRNA transcripts that are cleaved in the nucleus by the RNase III (Drosha). The end-product is a hair-pin precursor called a pre-miRNA. Pre-miRNA is then exported from the nucleus to the cytoplasm by Exportin 5. Once in the cytoplasm, it is processed by the Dicer enzyme which produces a transient

miRNA duplex. Next, a single strand of the miRNA duplex is incorporated into a protein complex called the RNA-induced silencing complex (RISC)[113]. Once in this functional state, they are ready to regulate their target gene expression transcriptionally and post-transcriptionally via interaction with complementary mRNAs. In mammals, miRNAs are negative regulators of gene expression[114]; they can inhibit protein translation, leading to a subsequent downregulation of their target protein expression. Notably, a single miRNA can potentially regulate a wide range of target genes resulting in a global impact on gene expression[115].This characteristic implies that by inhibiting one miRNA, we can potentially suppress multiple genes, subsequently silencing whole pathways[116].

FIGURE 6: Schematic diagram outlining miRNA biogenesis in a mammalian cell. (adapted from Rothschild 2013[113])



In the context of GBM, the targets and modulators of therapy have traditionally been DNA, mRNA and proteins. Therefore, mutations, copy number changes, and epigenetic variables at the DNA level and expression changes at the mRNA and protein levels have been previously studied to probe mechanisms that determine the pharmacologic response[117-121]. Work from different groups studying global miRNA expression profiles and in-depth individual miRNA function has established that miRNAs have important roles in different aspects of gliomagenesis, including chemo- and radiation resistance[114]. Some miRNAs have more implications in GBM pathogenesis due to their wider targets, more robust miRNA binding sites, broader functional coverage and their multiple roles in comparison to other miRNAs[114].Thus, miRNA-mediated mechanisms by connecting and establishing cross-talk between wide arrays of aberrant pathways and cellular functions may provide a global perspective to confront heterogeneous and challenging tumours like GBM[114].

Approximating miRNA function through mRNA target prediction

In recent years, the development of miRNA high-throughput platforms such as hybridization-based microarrays, next-generation sequencing (NGS)[122] and amplification assays (RT-qPCR)[123] has been effectively utilized for miRNA expression profiling[124,125]. Building on this and with the valuable input of bioinformatics, the application of miRNA target prediction offers an optimistic route to the discovery of associated mRNA function, and vice versa[103,126-129]. Depending on the prediction database of choice, each will use

its own algorithm to predict miRNA targets from mammalian genomes via conserved sites in the 3'-UTRs of the complementary genes[130]. However, despite the attractive convenience of such programmes, a note of caution: it remains an open question as to how many verified miRNA targets in these heterologous systems are actually important targets in vivo[108]. Thus, a conscientious effort is still required to experimentally validate the predicted targets from such mapping programmes for biological certainty.

1.5. Gaps in disease knowledge

At present, it is difficult to discuss personalized medicine for GBM patients given the paucity of effective treatment options[64]. Up to date, the most conclusive prognostic factors for GBM are extent of tumour resection, age at diagnosis, and Karnofsky performance status[131,132]. Embarrassingly, these factors for GBM are considerably benighted, especially in present time, where other cancers can be prognosticated by molecular markers. Furthermore, targeted therapies aimed at survival pathways have achieved success in other cancers, whilst their efficacies remain stalemate in GBM[6].

At this point in time, the only predictors of patient outcome are age, WHO tumour grading based on histopathological subtype, and Karnofsky Performance Scale (KPS)[54]. The KPS index classifies patients according to their functional impairment. The scale compares effectiveness of different therapies and assesses prognosis in individual patients. Essentially, the lower the Karnofsky score, the poorer the survival for the illness[133,134]. Despite

research efforts in GBM, there is not yet a single or cluster of robust molecular markers that has been standardized for translational use in medical practice. Also, in the context of therapeutics, we have not significantly progressed beyond the Stupp Protocol initiated in 2005[30]. The obvious step forward is to achieve better understanding of the complex molecular and cellular mechanisms leading to GBM resistance—an important pre-requisite to the identification of more effective strategies[135].

TABLE 2: Karnofsky Performance Scale (KPS) (Adapted from Karnosky and Burchenal, 1949[134])

KARNOFSKY STATUS	KARNOFSKY GRADE
Normal, no complaints.	100
Able to carry on normal activities. Minor signs or symptoms of disease.	90
Normal activity with effort.	80
Care for self. Unable to carry on normal activity or to do active work.	70
Requires occasional assistance, but able to care for most of his needs.	60
Requires considerable assistance and frequent medical care.	50
Disabled. Requires special care and assistance.	40
Severely disabled. Hospitalisation indicated though death non-imminent.	30
Very sick. Hospitalisation necessary. Active supportive treatment necessary.	20
Moribund	10
Dead	0

1.6. Project outline

Owing to the elusive nature of the disease, it is impossible to study every possible bio-target. Incongruously, it is also unrealistic to concentrate on only one of its facets. In essence, this project aims to elucidate the issues underlying therapeutic resistance in GBM. Based on previous discussions, we will draw on current disease understanding, with the implementation of new biological knowledge in the field and up-to-date techniques as a whole, to approach our aims.

1.6.1. Hypotheses and objectives

The following summarises the hypotheses and objectives of the project:

Hypotheses

Primary Hypothesis:

There is a subpopulation of GBM cells that is resistant to standard chemo-radiation therapy.

Secondary Hypothesis:

Therapeutically-resistant GBM cells are a molecularly distinct subpopulation whose properties are regulated by miRNAs.

The primary hypothesis is founded on the fact that GBM as a heterogeneous entity, consists of subpopulations with different genotypes and phenotypes that have divergent behaviours[136]. As a result, within the tumour itself, we postulate there is a subpopulation that is resilient to the effects of

standard therapeutics in use today. Based on the existence of such a subpopulation, our secondary hypothesis is that they are a unique subgroup of tumour cells whose resistance properties are influenced by miRNAs.

Objectives

1. To demonstrate a subpopulation of GBM cells is resistant to chemo-radiation therapy.
2. To examine whether different miRNA expression underlying GBM heterogeneity contributes towards resistance in chemo-radiation therapy.

Building upon our hypotheses, we state the key objectives for the project. Firstly, if such a therapeutically resistant subpopulation exists, it is imperative that the experimental models in our setup has to reflect what is observed *in vivo* as much as possible. Hence, they have to remain viable in the presence of clinically relevant treatment. Next, based on the assumption that this subpopulation will have its own genomic signature, we intend to use miRNA expression profiling to explicate these cells' pro-survival property.

CHAPTER TWO

Materials and Methods

Chapter 2

MATERIALS AND METHODS

2.1 Cell culture

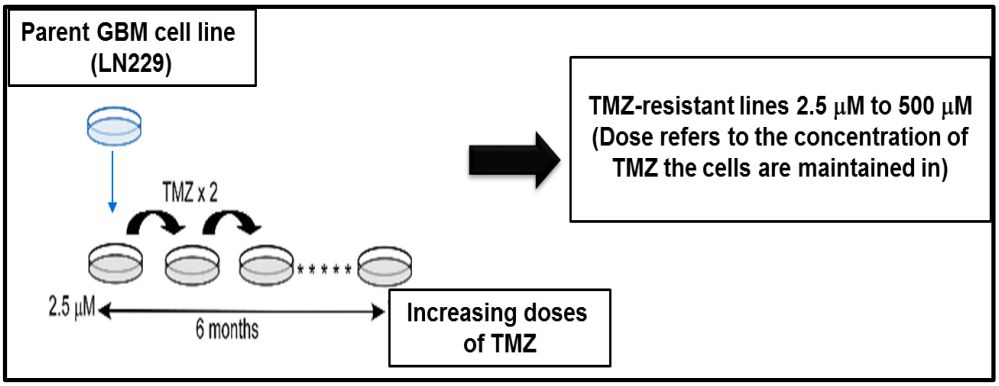
Immortalized human glioblastoma cell line LN229 from American Type Cell Collection (ATCC) was a kind gift from from A/Prof Too HP's laboratory (Department of Biochemistry, National University Singapore). The cell line was commercially authenticated using Short Tandem Repeat (STR) profiling. (See **Supplementary Data**). These cells were grown in DMEM (Sigma, USA), supplemented with 10% FBS (Hyclone, USA), 100 U/mL penicillin and 100 U/mL streptomycin (Life Technologies, USA). Subsequently, cells were maintained in a 37°C in a humidified incubator containing 5% CO2 and 95% air.

2.1.1 Development of temozomolomide-resistant glioblastoma cells

The TMZ-resistant (TMZ-R) cell lines used in this study are a kind gift from Dr Ho YK and A/Prof Too HP (Department of Biochemistry, National University Singapore). Here, they adapted a method described by Le Calve *et al* 2010[137] to generate TMZ-resistant cells of various drug resistance doses from GBM cell line LN229. Initiating treatment at 2.5 μM, cells were cultured in complete medium in the presence of incremental TMZ concentrations: 2.5, 5, 10, 20, 40, 80, 160 and finally 320 μM. During the selection of resistant clones, the cells adapted to TMZ for approximately 30 days before exposure to the next dose. Subsequently, the cell panel was cultured in complete media

containing respective doses of TMZ to maintain their acquired chemoresistance[138].

FIGURE 7: Schematic diagram showing method of generating TMZ-resistance cells of various resistance doses (adapted from Le Calve *et al*2010[137])



2.1.2. Selection of clinically relevant temozolomide-resistant glioblastoma cells for experiments

The selection of appropriate *in vitro* models from the LN229 TMZ-R panel was corroborated with relevant literature. From patient studies, the predicted peak concentrations of TMZ were found to be in the range of ***2.9 to 6.7 mg/ mL*** in human glioma tumours, and 1.8 to 3.7mg/mL in normal brain. This was calculated with the total drug exposure, as indicated by the tissue: plasma area under the curve (AUC) ratio, which was about 1.3 in tumour compared with 0.9 in normal brain parenchyma[139]. Next, it is also known that TMZ has 100% oral bioavailability in patients[140]. Based on these data, we calculated an *in vitro* equivalent to a parallel concentration to what a GBM patient is likely to receive during actual treatment. Using the higher end dose

range of what a glioma tumour is likely to receive, that is, **6.7 mg/ mL**, the *in vitro* concentration was calculated to be approximately **34 μM**. In order to simulate possible TMZ-resistance, the closest dosage and 2 more even more TMZ-resistant lines in the LN229 cell panel were selected—40 μM, 80 μM and 160 μM. These TMZ-R cell lines are used in Chapter 3 experiments. **The TMZ dose of 34 μM was set as the 'clinically relevant' dose for *in vitro* treatment of cells, as reflected in Chapters 3 and 4.**

2.1.3. Isolation of glioma 'stem cell-like' population from glioblastoma cells

The method used for the development of this subpopulation was adapted from the literature[141,142]. In essence, a chemically-defined media composed of 3:1 mix of DMEM (Sigma, USA) and Ham's F-12 Nutrient Mixture (Life technologies, USA), the following were added: 1x B27 serum-free supplement (Life Technologies, USA), 20 ng/mL EGF (Sigma, USA) and 20 ng/mL bFGF (Sigma, USA). Sub-selected cells were then split into 3 separate clonal populations, labelled 1 to 3 respectively. These 3 clones were used as biological replicates for subsequent experiments. These LN229 'GSC-like' cell lines were used in Chapters 4, 5 and 6.

2.2 Proliferation assay

The proliferation properties of LN229 parent and TMZ-R variant cells were compared using 2 different methods: cell counting to calculate their doubling times, and 5'bromo-2'deoxyuridine (BrdU)-incorporation into cellular DNA using a BrdU ELISA colorimeteric assay.

2.2.1. Cell counting

The cells are plated a 0.5 x 10^4 cells per well in 12-well plates. One plate is used for each time point. All cells are starved overnight and given serum media the following day. At the required time point, the cells are washed, trypsinzied and counted with a haemocytometer. Tryphan blue exclusion technique was used to ensure only the viable cells were included. This technique was applied throughout Chapters 3, 4, 5 and 6.

2.2.2. BrdU (5-bromo-2'-deoxyuridine) ELISA assay

The experiment was performed according to the manufacturer's instructions in Cell Proliferation ELISA, BrdU Colorimeteric kit (Roche, USA). BrdU is an analogue of thymidine that gets incorporated into the DNA of proliferating cells in place of thymidine. This assay is hence, based on the detection of BrdU incorporation into the newly-synthesized DNA during cell proliferation. Briefly, cells were plated at 0.2 x 10^4 cells per well in 96-well plates. Each cell type was plated in quadruplicates and one plate was used for each time point. To ensure cell number efficiency for each assay, the control cell line was additionally plated in incremental numbers (500 to 1.6 x 10^4 cells

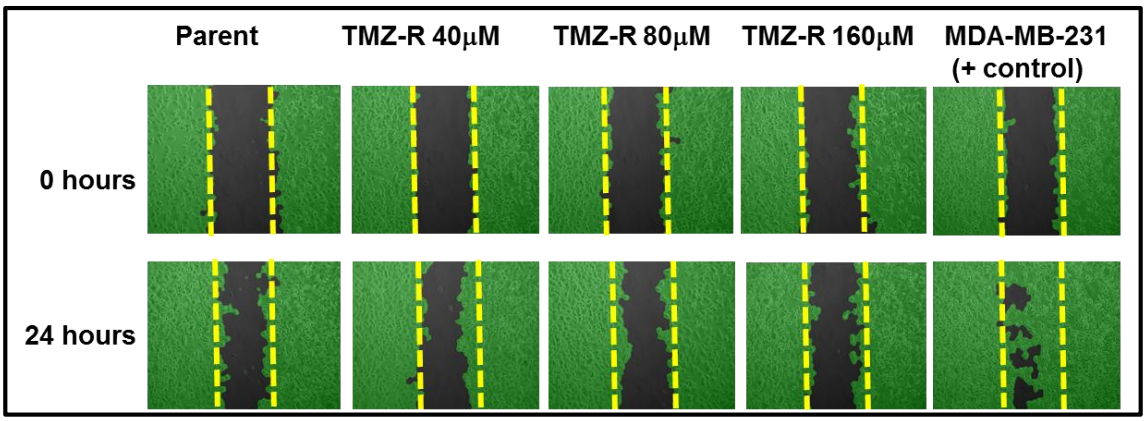
per well) on the same plate for a standard curve. All cells were serum-starved overnight and given BrdU (10nM) in serum culture media the following day. At each required time-point, absorbance values were measured using ASYS UVM340 microplate reader at 450 nm wavelength (reference wavelength: 690nm). Next, the raw values were quantified and normalized to the control cell line per time point. This assay was used in Chapter 3, Section 3.2.3.

2.3. Migration assays

2.3.1. Wound healing assay

Silicon inserts (ibidi®, Denmark) were used according to the manufacturer's instructions. The use of these inserts and subsequent data analysis for *in vitro* migration experiments has been previously described[143-145]. Essentially, 1 x 10^5 cells in 90 µL culture media were plated in each side of the silicon chamber. The cells were allowed to adhere overnight within each chamber insert. After 24 hours, the silicon inserts were removed. 2 mL of regular culture media is then allowed to fill each well. At the required time point, photographs of the wound healing assay in per sample were taken in triplicates at 20x magnification. The pictures were subjected to WimScratch™ software analysis (ibidi®, Denmark) where the results expressed as a ratio of %scratch area : %cell-covered area per sample. The cell line, MDA-MB-231 was used as a positive control for this assay. This assay was used in Chapter 3, Section 3.2.3.

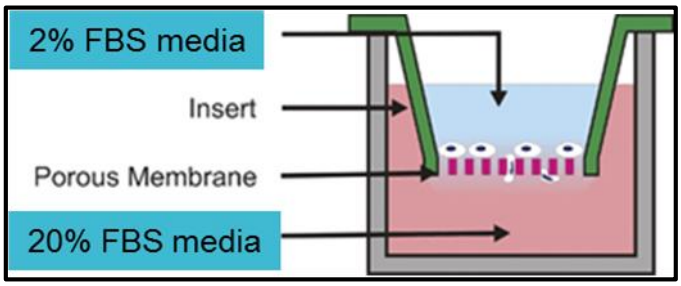
FIGURE 8: Representative pictures of each cell line from WimScratch™ analysis software



2.3.2. Transwell migration assay

The cells used for the experiment were serum-starved overnight in 1% FBS cell culture media. The following day, 5 x 10^4 cells in 1% FBS culture media were seeded on the surface 8 μm pore-size filters per transwell (Corning® Costar®, USA). Cell culture media with 20% FBS was used as a chemoattractant in the lower chamber. At 24 hours post-incubation, cells on the upper chamber were gently removed while cells which had migrated through the transwell filters were fixed with 75% analytical ethanol. Next, they were stained with 0.5μg/mL Hoescht 33342 (Life Technologies, USA) and washed with 1x PBS to remove any unbound fluorescent conjugates. Photographs of the stained cells are taken with an inverted microscope and analysed with ImageJ ver1.48s software. The cell line, MDA-MB-231 was used as a positive control for this assay. This assay was used in Chapter 3, Section 3.2.3.

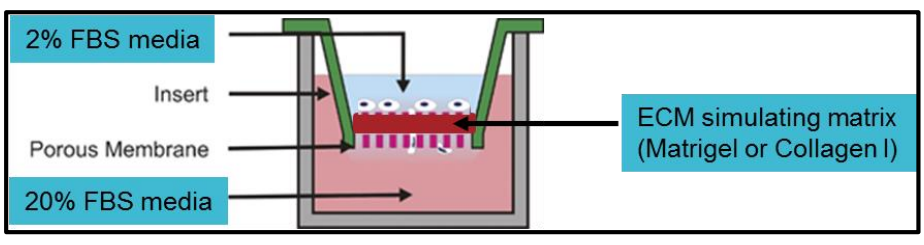
FIGURE 9: Schematic diagram depicting cross-sectional view of a transwell migration set-up



2.4. Transwell invasion assay

Two different types of matrices were used independently in this experiment: BD Matrigel (BD Biosciences, USA) and Collagen I (BD Biosciences, USA). Each ECM-simulation matrix was diluted in cell culture media containing 1% FBS and coated on 8 µm pore-size transwell filters (Corning® Costar®, USA). The transwells with the ECM-simulation coating were left to polymerize overnight in a 37°C in a humidified incubator containing 5% CO2 and 95% air. Prior to plating, the cells planned for the experiment were serum-starved overnight in 1% FBS cell culture media. The following day, 5 x 10^4 cells in 1% FBS culture media were seeded on the now-polymerized matrix in each transwell. Cell culture media containing 20% FBS was used as a chemoattractant in the lower chamber. At 24 hours post-incubation, cells on the upper chamber were carefully removed while cells which had invaded through the matrix-coated membranes were fixed with 75% analytical ethanol. Next, the cells were stained with 0.5µg/mL Hoescht 33342 (Life Technologies, USA) and washed with 1x PBS to remove any unbound fluorescent conjugates. Photographs of the stained cells are taken with an inverted microscope and analysed with ImageJ ver1.48s software. The cell line, MDA-MB-231 was used as a positive control for this assay.

FIGURE 10: Schematic diagram depicting cross-sectional view of a transwell invasion set-up



2.5. Cell viability assay

2.5.1. Cell viability to determine temozolomide dose-response

Cells were plated at 0.5 x 10^4 cells per well in a 96-well plate. The cells were allowed to attach overnight before drug treatment the following day. Incremental doses of TMZ (up to 800 μM) in cell culture media are administered to the cells for 72 hours. DMSO was used as the vehicle control for the experiment. CellTiter 96®AQueousOne Solution MTS Assay (Promega, USA) was added to the cells and incubated at 37°C for an hour, according to the manufacturer's instructions. Following this, the absorbance of the cells was read at 490 nm using a ASYS UVM340 microplate reader. All cell samples were plated and treated in triplicates. The cell viability absorbance results were analysed with GraphPad Prism ver6. This assay was used in Chapter 3, Section 3.2.4.

2.5.2. Cell viability assay post-clinically relevant treatment

Cells were seeded at 1 x 10^5 cells per well in 6-well plates. At 24 hours post-plating, they were subjected to one of the following regimens: DMSO control, TMZ only, RT only and combinatorial TMZ and RT. At the required time-point, the cells are washed, trypsinized and quantified using a haemocytometer. Cell viability was assessed using tryphan blue exclusion. This technique was applied throughout Chapters 4, 5 and 6.

2.6. Ionizing radiation assay

(This experiment was carried out with the kind assistance of Dr Keith Lim and Miss Tan Poh Wee, Department of Radiation Oncology, NUHS)

The protocol for this assay was adapted according to the literature[146,147]. Cells were seeded into either 12-well or 6-well plates (depending on the experiment), and subsequently placed in a water phantom to ensure dose homogeneity. This is a plastic water phantom with a density of 1.03 g/cm^3, which is almost water equivalent. The phantom mimics the human body as it would interact with radiation (CIRS Inc., USA). Essentially, it is a material with a quantified mass similar to human tissue that is used to investigate radiation beam effects of on humans. Next, the plated cells were irradiated with a 6-MV photon generated by the linear accelerator (Elekta Synergy) at a dose-rate of 8.7 Gy/ min, with different doses (2, 4, 6, 8 and 10 Gy). In order to achieve a final dose of 60 Gy to be delivered *in vitro*, we applied the Biological Effective Dose (BED) formula[148,149]. From the BED, the required IR dose for the experiment was calculated to be 30 Gy, to be given over 3 fractions at 10Gy per day. This gave us the biological effective dose (BED) of having given 60Gy to the cells or an Equivalent Dose in 2 Gy per day (EQD2) of 50Gy, more than sufficient radiation to deliver effective cell kill. This assay was applied in Chapters 3, 4, 5 and 6.

2.7. Quantitative RT-PCR

Total RNA from the harvested cells of interest was isolated with TRIzol reagent (Life Technologies, USA) according to manufacturer's instructions. The integrity of isolated total RNA was validated by denaturing agarose gel electrophoresis. One microgram of total RNA was reverse-transcribed using 400U of ImPromII reverse transcriptase (Promega, USA) and 0.5μg random hexamer (AIT Biotech, Singapore) for 60 minutes at 42°C. The reaction was terminated by heating at 70°C for 5 minutes. Next, gene expression levels were quantified by real-time PCR using gene-specific primers. Primers for the required genes of interest were listed as target cDNA. Real-time PCR (RT-qPCR) was performed on CFX96 (Bio-Rad, USA) in a total volume of 25μL in 1X Xtensa Mix (MIRXES, Singapore), containing 1μL of cDNA sample, 2.5mM $MgCl_2$, 100 nM of each primer, and 1.25U KlearTaq DNA polymerase (KBiosciences, UK). RT-qPCR was carried out after an initial denaturation for 10 minutes at 95°C, followed by 40 cycles of 30 seconds denaturation at 95°C, 30 seconds annealing at 60°C and 30 seconds extension at 72°C. Fluorescent detection was carried out during the annealing phase. Threshold cycles (Ct) were calculated automatically using the CFX Manager ver1.6 software (Bio-Rad, USA). The expression levels of the target genes were normalized to housekeeping genes in the same samples. This assay was applied in Chapters 3 (Section 3.2.2), 4 (Section 4.2.1), Chapter 5 (Sections 5.2.2 and 5.2.4) and Chapter 6 (Section 6.2.1).

TABLE 3: List of primers used for quantitative RT-PCR

GENE (*homo-sapiens*)	**FORWARD SEQUENCE (5'-3')**	**REVERSE SEQUENCE (5'-3')**
GAPDH	ATGGGGAAGGTGAAGGTCGG	GACGGTGCCATGGAATTTGC
β-actin	AAGATCAAGATCATTGCTCCTC	AACTAAGTCATAGTCCGCCT
RPL19	TCAGGCTTCAGAAGAGGCT	GGAGTTGGCATTGGCGATT
BMI-1	TTCATTGATGCCACAACCAT	CAGCATCAGCAG AAGGATGA
CD133	AGAGCTTGCACCAACAAAGTACAC	ACCAAGCACAGAGGGTCATTG
FABP7	TGACCAACAGTCAGAACTTT	GGAGGAGAGCAGAGAAGAGA
Nestin	GGAGGAGAGCAGAGAAGAGA	AAGCCAGGACAGCAGGAT
SOX2	TACAGCATGTCCTACTCGCAG	GAGGAAGAGGTAACCACAGGG
SOX9	CCCCAACAGATCGCCTACAG	TCTGGTGGTCGGTGTAGTCGTA
Vimentin	TGCAGGAGGCAGAAGAATGG	ATTTCACGCATCTGGCGTTC
MGMT	GTCGTTCACCAGACAGGTGTTA	ACAGGATTGCCTCTCATTGCTC
MLH1	TTCGTGGCAGGGTTTTG	GCCTCCCTCTTTAACAATCACTT
MSH2	GCTGGAAATAAGGCATCCAAGG	CACCAATGGAAGCTGACATATCA
MSH3	TGGAAAATGATGGGCCTGTTAAA	AGACATTCCCAGATCACTTCCT
MSH6	AGCTTAAAGGATCACGCCATC	AAGCACACAATAGGCTTTGCC
PMS1	GTTCTGGGGACTGCTGTTATG	GGTCTGCATCACACTTTGGAA
PMS2	GAAGGTTGGAACTCGACTGATG	CGCACAGGTAGTGTGGAAAA
ABCA3	GGAGAACTTCTATGCCTGGAG	GGTGTATAATTCTGTCAGTGTCC
ABCB1	TTCTACGGGAAATCATTGGT	GGTGTCAAATTTATGAGGCAG
ABCC3	GAATTCTGCAAGGGTTCTTGG	GGTGTCAAAGAAGGACTGTG
ABCG2	TCATGTTAGGATTGAAGCCA	TTGTGAGATTGACCAACAGAC
RFX1	GAAGCGACTGCCCAAAGC	CACGTCGGGAATGAGGATTT
FLVCR2	CCCAGCAGGGCCCAAT	CGGGCGATGGAACCTAAGT

2.8. Immunoblotting

The cells were washed with 1x PBS and subsequently lysed in 2% SDS lysis buffer as previously described by Wan *et al*[143]. Protein concentrations are quantified using the MicroBCA assay (Pierce, USA). Each protein sample was then separated by 8% to 15% SDS-PAGE gel electrophoresis, transferred onto nitrocellulose membranes (Bio-Rad, USA) and blocked with 5% low-fat milk at room temperature. Next, the membrane was probed with primary antibodies to detect each protein of interest. Secondary antibodies appropriate for each primary antibody were probed for an estimated 60 minutes at room temperature. For both primary and secondary antibodies, the antibody dilution used followed as per the manufacturer's recommendations, and adjusted accordingly if required. The protein bands were developed with Immobilon Western Chemiluminescent HRP Substrate (Millipore, USA) on a ChemiDoc™ XRS+ system (Bio-Rad, USA). Subsequently, the band intensities were quantified using Image Lab v4.1 software (Bio-Rad, USA). This assay was used in Chapters 3 (Section 3.2.2) and 4 (Section 4.2.1).

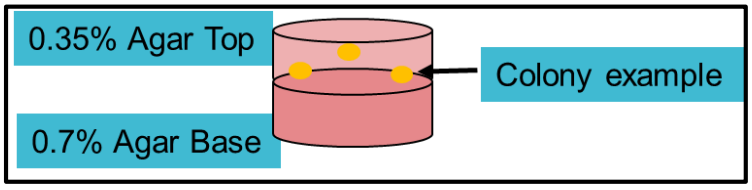
TABLE 4: List of antibodies used for immunoblotting

PRIMARY ANTIBODY	SOURCE	SECONDARY ANTIBODY
β-actin	Sigma, USA (AC-15)	mouse
β-tubulin	Santa Cruz Biotechnology, USA (H-235)	rabbit
MGMT	Cell Signaling Technology, USA (#2739)	rabbit
FABP7	Cell Signaling Technologies, USA (#9597)	rabbit
MSI-1	Abcam, USA (ab97959)	rabbit
SOX-2	Abcam, USA (ab52865)	rabbit

2.9. Soft agar colony forming assay

Briefly, 1% gum agar (Sigma, USA) was melted in a microwave and cooled to 40°C in a water bath. Under sterile conditions, the melted agar was mixed with equal volumes of culture media to form the base mixture. This final mixture concentration consisted of: 0.7% agar + 3:1 (DMEM: F12) + 1x B27 + 20 ng/mL bFGF + 20ng/mL EGF. The base agar was allowed to solidify at room temperature for approximately 30 minutes. In meantime, the top agar layer was prepared using 1% melted agar mixed with equal volumes of culture media containing cells. The cells of interest were dissociated with Accutase® (Life Technologies, USA) to ensure single cell suspension and manually counted. Each top layer consisted of 1 x 10^4 cells in a final mixture concentration of 0.35% agar + 3:1 (DMEM: F12) + 1x B27 + 20 ng/mL bFGF + 20ng/mL EGF. Next, the cells were incubated at 37°C in a water-saturated incubator containing 5% CO2 and 95% air for 14 days. The cells were fed with culture media every 48 hours. The experiments were done in 12-well plates, using biological triplicates, each biological line with technical triplicates. Each plate included 2 wells containing only base and top agar layers (i.e. without cells) to serve as a background control for cell quantification. At the required time-point, the number of colonies were manually quantified using ImageJ ver1.48s software. This assay was used in Chapter 4 (Section 4.2.2).

FIGURE 11: Schematic diagram of soft agar colony assay set-up



2.10. MicroRNA-related experiments

2.10.1. MicroRNA profiling

(The workflow described below was developed by Dr Zhou Lihan and Dr Zou Ruiyang, MiRXES, Pte Ltd and was carried out by myself in μRSIC, an A*STAR facility dedicated to miRNA profiling.)

The following assay was utitlised in Chapter 5 (Section 5.2.1). MicroRNA profiles were determined using a facile high performance real-time RT-PCR assay (modified stem-loop mediated reverse transcription quantitative PCR; mSMRT-qPCR) that allowed specific and rapid detection of mature miRNAs using fast thermo-cycling profile (10 seconds per cycle).[123]These assays were developed using a proprietary *in-house* designed algorithm based on thermodynamics principles and parametrizations derived from data-driven computational modelling. A standard workflow protocol dictated the miRNA profiling process. Essentially, it consisted of 3 key components as per following:

I. Pre-analytics.

Total RNA from up to 200 μL of cells was isolated with the phenol-chloroform RNA extraction method as previously described (**2.7. Quantitative RT-PCR**). To reduce RNA loss and monitor extraction efficiency, isolation enhancers and spike-in control RNAs (MiRXES) were added to each sample prior to isolation.

II. Analytics.

The isolated total RNAs and 7 dilutions (10^8 to 10 copies) of synthetic template RNAs were converted to cDNA in multiplex (60-plex) reverse transcription reactions. Since the concentration of the total RNA could be reliably quantified, the RNA input to the RT reaction is normalized by volume instead of quantity. Typically, 5 to 10% (volume) of total isolated RNA was used for each multiplex reaction. To detect the presence of inhibitors and monitor the RT-qPCR efficiency, a second set of spike-in control RNAs was added to the total RNA prior to the RT step. These spike-in RNAs, together with the ones added during RNA extraction step, were reverse-transcribed and detected by specifically designed assays. In addition, the spike-in RNAs were added in different amounts to assess the efficiency of the whole workflow in detecting high, medium and low abundance target miRNAs. The synthesized cDNA was then subjected to a multiplex augmentation step that specifically augments the cDNA content by 1,000 to 10,000 fold, without changing the representation of the miRNA. Next, the augmented cDNA was diluted and quantified in SYBR Green based single-plex qPCR. The cDNA of the serial dilutions of synthetic templates are simultaneously quantified and used to construct a standard curve that allows absolute quantifications of target miRNAs.

III. Data processing and analysis.

Upon completion of profiling, the raw Threshold Cycles (CT) values were processed and the absolute copy numbers of the target miRNAs in each sample were determined through intra-polation of the standard curves. The copy numbers of the spike-in control RNAs were simultaneously quantified for each sample. The technical variations introduced during RNA isolation and

the process of RT-qPCR was then normalized by the geometric means of these spike-in control RNAs. With the normalized data, a set of endogenous reference miRNAs that were stably expressed across all samples could be identified using multiple validated mathematical models. A combination of several stably expressed endogenous reference miRNAs is then used to normalize the biological variations in the samples. The final miRNA expression levels after normalization of both technical and biological variations were analysed with multiple statistical methods to identify panels of miRNAs with the highest discriminatory power between control and treated cells. For the purpose of increasing the robustness as well as reducing the chance of over-fitting, the features (miRNAs) were filtered by 2 criteria to form the candidate feature pool for the miRNA panel selection. The features with individually low ROC values (<0.7) of the ROC and signal-to-noise ratio (the distance to detection limit) were excluded.

2.10.2. Overexpression of selected miRNAs

As per manufacturer's instructions, 5 nM of miRNA mimic (Qiagen, USA) was used to overexpress the individual expression of the miRNA of interest in cells. Each miRNA was mixed with Lipofectamine RNAiMAX® transfection reagent (Life Technologies, USA) in Opti-MEM reduced serum media (Life Technologies, USA), and allowed to stand at room temperature for 15 minutes before transfection. Approximately, six hours post-transfection, the transfection media was replaced with regular cell culture media. Medium GC content negative control (Life Technologies, USA) was used as experimental control. This experiment was performed in Chapter 5 (Section 5.2.3).

TABLE 5: Oligoribonucleotide sequences of miRNA mimics used

miRNA of interest	miRNA mimic sequence
hsa-miR-125a-3p	5' ACAGGUGAGGUUCUUGGGAGCC
hsa-miR-629-5p	5' UGGGUUUACGUUGGGAGAACU
hsa-miR-19b-5p	5' UGUGCAAAUCCAUGCAAAACUGA

2.11. Microarray data processing and analysis

(The following was carried out with the kind assistance of Dr Vivien Koh, Biopolis Shared Facilities, A*STAR)

Total RNA was isolated from cell types of interest and subjected to microarray gene expression analysis using Illumina HT-12 v4 Expression BeadChips (Illumina, USA). Three independent biological replicates were profiled for each cell type of interest. The RNA was first biotinylated and amplified using the Illumina® TotalPrep-96 RNA Amplification Kit according to the manufacturer's instructions, followed by cDNA synthesis, cDNA purification, cRNA synthesis and cRNA purification. Next, the samples were hybridized onto the arrays for 16 hours at 56°C. Arrays were washed and scanned using the Illumina BeadArray Reader. Data was then exported and analyzed using GenomeStudio v2011.11 software (Illumina, USA). This assay was used for Chapters 4 (Section 4.2.1) and 5 (Section 5.2.2).

2.12. Predictive database analysis of miRNA-mRNA mapping

(This analysis was carried out with the kind assistance of Dr Zhou Lihan and Dr Zou Ruiyang, MiRXES, Pte Ltd)

The following analysis was utilised in Chapter 5 (Section 5.2.2). The online interactive tool miRror 2.0, was used for analysing experimental results under the notion of coordination in miRNAs regulation[127]. For a set of miRNAs, miRror 2.0 outputs a ranked list of gene targets according to their likelihood to be targeted by the miRNA ensemble and vice versa for a set of regulated genes. The miRror 2.0 algorithm then designates statistical criteria that were uniformly applied to a dozen miRNA-target prediction databases. Users are able to select the preferred databases for predictions and numerous optional filters that restrict the search to predictor scores[150]. Next, miRror 2.0 converts all predictor resources results into a unified platform by incorporating a statistical measure according to the hyper-geometrical distribution[127]. Here, we set the following parameters for our mRNA target prediction: 1) the mRNA targets must be common to all 3 selected miRNAs; 2) ≥2 mRNA targets have to be simultaneously found in ≥2 independent miRNA databases; 3) the statistical value of each predicted mRNA target has to be <0.05 (*p <0.05)*. The predicted list of mRNA targets was subsequently mapped against all upregulated genes in the mRNA microarray data. Each confirmed mRNA target was then individually validated using RT-qPCR to confirm its upregulated expression.

2.13. Bioinformatics analysis with human clinical databases

(This analysis was kindly assisted by Edwin Sandanaraj, Senior Research Fellow in Bioinformatics, NNI)

The mRNA expression of genomic markers was evaluated in two independent glioma databases including REMBRANDT and Gravendeel [94,151]. The REMBRANDT (Respository of Molecular Brain Neoplasia Data) patient glioma database contains data generated through the Glioma Molecular Diagnostic Initiative from 874 specimens[94]. The Gravendeel patient glioma database has cummulative data from 276 specimens from the Erasmus University Medical Centre tumour archive[151].The required raw data was extracted from REMBRANDT and GEO database archive (GSE16011). The cel files were processed in mas5 algorithm using 'affy' packages implemented in R bioconductor [152,153]. Clinical information of patients was collected from respective publication for statistical analyses. Probeset annotation for genomic markers was queried in ensemble human database with biomaRt packages [154]. A standard one-way ANOVA model was fitted to assess the variability in gene expression across histological subtypes of glioma patients and statistical significance was evaluated at the level of 5% ($p<0.05$). Survival association for gene expression was estimated using tercile-based stratification of patients. A terciles approach ranks patients based on mRNA expression of individual gene and stratifies the population into three groups as low-, intermediate- and high-expression patients. Kaplan-Meier survival curves were plotted for the patient groups (terciles) and the statistical significance was evaluated by log-rank test. In addition, a multivariate Cox Proportional-Hazards model was regressed to assess the independent association of covariates investigated. A p-

value of <0.05 was considered to be statistically significant. Graphical illustrations were generated using CRAN packages such as beeswarm, survival and graphics [155-157]. The results of these analyses are reflected in Chapter 6 (Section 6.2.2).

2.14. siRNA knockdown of selected genes

As recommended by the manufacturer, 20 nM of siGENOME SMARTpool siRNA duplex (Dharmacon, USA) was used to individually silence the expression of each gene of interest in the cells. Each siRNA was mixed with Lipofectamine RNAiMAX® transfection reagent (Life Technologies, USA) in Opti-MEM reduced serum media (Life Technologies, USA), and allowed to stand at room temperature for 15 minutes before transfection. Approximately, 6 hours post-transfection, the transfection media was replaced with regular cell culture media. Medium GC content negative control (Life Technologies, USA) and void-siRNA treatment were used as experimental controls. This assay was used in Chapter 6 (Section 6.2.1).

TABLE 6: Oligoribonucleotide sequences (4 in 1) in each SMARTpool siRNA

Gene of interest	Target sequence
Human RFX1	GGAACACUGUGAGGCCAUU
	GAUCCAAGGCGGCUACAUG
	GCAGAACACCGCACAGAUC
	CAACACAGGCGUAUACUGA
Human FLVCR2	CAACCCUGGUAGUCUAUAU
	GAGCUCACGUACCCAGAAU
	GAAUUGCGAUUGGGUUCUU
	UCAGGAAUCUGGCUGGAUA

2.15. Statistical analysis

Statistical analyses were performed using 2-tailed Student's t-test or Analysis of variance (ANOVA) test, depending on the experimental setup. Differences between sample means were considered statistically significant when *p-value*<0.05 (*) or *p-value*<0.001 (**).

CHAPTER THREE

A subpopulation of LN229 human glioblastoma cells survives long-term temozolomide treatment but is sensitive to high-dose radiation

'A theory has only the alternative of being right or wrong. A model has a third possibility: it may be right, but irrelevant'

M. Eigen (1927-present)

(Quote from 'The origin of biological information 1973')

Chapter 3

A SUBPOPULATION OF LN229 HUMAN GLIOBLASTOMA CELLS SURVIVES LONG-TERM TEMOZOLOMIDE TREATMENT BUT IS SENSITIVE TO HIGH-DOSE RADIATION

3.1. Introduction

Since the emergence of reports showing a survival benefit with the addition of TMZ to RT as a first-line treatment , TMZ has been the most studied agent in recurrent GBM, either as monotherapy or as the mainstay of a combination regimen[158]. As a result, there is a surge in the exploration of mechanisms underlying acquired TMZ-related resistance in GBM[159-164]. As a result, a popular *in vitro* method described to do so is the utilization of TMZ-resistant (TMZ-R) GBM cell lines. These techniques of developing TMZ-R GBM cell lines have been well-described in literature[137,161,165,166]. Although this approach may not completely reflect the situation *in vivo,* it allows assessment of possible mechanisms triggered by repeated pulse exposure to TMZ[167]. In this chapter, the approach describes the assessment of LN229 parent cell versus LN229 TMZ-R cell lines in the context of common cancer hallmarks and response to clinically relevant treatments.

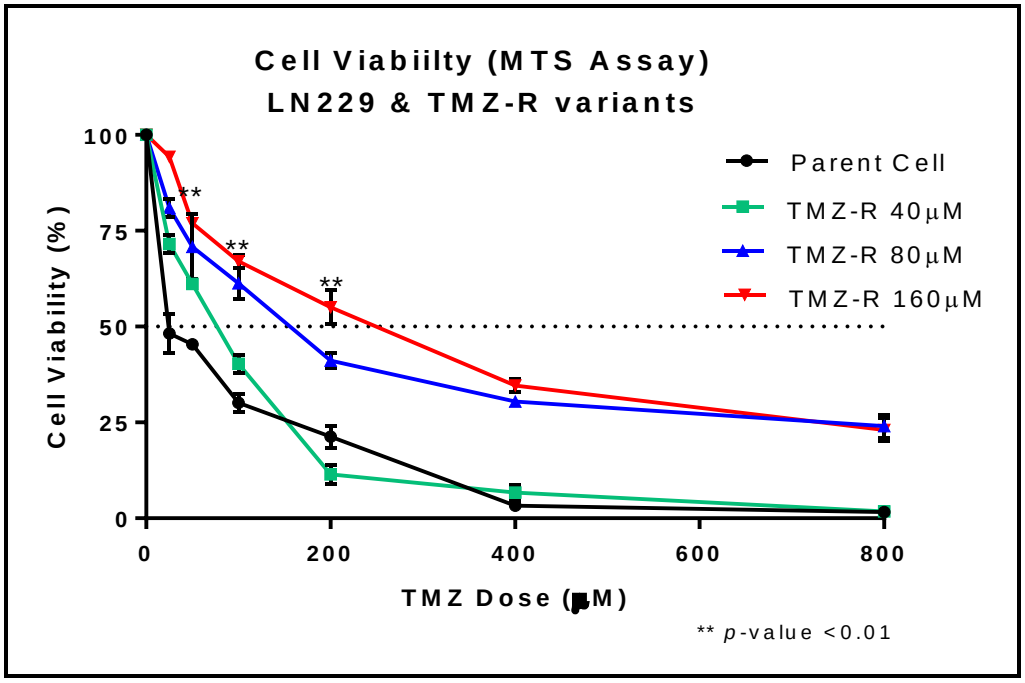
3.2. Results

3.2.1. A subpopulation of LN229 cells demonstrates resistance towards commonly used GBM chemotherapeutics

During a screen of a panel of human glioblastoma cell lines, LN229 was noted to survive long-term incremental doses of TMZ treatment. To assess their resistance potential, LN229 TMZ-R variants were subjected to a TMZ treatment cell viability assay. In order to account for reproducibility, the results were counter-checked for cell viability doses at 50% in TMZ and BCNU against what is reported in the literature for TMZ-sensitive LN229 cells. We found that our findings concurred to a similar range for both drugs in the literature[168,169]. For the LN229 TMZ-R variants, the panel displayed higher cell viability in comparison to the LN229 parent cells, in the presence of incremental doses of TMZ. (See **Figure 12A**). Under the same circumstances, it was also noted that LN229 TMZ-R cells cultured in higher doses of TMZ (hence, presumably more chemo-resistant) maintained better cell viability at higher doses of TMZ, in comparison to LN229 parent cells. In addition, a similar trend was observed when the same LN229 cell panel was treated with BCNU, another DNA-methylation agent. (See **Figure 12B**). The cross-resistance across 2 commonly cited GBM chemotherapeutics suggests that in the presence of TMZ-resistance, the use of another agent may likely be futile unless high enough concentrations are achieved.

FIGURE 12: A. LN229 TMZ-resistant variants demonstrate higher cell viability when treated with TMZ, as compared to the LN229 TMZ-sensitive parent cells. B. In a similar trend, LN229 TMZ-resistant variants demonstrate higher cell viability when treated with BCNU, as compared to the LN229 TMZ-sensitive parent cells.

A



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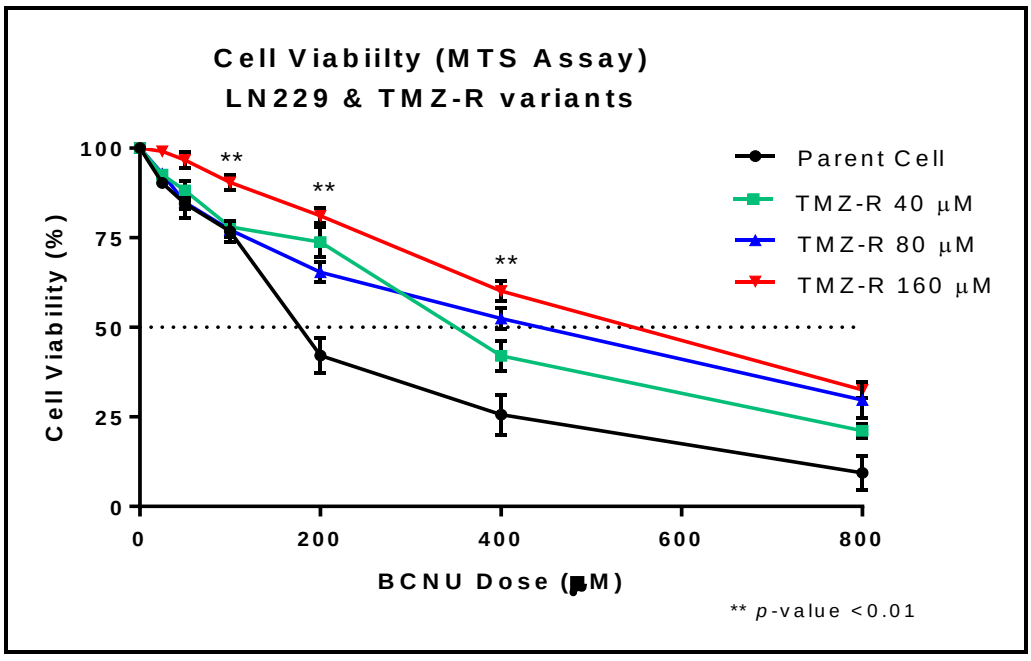
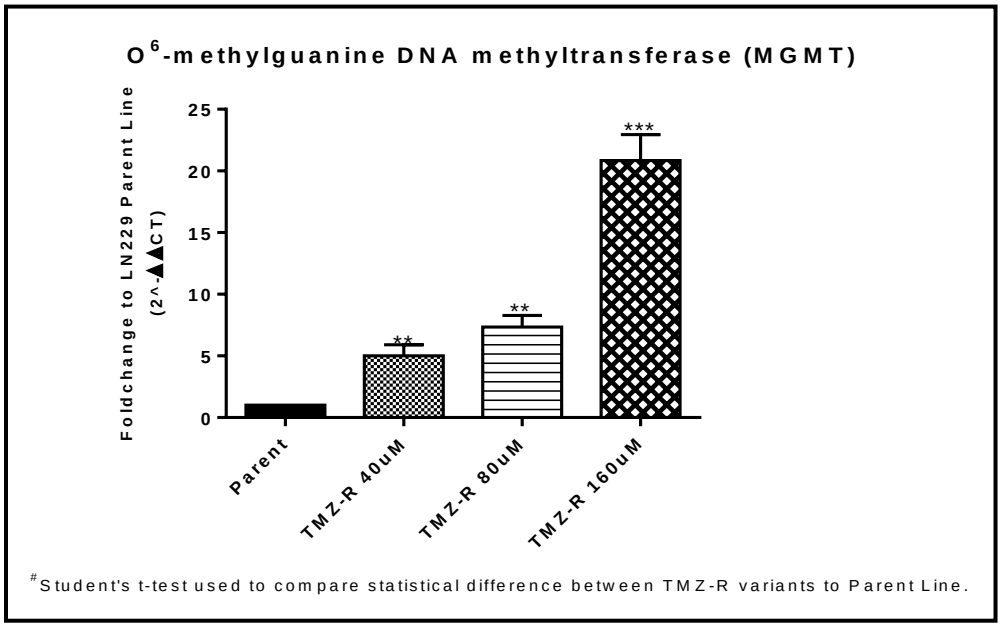


3.2.2. Acquired temozolomide resistance in LN229 human glioblastoma cells correlates with MGMT expression

From the previous results (See **Section 3.2.1**), the cross-resistance of LN229 TMZ-R variants to both TMZ and BCNU was observed. As both chemotherapy agents are DNA methylation agents, such a phenomenon is not entirely surprising. The expression of MGMT, a gene well-studied in TMZ resistance for GBM patients was examined in the LN229 cell panel. (See **Figure 13**). MGMT is consumed when counteracting TMZ-induced damage, thus, intracellular levels of MGMT are expected to correlate with chemoresistance[167]. Our results identified 2 key findings: firstly, we were unable to detect MGMT at 35-40 cycles using RT-qPCR in TMZ-sensitive LN229 parent cells, concurring with our immunoblotting findings. The equation 2^-ΔΔCT was used to quantify the PCR findings, thus assigning a relative value of 1 to LN229 parent cells. Next, there was an incremental expression of MGMT at both transcriptional and protein levels as LN229 TMZ-R cells become more TMZ-resistant. In affected patients, there is already awareness that GBM tumours that actively express MGMT are more resistant to TMZ than tumours where the MGMT gene is absent[170]. Furthermore, there is some persuasive data supporting that a patient's MGMT status influences GBM recurrence, to the point of prompting clinical studies to use a 're-challenge TMZ' metronomic approach in these patients[171-173]. This practice is currently used for a select group of GBM patients with recurring tumours today in our local institution. Putting it all together, the preliminary data is consistent with a role for MGMT in chemoresistance of LN229 human glioblastoma cells.

FIGURE 13: A. mRNA expression of MGMT from RT-qPCR. B. Western blot showing protein expression of MGMT in LN229 parent and TMZ-R variant cells. (Jurkat cell line was used as a positive control for the MGMT antibody in the Western blot [174].)

A



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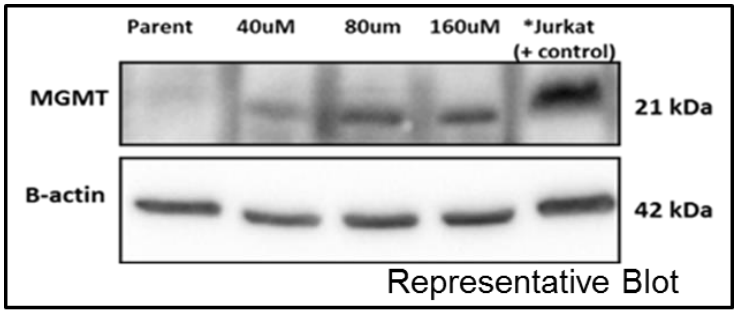
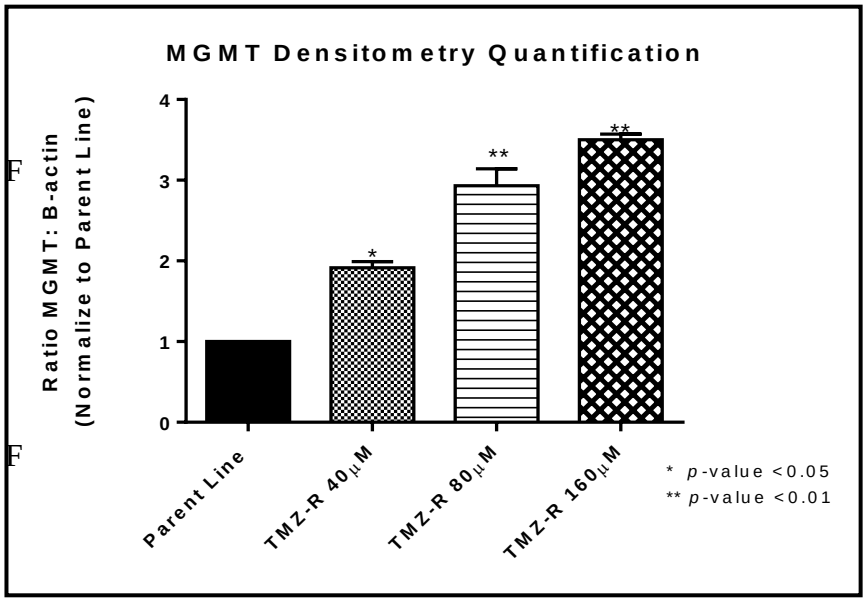




Following the course of the previous findings, the mRNA expression levels of other transcription factors commonly related to therapeutic resistance in GBMs, such as the mismatch repair (MMR) and ATP-binding cassette transporters (ABC transporter family) genes was investigated. (See **Table 7**).

TABLE 7: List of other genes investigated in the LN229 cell panel

MMR genes	ABC transporter family genes
MLH1	ABCA3
MSH2	ABCB1
MSH3	ABCC3
MSH6	ABCG2
PMS1	

In this experiment, some of these genes showed changes at mRNA level. (See **Figure 14**). The decreased levels of MMR proteins has been reported to be involved in resistance to alkylating agents, including gliomas[167]. For example, MLH1 and MSH2 have been previously demonstrated to be the commonly mutated in human malignancies[175].The absence of MLH1 was often associated with a high degree of TMZ resistance, and its resistance-related mechanisms was also known to be independent of O^6-alkylguanine DNA alkyltransferase levels[175]. These findings were also similar for MSH2, however, MLH1 deficiency was more common than MSH2 deficiency in the context of TMZ resistance[175].

In the LN229 cell panel, MLH1 and MSH2 were not detectable at 40 cycles in RT-qPCR in all lines, including the LN229 parent cells. Since the

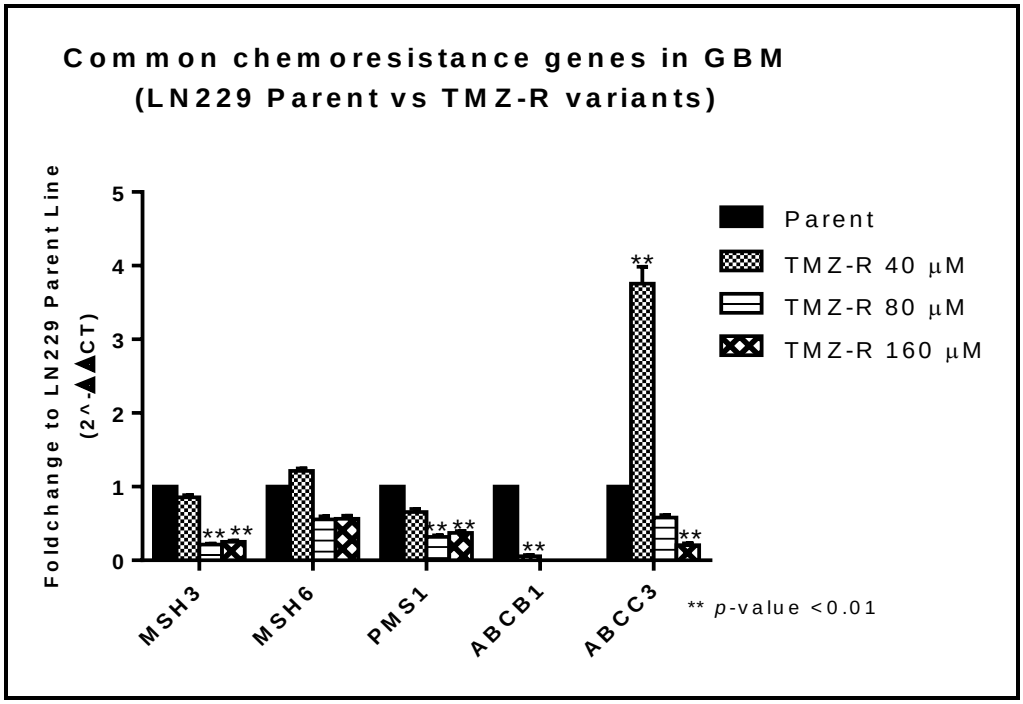
LN229 parent cells were able to survive incremental TMZ concentration to generate their resistant counterparts while other cell lines succumbed, it is hence not unexpected that the LN229 TMZ-R variant cells maintained this feature in their phenotype. In contrast, the mRNA expression of MSH3 and PMS1 was found to be significantly downregulated in both LN229 TMZ-R 80 μM and 160 μM cells, in comparison to the LN229 parent cells. However, for the LN229 TMZ-R 40 μM line, the reduction in expression was not statistically significant. The reduction in MSH3 and PMS1 for the LN229 TMZ-R 80 μM and 160 μM cells concur with what has been previously reported in the literature[167]. In addition, although the inactivation of MSH6 had been previously shown to mediate chemoresistance in glioblastoma cells[176], a statistically significant reduction in its expression for all the LN229 TMZ-R variant cells, in comparison to LN229 parent cells was not observed.

High expression of drug efflux pumps, including the ABC transporter family of genes, has been previously shown to have the capacity to expel cytotoxic drugs, resulting in better cancer cell survival and thus promoting chemoresistance[177,178]. In this experiment, the majority of the ABC transporter family genes were either not detectable at 40 cycles in RT-qPCR (ABCA3 and ABCG2), or had reduced expressions (ABCB1 and ABCC3) in the LN229 cell panel, with the sole exception of ABCC3 in LN229 TMZ-R 40 μM cells.

Putting it all together, the results demonstrated that as LN229 cells become progressively more chemoresistant, MGMT expression was noted to

be incremental. However, the other chemoresistant genes did not show a similar trend to MGMT. In particular, LN229 TMZ-R 40 μM was noted to be different its expression of MSH3, PMS1 and ABCC3 in comparison to its LN229 TMZ-R 80 μM and 160 μM counterparts. Nonetheless, the aim of the mRNA screening of these transcriptional factors was to seek a dose-dependent consensus in the expression of the TMZ-resistant LN229 cell lines, as previously demonstrated by MGMT expression at both mRNA and protein level. In this context, the RT-qPCR experiments for the undetectable genes can be repeated using the following cells lines U251MG for MLH1 and MSH2, and U87MG for ABCA3 and ABCG2, as positive controls, as verified from the literature. Overall, these findings suggest that the possible interplay and or, contributions by other genes, whether if they are independent of MGMT-related mechanisms, in LN229 TMZ-R cells in the context of chemoresistance, are not as straightforward at this stage.

FIGURE 14: RT-qPCR showing mRNA expression of common genes cited in GBM chemoresistance. MLH1, MSH2, ABCA3 and ABCG2 were not detectable at 40 cycles.



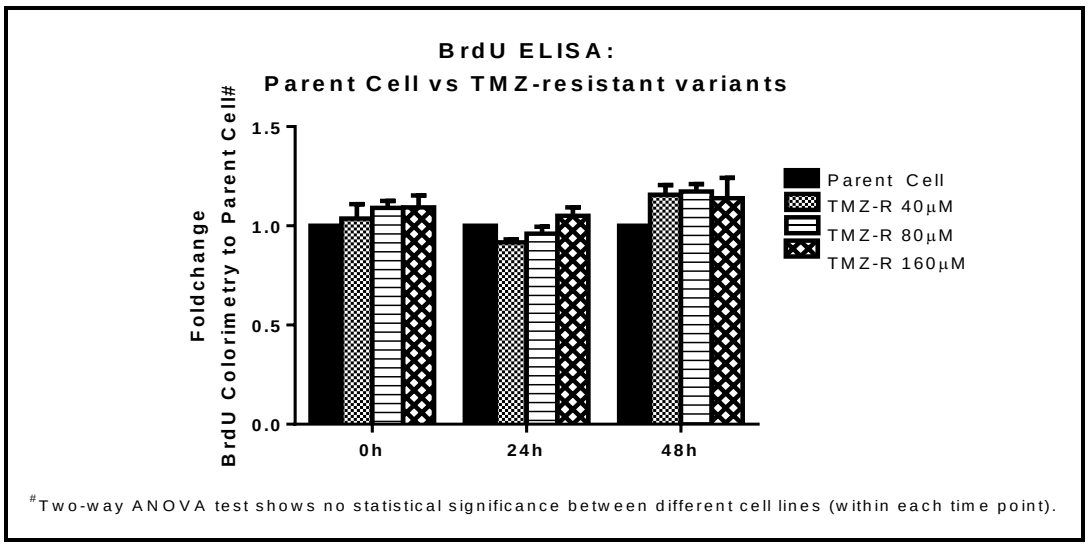
3.2.3. Temozolomide-resistant LN229 human glioblastoma cells show similar tumorigenic hallmarks to temozolomide-sensitive LN229 cells

A main feature of malignant tumours involve their ability to sustain chronic proliferation. In the case of normal tissues, homeostasis is maintained via the control of signals that initiate entry into and progression through the cell cycle. However, GBM cells have been known to dysregulate such signals, in order to continue cancer cell growth[179]. This experimental setup demonstrated the ability of LN229 TMZ-R variants to maintain similar proliferative rates as LN229 parent cells. (See **Figures 15A** and **15B**). In

addition, migrating GBM cells are known to be able to generate forces and subsequently distort into shapes, hence allowing them to move through brain structures[27]. It was that noted the LN229 cell panel showed similar migratory properties between parent and TMZ-R variant cells, another indication of their ability to conserve this aspect of neoplastic behaviour during acquisition of TMZ resistance (see **Figures 16A** and **16B**). Another crucial feature of GBMs is their diffusely infiltrative nature. In clinical practice, we often encounter GBM tumours invading functional brain areas that make difficult for pathological tissues to be fully resected, inevitably affecting the patient's outcome[180]. Owing to this malignant cell invasion, many treatments for GBM remain elusive[181]. These results demonstrate that LN229 TMZ-R variants retain the invasive properties as LN229 parent cells, in both types of simulated ECM matrices. (See **Figures 17A** and **17B**). To conclude the findings in this section, it was observed that the *in vitro* ability of LN229 TMZ-R cells preserved their innate properties of 3 known cancer hallmarks, in comparison to LN229 parent cells: proliferation, migration and invasion, in the face of resisting cell injury by chemotherapeutics used for GBM.

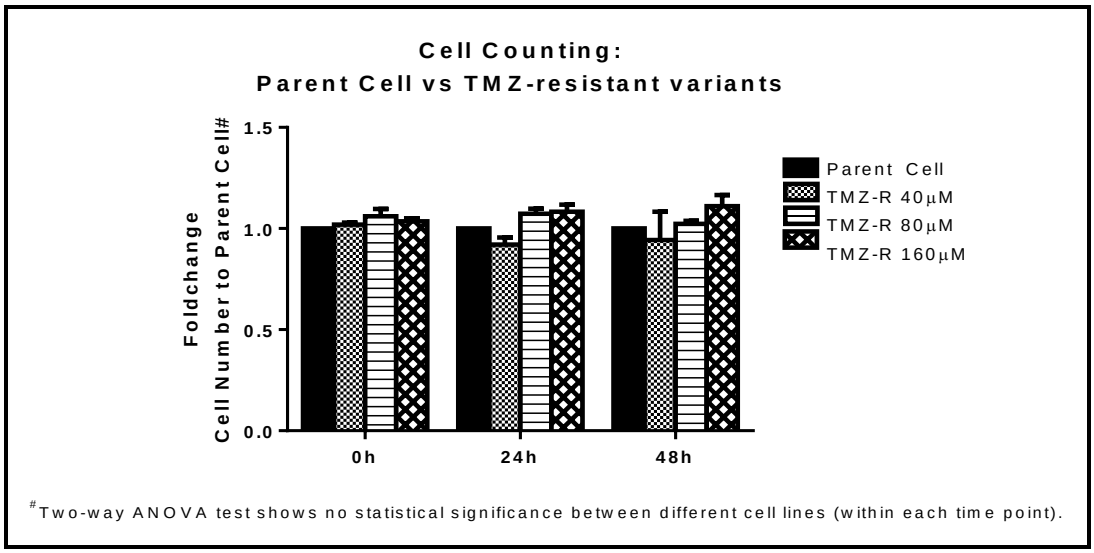
FIGURE 15: A. BrdU ELISA cell proliferation assay of LN229 parent versus LN229 TMZ-R variant cells. B. Cell counting experiment comparing LN229 parent versus LN229 TMZ-R variant cells. C. Cell cycle profile of LN229 parent versus LN229 TMZ-R variant cells.

A



#Two-way ANOVA test shows no statistical significance between different cell lines (within each time point).

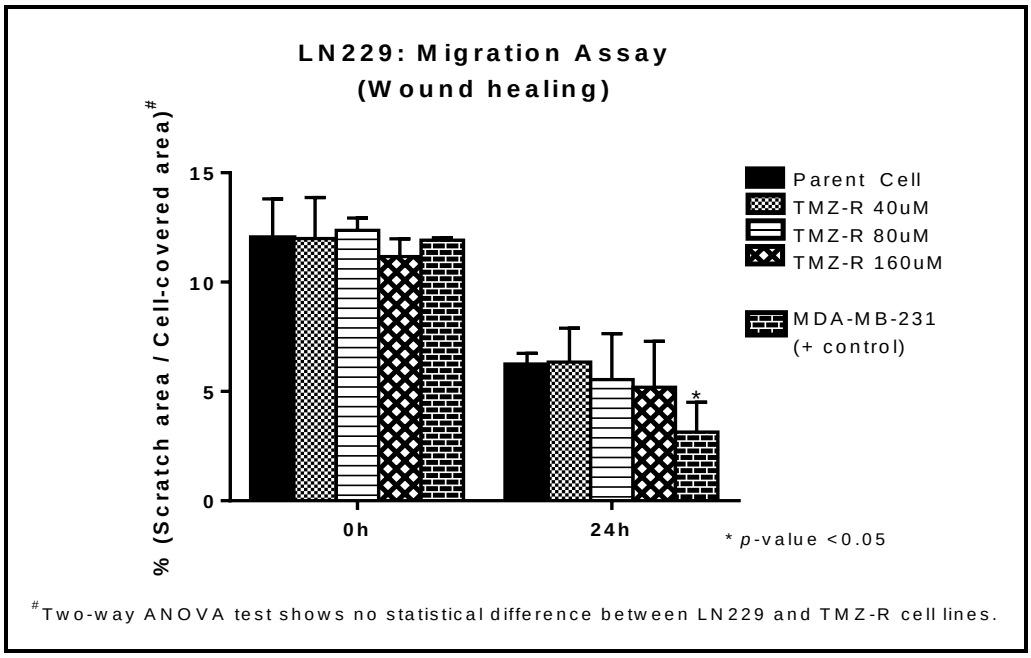
B



#Two-way ANOVA test shows no statistical significance between different cell lines (within each time point).

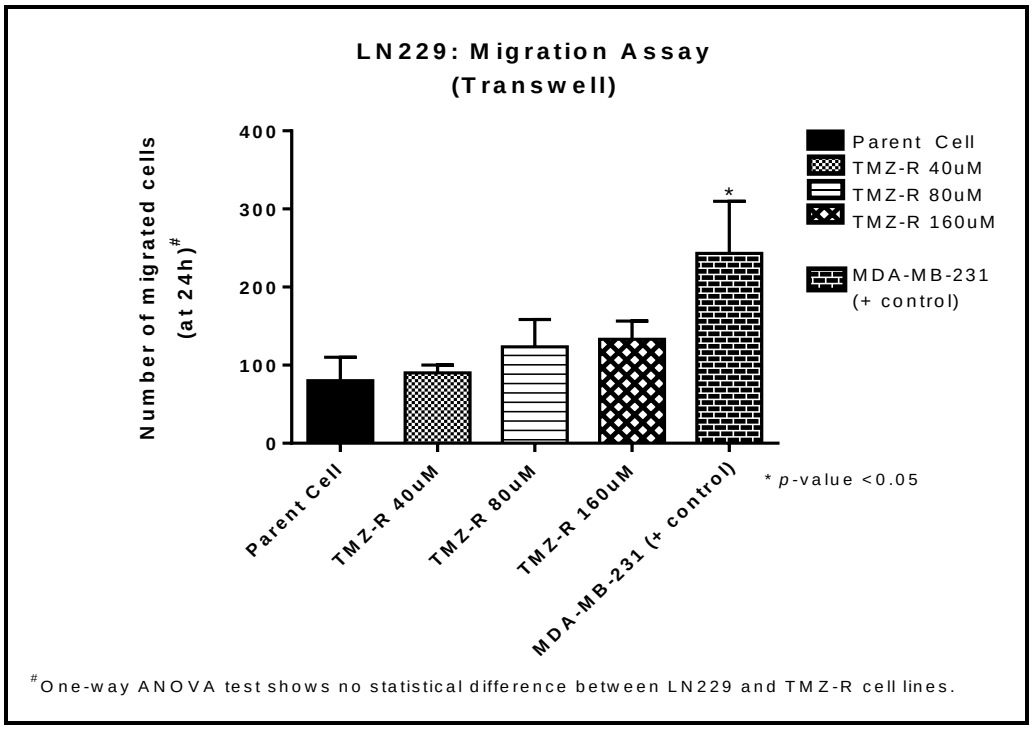
FIGURE 16: A. Wound healing assay comparing LN229 parent versus TMZ-R variants. B. Transwell migration assay comparing LN229 parent versus TMZ-R variants.

A



#Two-way ANOVA test shows no statistical difference between LN229 and TMZ-R cell lines.

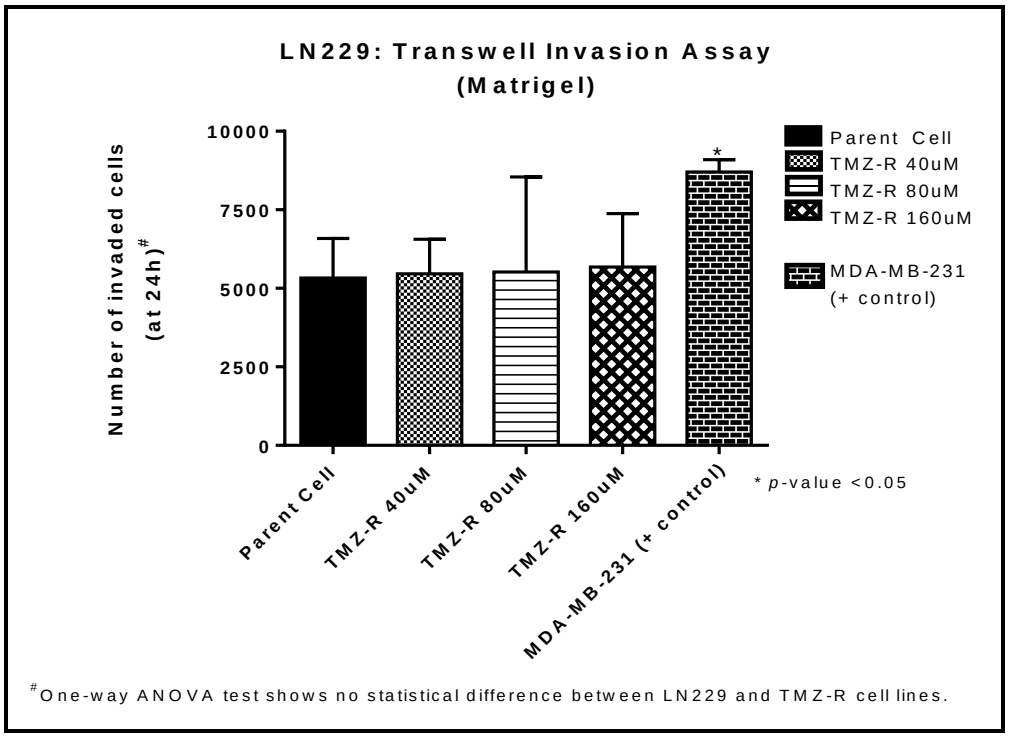
B



#One-way ANOVA test shows no statistical difference between LN229 and TMZ-R cell lines.

FIGURE 17: A. Transwell invastion assay results of LN229 parent versus TMZ-R variants (Matrigel matrix). B. Transwell invastion assay results of LN229 parent versus TMZ-R variants (Collagen I matrix).

A



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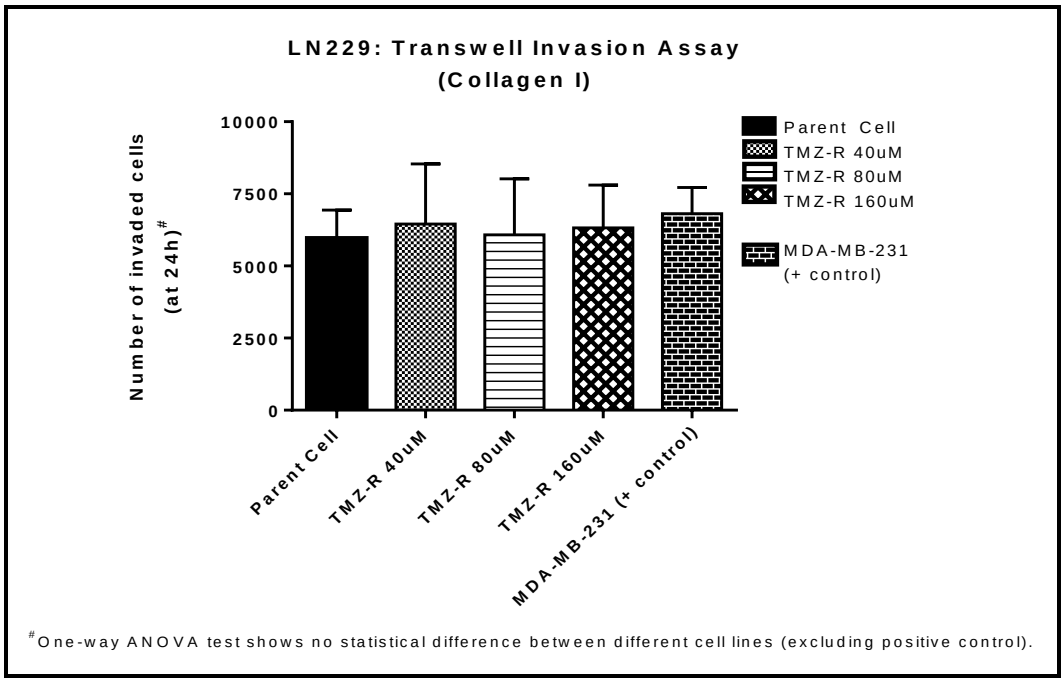
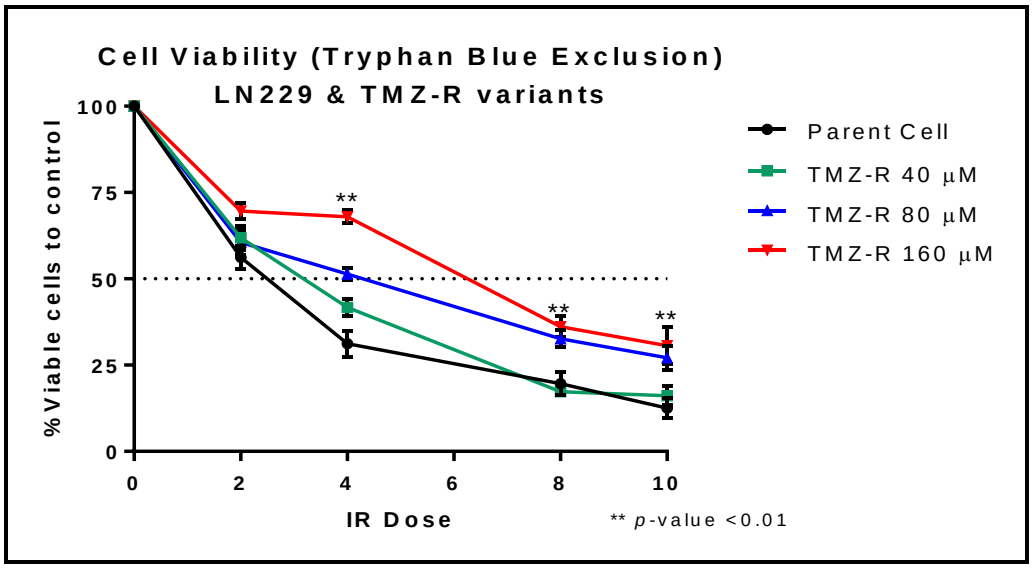


3.2.4. Acquired temozolomide resistance in LN229 human glioblastoma cells demonstrates higher resistance to ionizing radiation compared to temozolomide-sensitive LN229 cells

In this cell viability assay, the interest was to know if the panel of LN229 TMZ-R cells was able to withstand the effects of IR. The results demonstrated that the TMZ-resistant LN229 cells were generally more resilient to IR treatment in comparison to LN229 parent cells. Furthermore, within the LN229 TMZ-R variants, we noted that as the cells became increasingly TMZ-resistant, they were more viable in the presence of single-dose IR treatment (2, 4, 6, 8 and 10 Gy). (See **Figure 18**). As IR causes cell damage by DNA strand breaks, and that MGMT functions as a DNA damage repair entity[76], such findings were not unexpected. Putting these experimental outcomes together, an IR treatment dose-dependent relationship between LN229 TMZ-R cells that seemed to correspond to their respective MGMT expressions was observed. Here, as the MGMT expression increased in the cells from 40 μM to 80μM and finally, 160 μM, there was likewise, a parallel increase in their pro-survival response to IR treatment.

FIGURE 18: Cell viability assay demonstrating the effects of incremental IR doses on LN229 parent versus LN229 TMZ-R variants. A single dose of IR (2, 4, 6, 8 and 10 Gy) was delivered to the cells at a dose-rate of 8.7 Gy/min using irradiation with a 6-MV photon generated by the linear accelerator (Elekta Synergy). The cells were harvested and assessed for viability at 48 hours post-treatment.

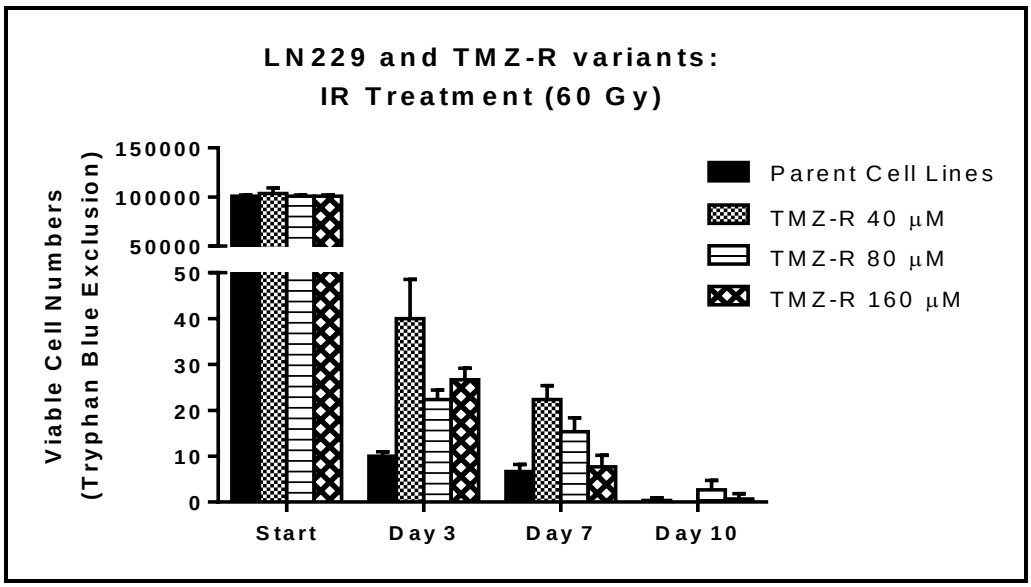


3.2.5. Temozolomide-resistant LN229 human glioblastoma cells do not survive the high-dose radiation treatment

As the previous findings in **Section 3.2.4** showed TMZ-R cells acquire partial resistance to low dose radiation (up to 10 Gy), it would be interesting to see the effects of high-dose radiation on the LN229 TMZ-R cell panel, in comparison to the LN229 parent cells. The results showed that under such circumstances, the cells succumbed to the high-dose radiotherapy (HDRT) of 60 Gy routinely used in the clinical setting. It was also noted that the LN229 TMZ-R cells did display more resistance to the treatment effect in the initial stages. However, when the cells were harvested at Days 3, 7 and 10 after

completion of treatment, the number of viable cells became increasingly negligible. (See **Figure 19**). The findings in this context highlighted a couple of key points: firstly, LN229 TMZ-R cells in spite of their MGMT status and acquired chemoresistance properties, were unable to survive HDRT. Next, the use of HDRT as a treatment modality for GBM patients retains its importance[182], as a majority of chemoresistant GBM cells do succumb to HDRT.

FIGURE 19: LN229 parent and TMZ-R variant cells succumb to clinically relevant dose of radiation treatment (60 Gy). To achieve a final dose of 60 Gy to be delivered *in vitro*, the Biological Effective Dose (BED) formula was applied. From the BED, the required IR dose for the experiment was calculated to be 30 Gy. This was delivered over 3 fractions at 10 Gy per day to the cells.



3.3. Discussion and conclusions

In this chapter, there was derivation of therapeutically resistant GBM subpopulations (i.e. LN229 TMZ-R variants. 40 μM, 80 μM and 160 μM) that expressed MGMT, a gene well-associated with TMZ-resistance in the clinical setting. The LN229 TMZ-R variant cells, in particular LN229 TMZ-R 80 μM and 160 μM also demonstrated significant reduction in some of the MMR proteins. These TMZ-R variant populations were able to maintain some of the aggressive hallmarks in comparison to its TMZ-sensitive, MGMT-negative counterpart (LN229 parent cell). The correlation of MGMT in glioma resistance has been paralleled in large-scale patient studies where the role of MGMT in glioma resistance has been shown to be clinically relevant in present-day practice[79,183]. In addition, the incremental expression of MGMT in a TMZ dose-dependent fashion further augments its role in TMZ resistance. As mentioned before, these *in vitro* induced chemoresistance models have been previously reproduced by other publications[184] to understand drug-related mechanisms in gliomas. However, this project included the novel use of HDRT to examine therapeutic resistance further, especially so in a clinically relevant setting. Furthermore, the subsequent results from the IR-related experiments demonstrated that LN229 TMZ-R subpopulations examined here were unable to sustain cell viability in the face of HDRT, in spite of their acquired chemoresistance. Thus, from these findings, it was surmised that yet another probable therapeutically resistant subpopulation is important in GBM tumours, based on current disease understanding. As discussed in **Chapter 1.2**, glioma stem cells (GSCs) have been previously implicated in chemo- and radiation resistance. Therefore, in the following chapter, GSCs were

developed from LN229 cells and characterized for their roles in therapeutic resistance.

CHAPTER FOUR

A glioma ‘stem cell-like’ population in LN229 human glioblastoma cells demonstrates therapeutic resistance to temozolomide and high-dose ionizing radiation

Chapter 4

A GLIOMA 'STEM CELL-LIKE' POPULATION IN LN229 HUMAN GLIOBLASTOMA CELLS DEMONSTRATES THERAPEUTIC RESISTANCE TO TEMOZOLOMIDE AND HIGH-DOSE IONIZING RADIATION

4.1 Introduction

The human brain, for many years previously thought to be a static, fully-differentiated organ, is now considered to be a dynamic environment driven by multiple neuronal and glial progenitor-cell populations[66]. As previously discussed, recent studies have suggested a population of GSCs with the capacity to repopulate tumours and mediate chemo- and radiation resistance. This unique subpopulation is generally characterized by low rates of division and proliferation, hence conferring resistance to conventional chemotherapy and radiation treatment that target actively cycling cells[185,186]. Putting it all together, the presence of this GSC subpopulation seems to be especially applicable in GBM where tumour recurrence is a clinical reality.

As a whole, the GSC concept whereby an aberrant stem cell-like subpopulation is a sole perpetrator of therapeutic resistance in GBM is indeed tempting. However, a note of caution before we proceed: the existence of GSCs and their role in tumorigenesis is not universally accepted by all, and several aspects of this model remain controversial[136]. For instance, as neural stem cells and their progenitors share features similar to GSCs, there is a tendency to interpret lineage derivation of GSCs from NSCs or their

progenitor cells. One should note that it is still conceivable that more differentiated cells can, through multiple mutagenic events, acquire self-renewal characteristics reminiscent of GSCs[187]. However, despite the ongoing polemics surrounding their origins, their existence and contribution to therapeutic resistance is still highly accepted.

This chapter will attempt to describe in detail, experiences in the development of a ‘GSC-like’ population, establishment of its phenotype and molecular characteristics, and more importantly, its response to clinically relevant treatment.

4.2. Results

4.2.1. A 'GSC-like' population in LN229 human glioblastoma cells form neurospheres and express pluripotency markers

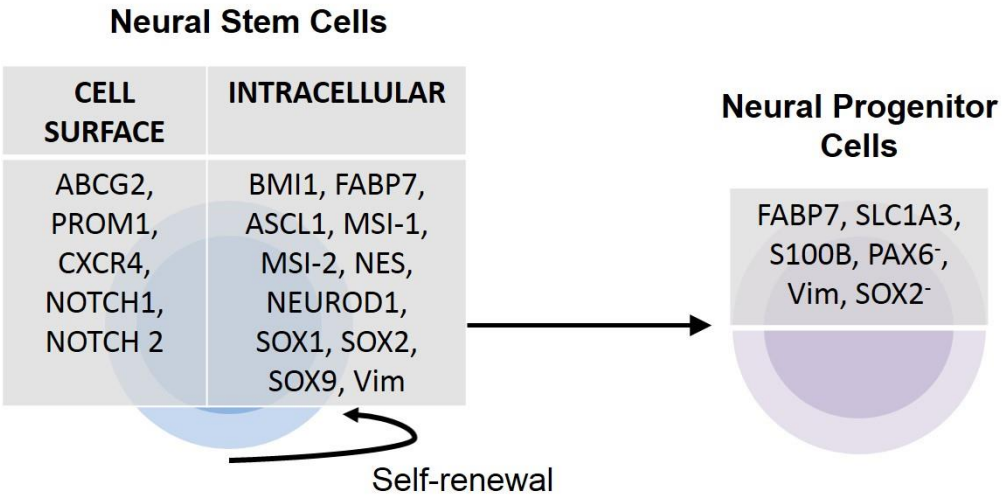
Glioblastoma-derived stem cell-like spheroid cultures is an acceptable method used to study stem cell-like behaviour and tumour biology[135]. Such 'GSC-like' cells have been reproducibly reported to display increased tumorigenicity and resistance to therapeutics in both *in vitro* and *in vivo*models[188]. Thus by inference, this suggests that isolating this population will generate a highly potent population of tumour-initiating cells that can be used to study therapeutic resistance[90,189]. In this field, a range of different techniques has been described to isolate and culture cancer stem cells[190-193]. A common *in vitro* method is the neurosphere assay, where populations of 'GSC-like' cells are able to continually divide and form multipotent, clonal neurospheres[135,189] when cultured in the presence of EGF and bFGF under serum-free conditions[194]. This approach is built on the foundation that neural stem cells were originally characterized and identified by their growth as neurospheres in a minimal medium containing growth factors[195,196]. Furthermore, the derivation of such a population from glioblastoma cell lines, including LN229, has been previously described in the literature[197]. In this setup, the morphology of LN229 parent cells was observed to change from an adherent population to a more spheroid-like one over a period of 12 to 24 weeks. (See **Figure 20**).

FIGURE 20: Representative photograph of LN229-derived neurospheres after prolonged culture in serum-free media with mitogens.

At present, there is still an overall lack of standardization regarding the assessment of this so-called "stemness" phenomenon[114,198,199]. During the early works on GSCs, CD133 was proposed as a marker for brain tumour-initiating cells. With the insight of new knowledge, researchers have come to realise that using CD133 as a definitive marker is too restrictive. Furthermore, there are now publications that demonstrate CD133-negative cells are too, capable of self-renewal and instigate tumours[200,201]. In order for this project methods to remain consistent with current literature, a panel of commonly-used neural stem cell developmental markers to profile our LN229 'GSC-like' population was curated.

Next, the cells were subjected to mRNA analysis to examine if these LN229-derived neurospheres were able to demonstrate gene expression changes in the list of known neural stem cell pluripotency markers (See **Figure 21**). The mRNA results were achieved using microarray, followed by individual validation using RT-qPCR. (See **Figures 22A** and **22B**). Following that, the LN229 'GSC-like' cells that showed higher mRNA expression of neural stem cell markers in comparison to LN229 parent cells were checked for protein expression using immunoblotting. (See **Figure 23A** and **23B**). The results demonstrated significant increases in relative foldchange of MSI-1, SOX9, SOX2 and FABP7 mRNA expressions in LN229 'GSC-like' cells. Most GBM tumours have been found to contain considerable number of cells expressing the markers Musashi-1 (MSI-1) and Sex determining region Y-box 2 (SOX2)[202,203], and the increase in their expressions has been shown to be prognostic of decreased overall survival for glioma patients[204,205]. In addition, the transcription factor Sex determining region Y-box 9 (SOX9) is known to play an important role in the development and differentiation of multiple cell lineages. In malignant gliomas, the overexpression of SOX9 has been associated with poorer clinical outcome of patients[206]. Also, the fatty acid binding protein 7 (FABP7) from the fatty acid binding (FABP) family, is known to be abundant in the foetal brain, and its function includes the development of brain cortical layers[207,208]. Higher expression of FABP7 has also been associated with poorer survival in GBM patients[91].

FIGURE 21: Schematic diagram of some of the commonly cited neural stem cell markers. (Modified and adapted from http://www.rndsystems.com)



(Note: the list here is not exhaustive. For the purposes of this project, the focus was on neural stem cell markers involved in neuronal differentiation)

FIGURE 22: A. Microarray (Illumina HT-12 v4) heatmap of neural stem cell markers (from FIGURE 21) in LN229 'GSC-like' population compared to LN229 parent cells. Parent 1, 2, 3 and GSC-like 1, 2, 3 represent biological triplicates per cell type, respectively. B. Validation of individual neural stem markers from microarray data using RT-qPCR.

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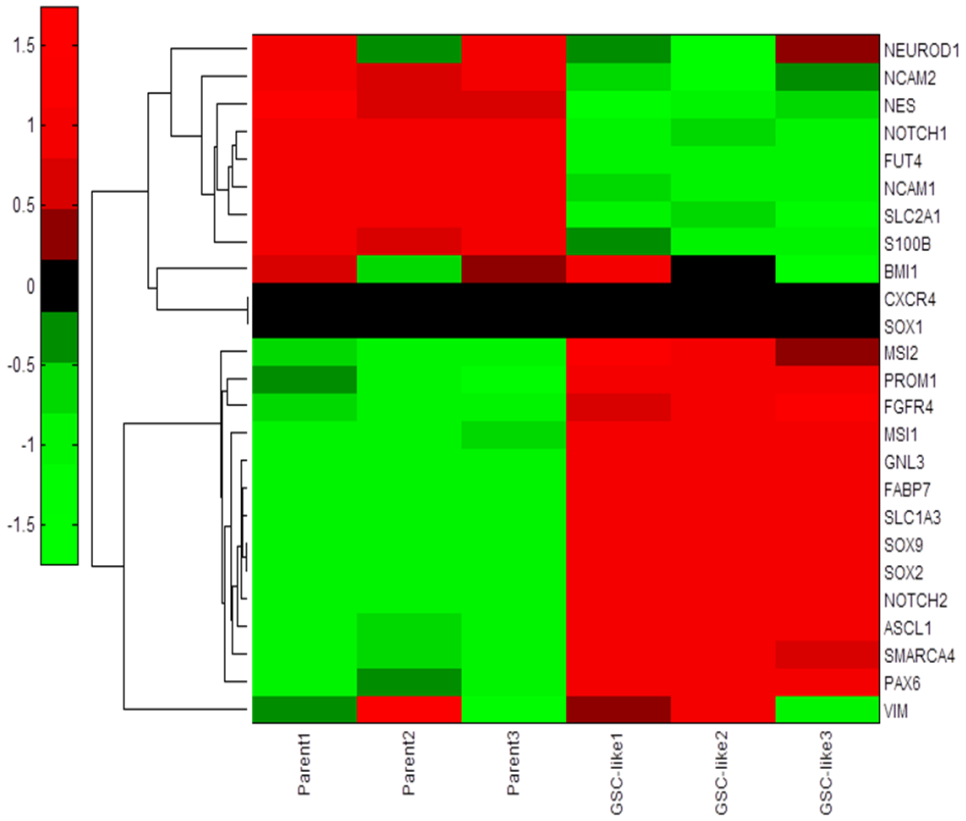

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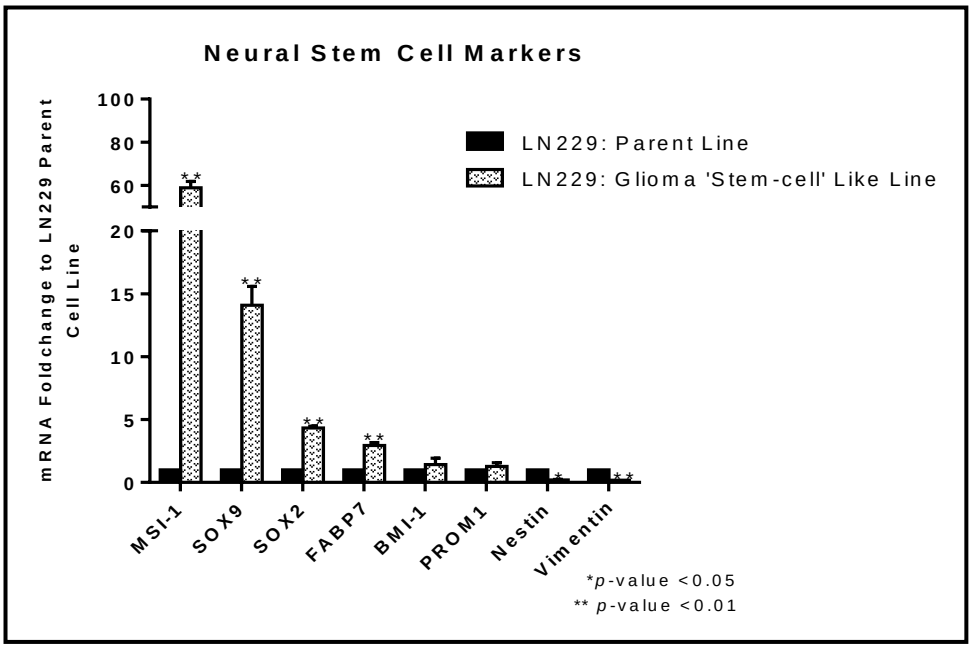
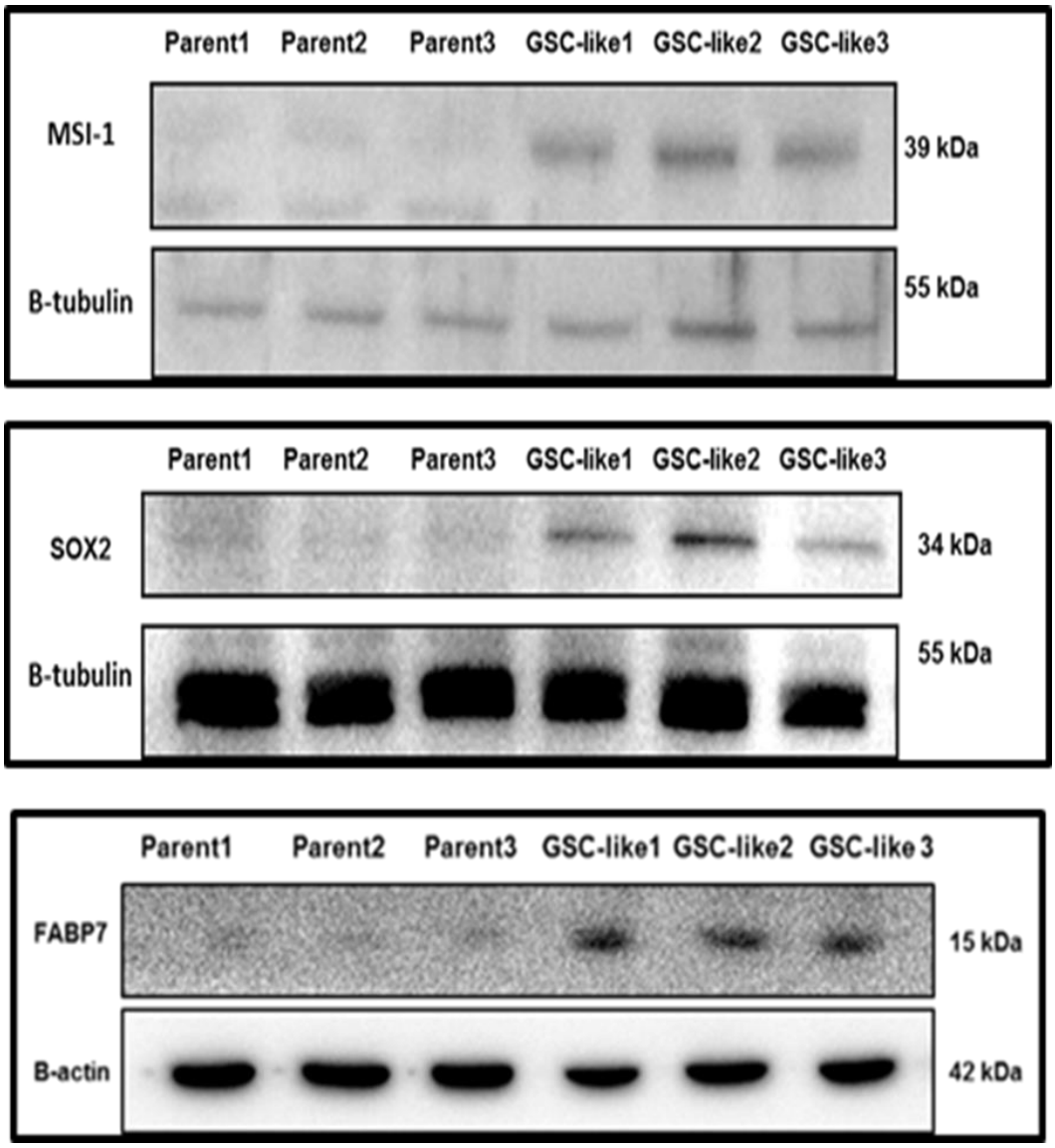


FIGURE 23: A. Immunoblotting results of neural stem cell markers in LN229 'GSC-like' cells. B. Densitometry quantification results of the protein bands from western blots using Image Lab v4.1 software.

A



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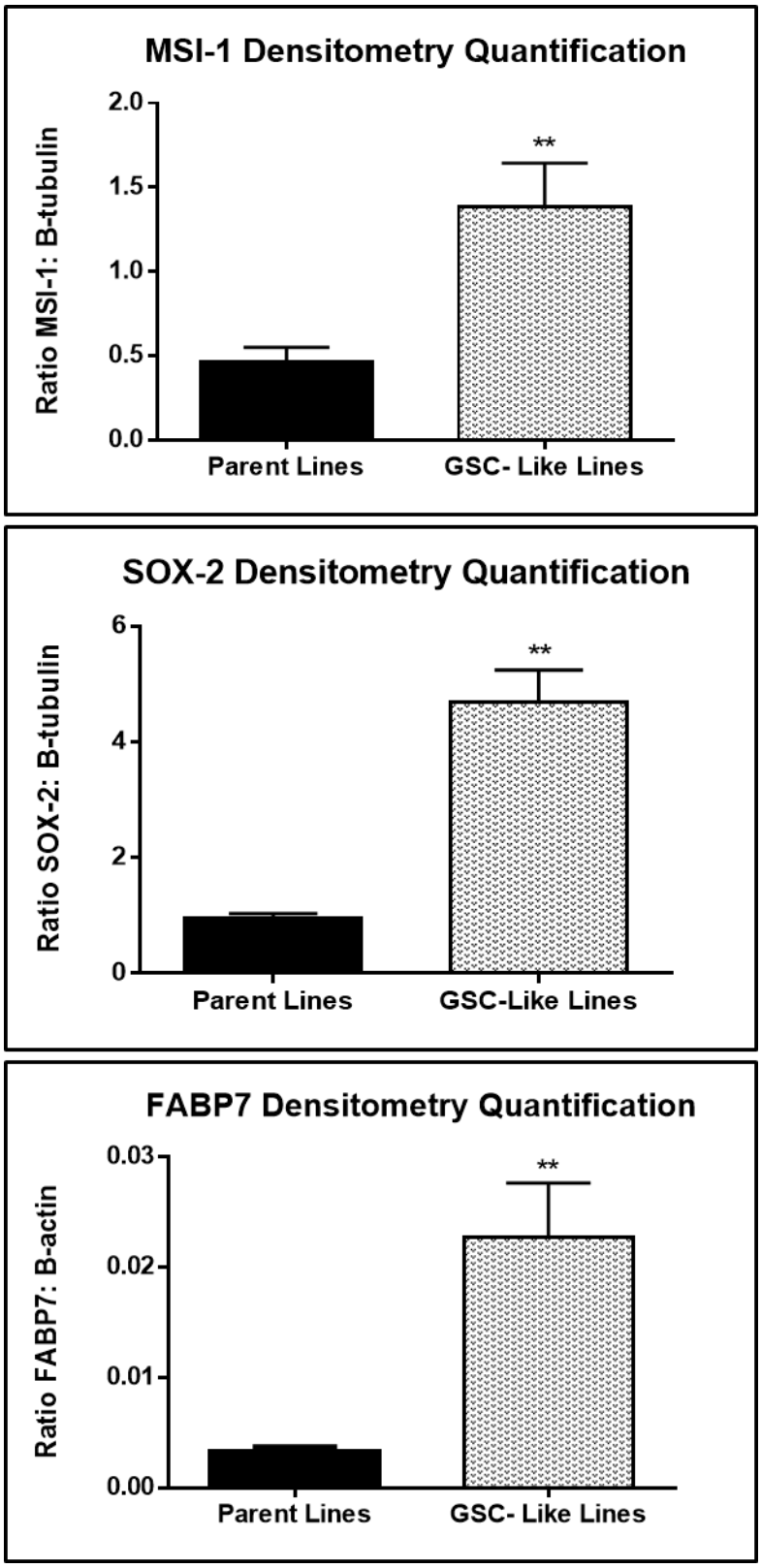
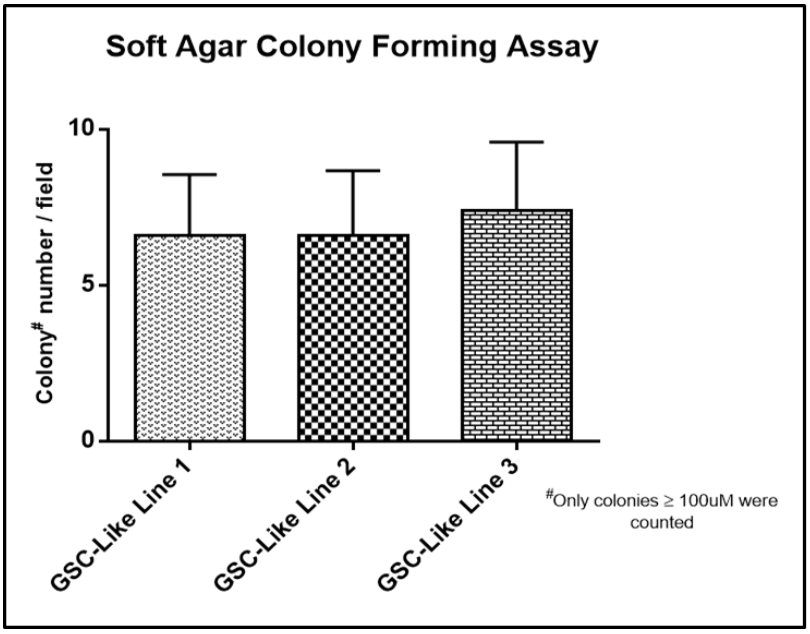
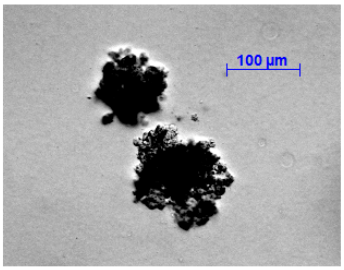

MSI-1 Densitometry Quantification
Ratio MSI-1: B-tubulin
2.0
1.5
1.0
0.5
0.0
**
Parent Lines
GSC- Like Lines
SOX-2 Densitometry Quantification
Ratio SOX-2: B-tubulin
6
4
2
0
**
Parent Lines
GSC-Like Lines
FABP7 Densitometry Quantification
Ratio FABP7: B-actin
0.03
0.02
0.01
0.00
**
Parent Lines
GSC- Like Lines

4.2.2. 'GSC-like' LN229 cells are able to form colonies in an anchorage-independent environment

The soft agar assay for colony formation is considered one of the most stringent assays for detecting malignant transformation of cells. In this setup, the aim was to examine the behaviour of cells that undergo *in vitro* transformation, where phenotypic changes such as anchorage independence and loss of contact inhibition were observed. (See **Figure 24**). This system is accepted to be a good predictor of *in vivo* carcinogenesis[209,210]. Concordantly, the successful use of this assay in LN229-derived neurospheres was previously described by Wang *et al* 2012[211].

FIGURE 24: Soft agar colony forming assay for LN229 'GSC-like' cells. Colonies were quantified on Day 14 post-plating. On Day 14, LN229 parent cells were observed not to have formed colonies.





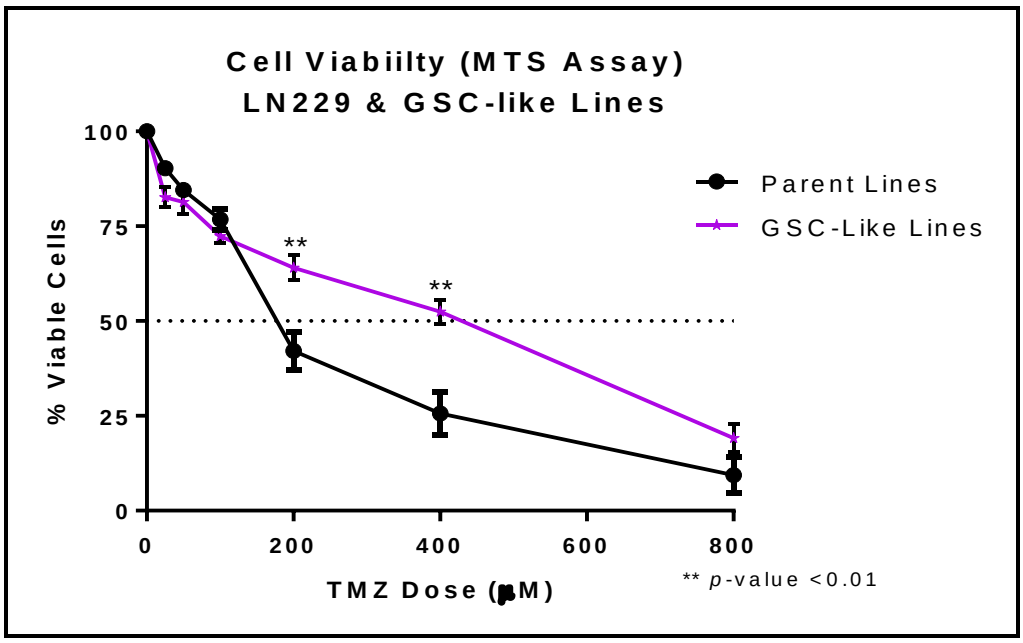
(Representative picture)

4.2.3. 'GSC-like' LN229 cells demonstrate temozolomide resistance

It was observed that LN229 'GSC-like' cells remained more viable when treated with incremental doses of TMZ for 72 hours when compared to LN229 parent cells. (See **Figure 25A**). In addition, when LN229 'GSC-like' cells were subjected to regular doses of TMZ (34 μM) over the course of 10 days, these cells continued to survive. As described in **Chapter 2.1.2**, the *in vitro* TMZ dose of 34 μM was calculated to reflect the peak concentration of TMZ reached in patient glioma tissues from clinical studies[139]. Similarly, in this second experiment, LN229 'GSC-like' cells remained viable in contrast to LN229 parent cells. (See **Figure 25B**). These findings were consistent with the notion whereby GSC populations are known to be resistant to chemotherapy[86,170,212-214], and most likely to be responsible for tumour chemoresistance. This is in the context of their capacity for unlimited self-renewal, ability to initiate and drive tumour progression *in vivo*[215].

FIGURE 25: A. Cell viability assay of LN229 'GSC-like' cells subjected to to a single doses of TMZ for 72 hours in incremental concentrations (0, 25, 50, 100, 200, 400 800 μM)[216]. [sl2]DMSO treatment was used for the vehicle control. B. Cell viability assay of LN229 'GSC-like' cells and parent cells treated with regular doses of TMZ 34 μM over the course of 10 days. DMSO treatment was used for the vehicle control.[sl3]

A



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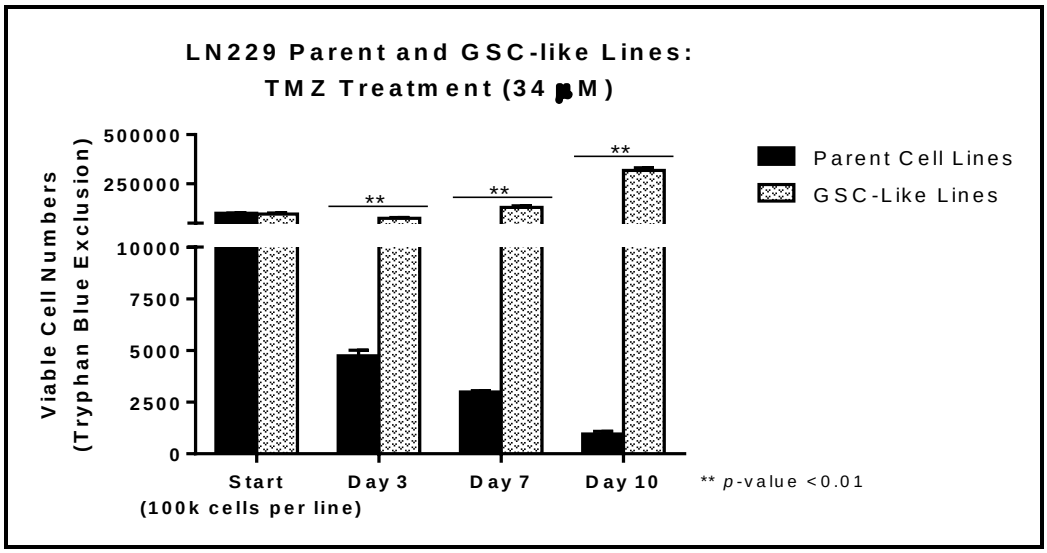

These findings also demonstrated an interesting point: LN229 'GSC-like' cells, despite being TMZ-resistant, did not express MGMT, unlike the previous LN229 TMZ-R cells, 40 μM, 80 μM and 160 μM as described in **Chapter 3**. This is contrary to the expectation will be that LN229 'GSC-like' cells may express MGMT, especially in the context of TMZ resistance[177,217]. Of noteworthy mention, Blough *et al* 2010's study found that their panel of unmethylated MGMT promoter GSC-like lines displayed at times unpredictable responses to TMZ[170]. Here, the results indicate that LN229 'GSC-like' cells do not express the same GBM chemoresistance markers as the LN229 TMZ-R panel. (See **Table** 8). This is shown in our microarray data (See **Figure 25A**). These include the MMR-related genes (MSH3, MSH6 and PMS1), ABC-transporter gene family members (ABCC3, ABCA3), other reported genes in the literature such as those involved in the cell cycle (TP53 and RB1), and base excision repair (APEX1).Some of these genes were validated using RT-qPCR (See **Figure 25B**). Given that GBM biology is complex, it is thus not inconceivable that other mechanisms can be driving the cells' resilience to treatment.

TABLE 8: List of genes commonly associated with chemoresistance in GBM

MMR-related genes	BER-related genes	ABC transporter family genes	Others
MLH1	MPG	ABCA3	CCNE1
MSH2	XRCC1	ABCB1	CCND1
MSH3	XRCC2	ABCC3	CDKN1A
MSH6	APEX1		CDKN2D
PMS1			CDKN1B
PMS2			TP53
			ATM
			ATR
			MGMT
			PARP1
			RB1
			IL23A

FIGURE 26: A. Microarray (Illumina HT-12 v4) heatmap data of common chemoresistance markers. Parent 1, 2, 3 and GSC-like 1, 2, 3 represent biological triplicates per cell type, respectively. B. RT-qPCR validation of genes from microarray data

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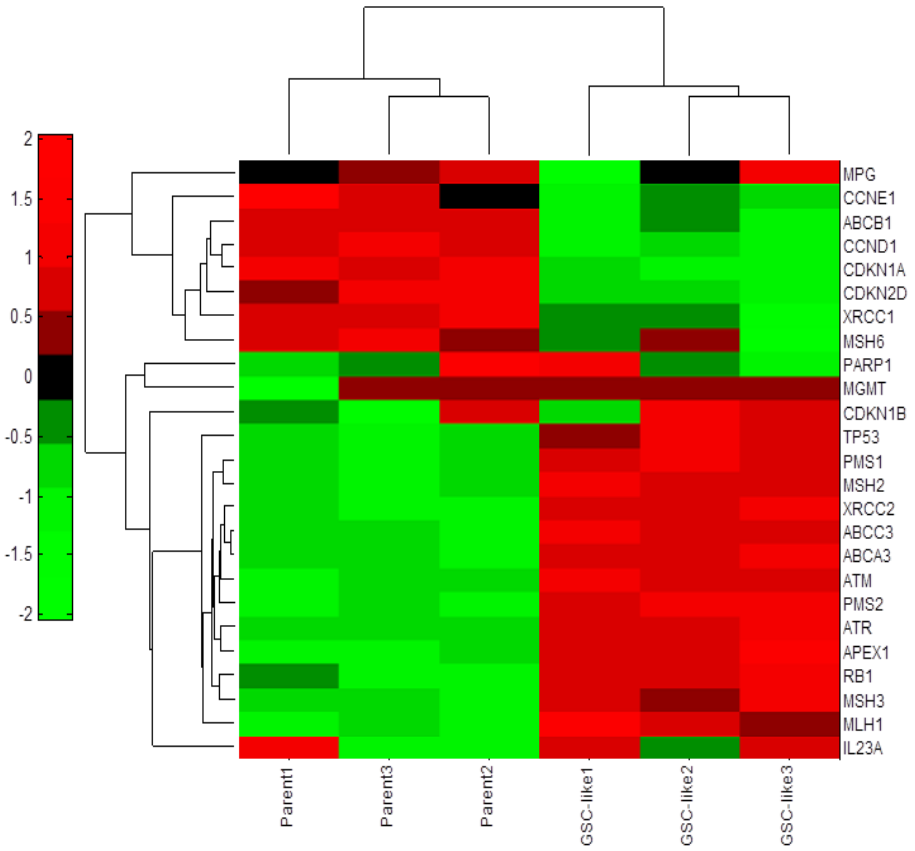

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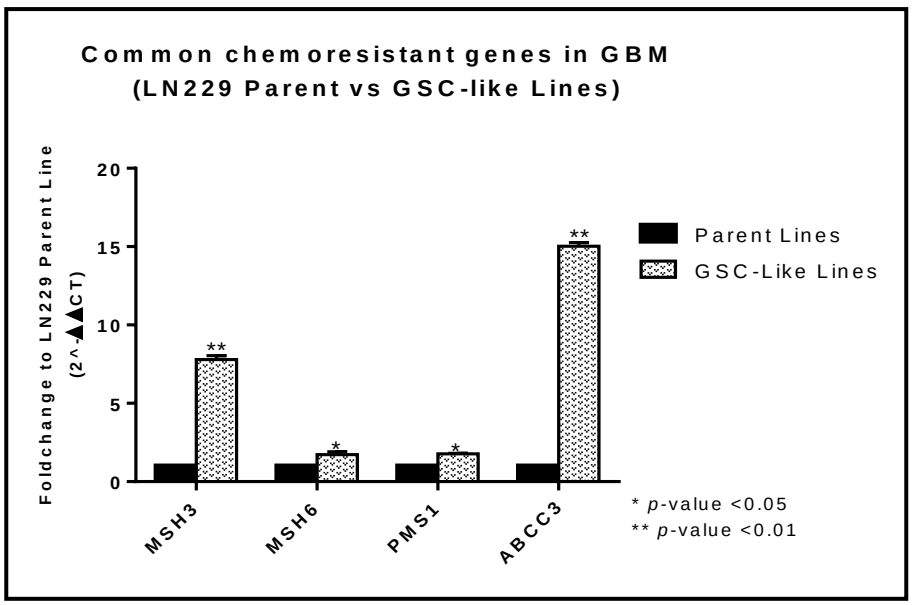

4.2.4. 'GSC-like' LN229 cells demonstrate radioresistance and continue to proliferate in the presence of ionizing radiation

In this experiment, the effects of both low (2 to 10 Gy) and high-dose radiation (60 Gy) in LN229 'GSC-like' cells were observed. The use of 60 Gy in this experiment was as previously described (See **Chapter 2.7**) and carried out in the LN229 TMZ-R cell panel (See **Chapter 3.2.5**). The results in this setup demonstrated 2 novel findings in comparison to those found in the LN229 TMZ-R cell panel: firstly, LN229 'GSC-like' cells were able to remain viable under the stresses of IR. This is in contrast to LN229 parent cells, whereby they were sensitive to the same IR doses. (See **Figure 27A**). Furthermore, IR treatment was noted to be associated with increased proliferation of LN229 'GSC-like' cells, as compared to their LN229 'GSC-like' untreated controls. (See **Figure 27B**).

With regards to the first finding, the property of radioresistance in cancer stem cells, including GBMs, is not entirely novel. Previous work by Bao *et al* 2006 had demonstrated that radiation resistance is mediated from GSCs within the GBM mass via preferential activation of DNA damage checkpoint response[188]. A parallel observation was seen in breast cancer cells, where cells maintained in spheroids were more radioresistant, demonstrating an absolute difference in mean survival fraction of approximately 20% when subjected to 2 Gy IR treatment[218]. There are also recent *in vitro* studies suggesting that therapeutic stressors, including IR, can induce a cancer stem-like phenotype in differentiated tumour cells—an indication of stem cell

plasticity as a plausible mechanism for survival[219]. In contrast, literature supporting the results from this second finding is scarce. However, a paper by Kil *et al* 2012 noted the significant increase of secreted VEGF after *in vitro* GBM cells were subjected to low-dose IR[220]. Although this study did not include cell proliferation as an endpoint, its outcomes do insinuate the likelihood of GBM cells secreting growth factors as a response to IR, hence promoting continued proliferation.

FIGURE 27: A. Cell viability assay of LN229 'GSC-like' cells when subjected to single incremental doses of IR (0, 2, 4, 8, 10 Gy). B. Cell viability assay of LN229 'GSC-like' cells treated with HDRT (60 Gy). The starting number of cells per line was 100k. LN229 parent cells were used as experimental control.

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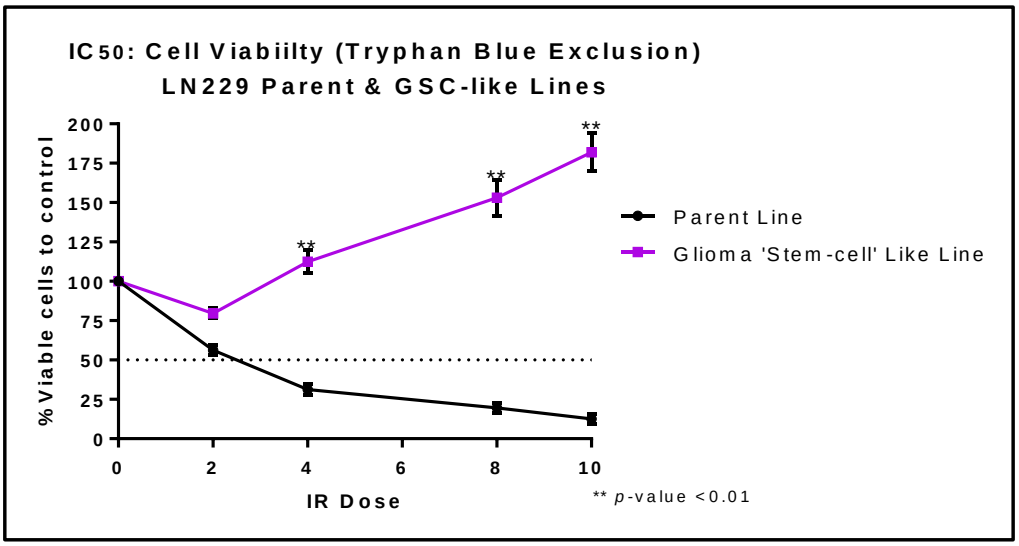

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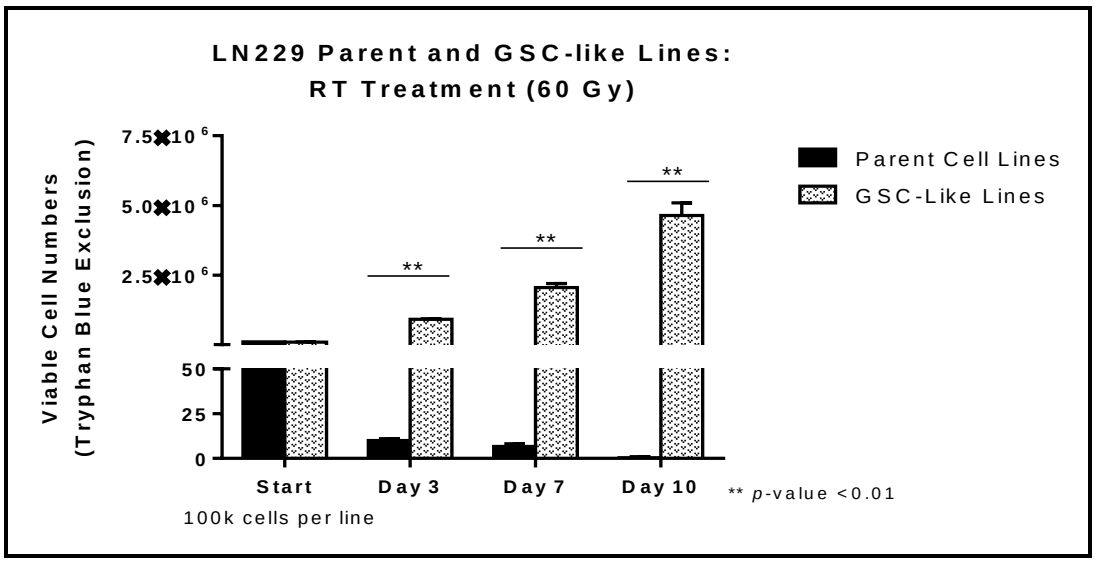


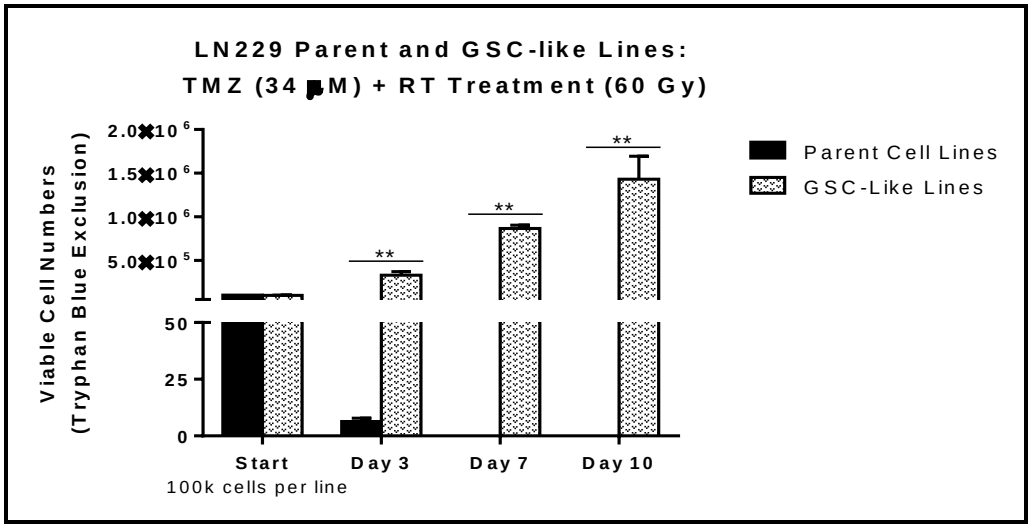
4.2.5. 'GSC-like' LN229 cells survive clinically relevant chemo-radiation treatments used for glioblastoma tumours

After subjecting LN229 'GSC-like' cells to clinically relevant treatment, the cells continued to remain viable. However, it was also noted that under different treatment conditions, they displayed variable growth rates. For instance, when subjected to prolonged TMZ alone, the cell numbers were noted to be less in comparison to DMSO control. (See **Figure 28A**).Our results demonstrating a decrease in proliferation in LN229 'GSC-like' cells concurs with a previous study by Blough *et al* 2010[170]. There, and in other similar reports, authors had found that some GSC populations can be susceptible to the effects of TMZ[170,221].

Furthermore, when treated with RT alone, the cell numbers were exponentially higher compared to DMSO control. However, when treated with simultaneous TMZ and RT, despite continued growth, the cell numbers were still less than the cells which underwent the RT treatment arm only. From these findings, it was concluded that although TMZ as a monotherapeutic drug does not kill LN229 'GSC-like' cells, it has some effect in reducing its growth numbers. In addition, TMZ when combined with RT, is able to slow down overall tumour cell growth in comparison to RT alone. (See **Figure 28B**). Importantly, these results are in concordance with real-life patient outcomes, whereby concomitant TMZ and RT confers better survival than RT only[30,222].

FIGURE 28: A. Cell viability results of LN229 'GSC-like' cells after treatment with TMZ (34 μM) and HDRT (60 Gy) as per clinically relevant treatment based on the 'Stupp Protocol'[30]. LN229 parent cells were used in the same experimental setup for comparison. The starting number of cells per line was 100k. B. Cell viability results demonstrating effects of TMZ (34 μM) on LN229 'GSC-like' cells when administered with, and without HDRT (60 Gy). LN229 'GSC-like' cells in DMSO were used as the control for TMZ treatment.

A



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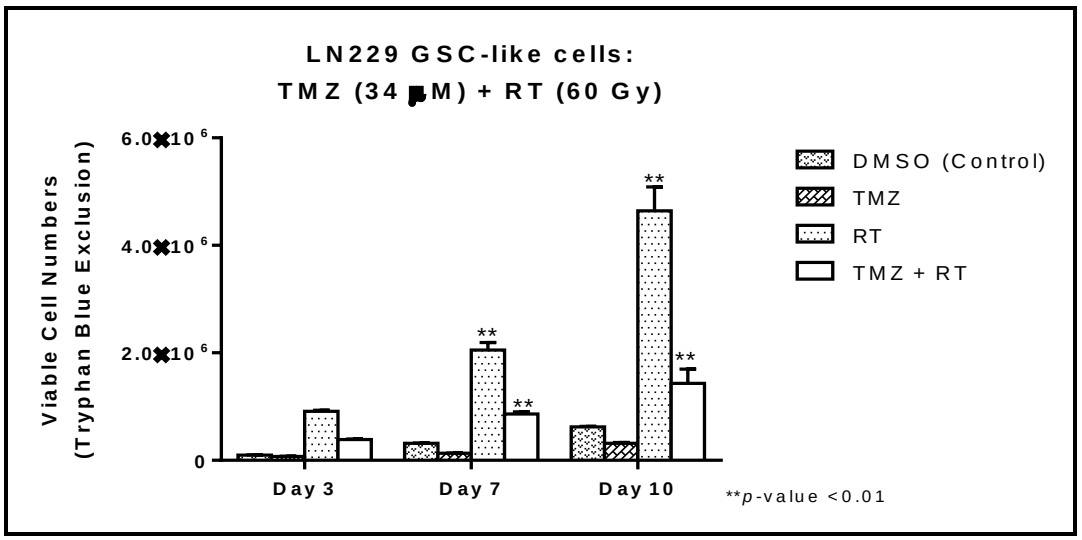

4.3. Discussion and conclusions

The current perception is that most malignant tumours, such as GBM, are postulated to originate from a subpopulation within themselves. Here, these so-called 'GSCs' are thought to possess an immature phenotype, with chemo-radiation resistance capacity as one of its key characteristics[34,215]. Accordingly, in this chapter, the existence of such a subpopulation was explored, with methods reproduced from scientific literature and subjected these cells to clinically relevant treatment using TMZ (34 μM) and HDRT (60 Gy). So far, the observations correlated with current concepts underlying therapeutic resistance, and also remain in congruency with our primary hypothesis, whereby a subpopulation of GBM cells would be resistant to standard chemo-radiation therapy. These findings demonstrated that LN229 'GSC-like' cells had shown to be remarkably resistant to radio- and chemotherapies. Hence, it was postulated that their resistance property would likely concurs with genomic and proteomic profiles distinct from their non-GSC population, as previously suggested in the literature[7]. As clearly laid-out, the understanding of chemo-radiation resistance of GSCs, and their persistent propagation in the face of treatment may hold the answer to improve curability for this disease. Drawing from these results, the priority interest will be to know if the properties underlying pro-survival effect of LN229 'GSC-like' cells when treated with TMZ, RT or concomitantly, are synergistic or independent. Therefore, for the following chapters, the aim is to investigate the potential involvement of miRNAs as the underlying contribution towards therapeutic resistance in LN229 'GSC-like' cells.

CHAPTER FIVE

A distinct group of miRNAs was observed to have expression changes in LN229 ‘GSC-like’ cells induced by clinically relevant treatment

‘You have to learn the rules of the game. And then you have to play better than anyone else.’

A. Einstein (1879-1955)

Chapter 5

A DISTINCT GROUP OF MIRNAS WERE OBSERVED TO HAVE EXPRESSION CHANGES IN LN229 'GSC-LIKE' CELLS INDUCED BY CLINICALLY RELEVANT TREATMENT

5.1. Introduction

In this chapter, the aim was to test the project's secondary hypothesis (i.e. the surviving LN229 'GSC-like' cells are a molecularly distinct subpopulation), and to use a miRNA-centered approach to fulfill the objectives.

Based on current knowledge, one is now aware that miRNAs are important regulators of mammalian stem cell division and development[223]. Similarly, for neural stem cells, the evidence seem to suggest that miRNAs are important for stem cell differentiation and proliferation[224]. In a parallel context, GSCs in Kim *et al* 2011's previously mentioned taxonomy study (Chapter 1) demonstrated that miRNAs determine GBM subclasses through their abilities to regulate developmental growth and differentiation programs in transformed neural precursor cells[104]. This aspect of their involvement in neural precursor cells is now apparent and applicable in this project. This chapter attempts to provide an in-depth discussion of the miRNA changes observed in LN229 'GSC-like' cells after clinically relevant treatment, and follow through with a discussion on the relevance of the miRNAs that were used to map for mRNA targets. Furthermore, it is common knowledge that mammalian miRNAs

perform gene regulatory roles by base-pairing to mRNAs to specify post-transcriptional repression of these messages[225].

5.2 Results

5.2.1. Clusters of miRNAs were observed to have significant expression changes during clinically relevant treatment

It was observed that LN229 'GSC-like' cells demonstrated significant miRNA expression changes during the course of independent treatment(s) with TMZ (34 μM), HDRT (60 Gy) or, combinatorial TMZ (34 μM) and HDRT (60 Gy), in comparison to the LN229 'GSC-like' DMSO control cells. Given the diverse roles of miRNAs in the regulation of different cell functions, including survival, these patterns of treatment-induced changes in miRNA expression were not unexpected. During the analysis of the miRNA data, particular attention was given to the miRNAs that were simultaneously downregulated in the presence of TMZ, RT and combined TMZ and RT. (See **Figure 29A**). The reasons for this selection were firstly, miRNAs reported to be suppressed in brain tumours are usually involved in the regulation of functions which may benefit for cancer growth and propagation[38]. Furthermore, previous analyses of miRNA expression in several human cancers had shown a general downregulation trend for miRNAs in tumours, in comparison to normal tissue counterparts[226]. This is postulated to be related to presumed defects in the molecular machinery used for miRNA

processing[224], such as defects in Drosha-mediated pri-miRNA cleavage [227]. Corroboratively, the observation that a global reduction of miRNA expression, biogenesis and loss of miRNA function disrupts stem cell differentiation, suggests that functional miRNA loss promotes glioma formation by arresting GSC differentiation[224]. Next, owing to the inverse relationship in the miRNA-mRNA connection, the downregulation of a miRNA implies that the increase in expression of its related mRNA may be by either via direct or indirect regulation. Moreover, the search for upregulated genes that perpetuate oncogenicity is cogent for potential biomarkers and, or the development of targeted molecular therapeutics. Following this, as previously mentioned in **Chapter 4**, the project's aims include the interest to know if the molecular targets affected by individual monotherapy (i.e. TMZ or HDRT) versus combinatorial TMZ and HDRT are similar in all 3 treatment arms. Finally, to be more stringent, the miRNAs of interest were part of the GBM miRNA subtyping taxonomy list reported by Kim *et al* 2011, as their selected miRNAs stratified with patient survival[104]. As a result, miR-125a-3p, miR-629-5p and miR-19b-3p were found to fit into the criterion established. Interestingly, when matched against the miRNA-based subtyping taxonomy, their pre-miRs were observed to be downregulated in the subtypes associated with lower median survival and poorer response to standard GBM treatment[104]. (See **Figure 29B**[CTY4]).

FIGURE 29: A. Heatmap of RT-qPCR of miRNA profiling panel. The regions boxed in yellow highlight the clusters of miRNA changes. B. The 3 selected miRNAs were chosen from the raw data using the criteria, where the miRNA of interest is consistently downregulated in all 3 arms of treatment: TMZ only, RT only and combinatorial TMZ and RT, in comparison to DMSO control.

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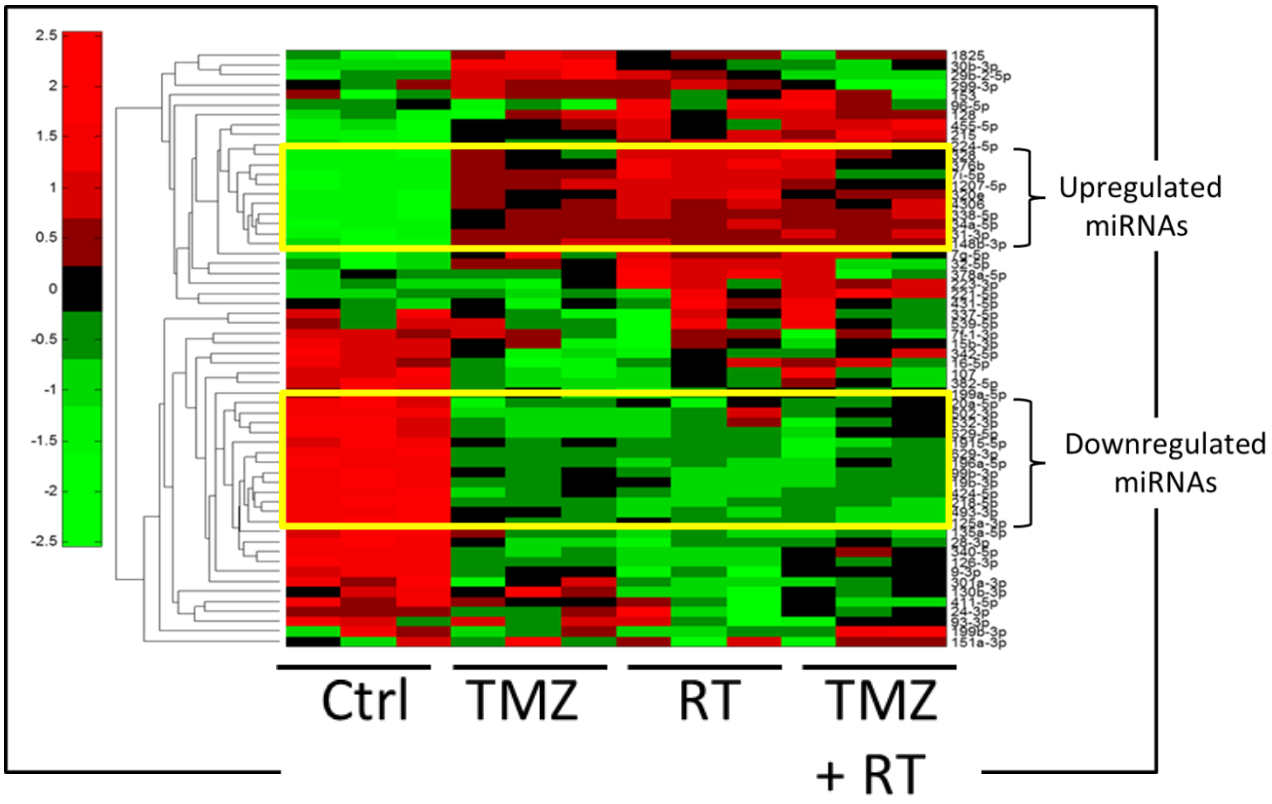
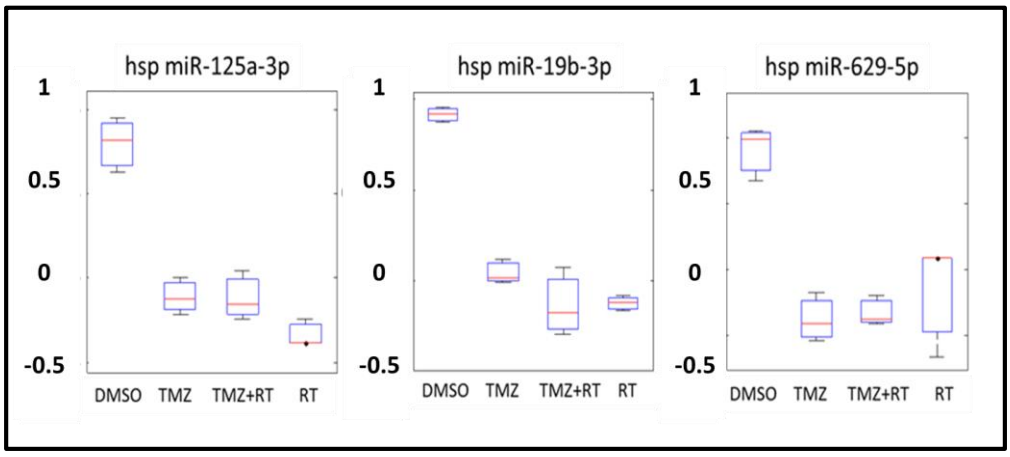


B

Criteria for selection of downregulated miRNAs:

- foldchange comparted to DMSO control where ***p-values*** **<0.001** in the presence of treatment(s)



Background of downregulated miRNAs:

miR-125a-3p

Downregulation of miR-125a-3p has been previously noted by Jiang *et al* 2010 in non-small cell lung carcinoma, and is associated with lymph node metastasis[228]. This observation has also been reported in gastric cancer where a lower expression of miR-125a-3p is associated with enhanced malignant potential and tumour dissemination in a matched patient cohort study[229]. Correspondingly, there are studies that have observed downregulation of its mature counterpart, miR-125a-5p in medulloblastomas[230] and other visceral tumours[231,232]. In addition, its pre-miR 125a has been previously reported to be downregulated in gliomas in comparison to normal brain tissue[38]. However, the specific mRNA targets and pathways regulated by miR-125a-3p remains uncertain at this stage. Nonetheless, putting all current evidence together, there

is strong suggestion for a miR-125a-3p to have a tumour suppressor role in human malignancies.

miR-629-5p

In a similar context, the pre-miRNA 629 has been previously observed to be in a cluster of downregulated miRNAs for breast cancer with metastases versus breast cancer without metastases[233]. With the insight of translational work in miRNAs, we are now aware that the expression of distinct miRNAs seem to be associated with the efficacy of therapeutic interventions, such as radiation therapy[234,235]. Interestingly, a study by Niemoeller *et al* 2011 looking at miRNA expression profiles in a panel of different cancer cell lines noted that the human glioblastoma cell line LN229, (which was used in this study) expressed lower miR-625 levels post-irradiation treatment[236].

miR-19b-3p

Amongst all the 3 selected miRNAs, miR-19b-3p has the least known function in glioma biology at this point in time. This miRNA belongs to the mir-17-92 miRNA cluster; hence any reports of gene regulation usually involves the whole cluster. This is because unlike classic protein-coding oncogenes, whereby 1 transcript usually gives rise to 1 protein product, the mir-17-92 cluster produces a single cistronic primary transcript that can yield 6 individually mature miRNAs. Subsequently, the distinct miRNA sequence of the mir-17-92 components dictates the specificity of their target regulation, in order to eventually determine individual functional specificity[225]. As an

individual entity, the miR-19b has been implicated in immunological diseases, such as rheumatoid arthritis[237,238].

5.2.2. Downregulation of miR-125a-3p, miR-625-3p and miR-19b-5p correspond with upregulation of RFX1 and FLVCR2 genes in LN229 'GSC-like' cells

The 3 miRNAs (miR-125a-3p, miR-629-5p and miR-19b-3p) were input into miRror 2.0 target prediction software[150] to identify mRNA targets. As previously mentioned, the programme analysis is based on a 'many-to-one' approach in which a set of input miRNAs is optimized for a minimal set of gene targets that are known to be maximally regulated by this set[150].These results were matched against upregulated mRNA in the microarray data set of the same cell populations subjected to similar 3 treatment arms: TMZ only, RT only and combinatorial TMZ and RT, in comparison to DMSO control. There were 19 predicted genes in the list identified as mRNA targets using miRror2.0 target prediction software that matched the upregulated mRNAs from our microarray data. (See **Figure 30**). Next, each individual gene was validated with RT-qPCR. Out of all the genes, it was found that RFX1 and FLVCR2 were correspondingly upregulated in the same LN229 'GSC-like' cells that were treated with TMZ (34 μM), HDRT (60 Gy) or, combinatorial TMZ (34 μM) and HDRT (60 Gy). (**Figure 31**).

FIGURE 30: Schematic diagram demonstrating workflow of miRNA-mRNA target prediction using miRor 2.0. The list of predicted mRNA targets was mapped to the microarray data from the same panel of LN229 'GSC-like' cells which have undergone the same 3 treatment arms: TMZ only, RT only and combinatorial TMZ and RT, in comparison to DMSO control.

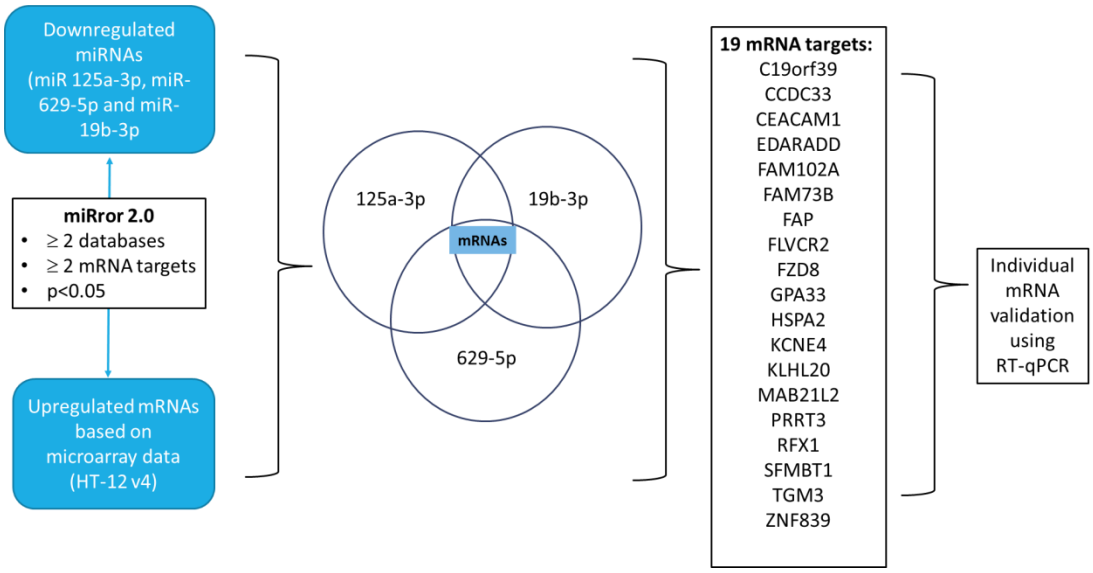
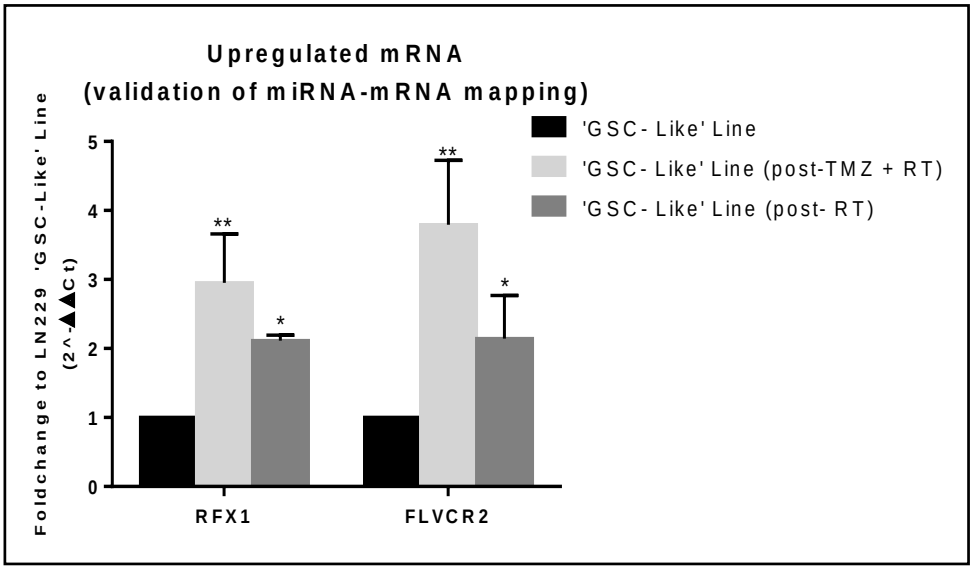


FIGURE 31: RT-qPCR results validating the upregulation of RFX1 and FLVCR2 in LN229 'GSC-like' cells 48 hours after treatment with TMZ (34 μM) and, or HDRT (60 Gy).



Background of upregulated mRNAs that are potential miRNA targets:

Regulatory factor X1 (RFX1)

The RFX gene family transcription factors were first reported in mammals as the regulatory factor that binds to a conserved *cis*-regulatory element called the X-box motif in 1988[239]. In this group, RFX1 was characterized as a candidate major histocompatibility complex (MHC) class II promoter binding protein[240]. Subsequent studies on the RFX family demonstrated that they are homodimeric and heterodimeric DNA-binding proteins[241] with critical roles in mammalian development[242]. In humans, RFX1 is highly expressed in the cerebral cortex[243]. This transcription factor also appears to be expressed in the other tissue types, such as the heart, eye and testis[242]. Other studies in human disease found RFX1 to be a functional trans activator of the hepatitis B virus enhancer[244].

At present, the knowledge of detailed mechanisms underlying RFX1 and gliomas remain limited. However, interestingly, there have been some reports that RFX1 may have a tumour suppressive role in gliomas. For example, Feng *et al* 2014 demonstrated that RFX1 directly downregulates CD44, a stem cell marker highly expressed in both normal brain[245] and in glioblastoma, especially of the mesenchymal subtype[93]. Broadly speaking, CD44 is a glycoprotein transmembrane receptor that is known to function as a cell adhesion and intracellular signaling molecule[246]. CD44-mediated adhesion is thought to have a role in maintaining the stem cell niche[247] and its overall expression is utilized for enrichment of GSCs[248]. Next, in Hsu *et al* 2010's

work, RFX1 was identified as a transcription suppressor of the human fibroblast growth factor 1 (FGF1) gene promoter[249]. The FGF1 gene has 4 known upstream untranslated exons[250-252], and they play roles in neurogenesis, cell growth and proliferation[249]. In this group, the exon FGF-1B is the major transcript within the human brain[251]. More significantly, in the context of the project's disease of interest, it has been shown that most malignant gliomas express FGF1 utilizing the 1B promoter. The study probing the association between RFX1 and FGF1 suggests that RFX1 negatively regulates GSC self-renewal via binding to the 18-bp *cis*-element in the FGF-1B promoter[249], to modulate overall FGF1 gene expression.

Feline leukaemia virus subgroup C cellular receptor family, member 2 (FLVCR2)

FLVCR2 is a cell surface membrane protein that belongs the major facilitator family (MFS), the largest and most diverse superfamily of membrane transporters[253,254]. Although its role in GBM has not been reported, germline mutations in FLVCR2 has been recently shown to be associated with Fowler Syndrome[255-258]. Fowler Syndrome, also known as proliferative vasculopathy and hydranencephaly-hydrocephaly (PVHH), is a proliferative vascular disorder of the brain[259]. Essentially, this is a rare but lethal prenatal disorder where there is glomeruloid vasculopathy throughout the CNS, with varying degrees of calcification and necrosis in both white and grey matter[258]. Following this, Duffy *et al* 2010's work reinforced the association between FLVCR2 and Fowler Syndrome by firstly, functionally demonstrating that

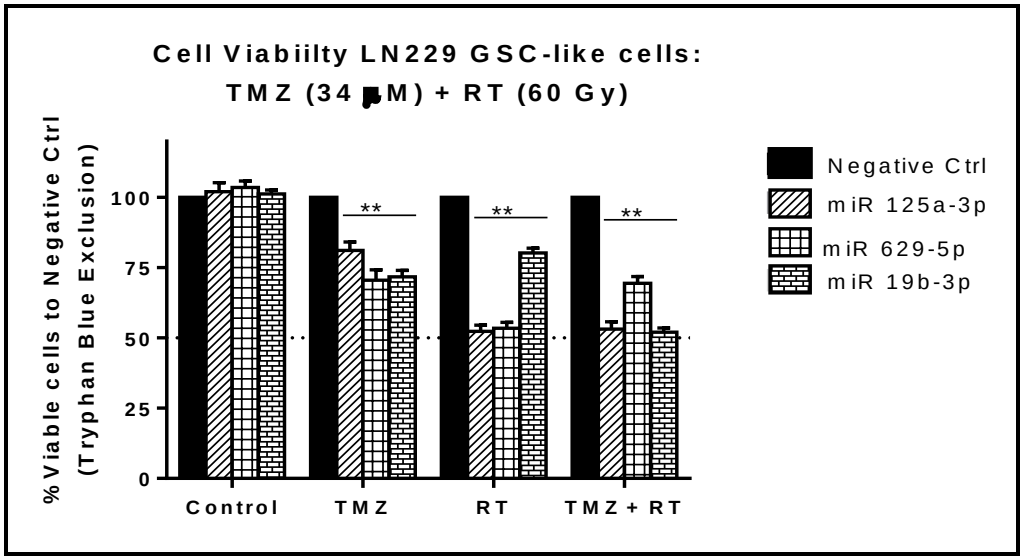
FLVCR2 binds to and imports haem. Next, they also showed that FLVCR2 is abundantly expressed in a large range of normal human tissues, especially in the brain[260]. Therefore, given its important physiological role and strong presence in the brain, it is not implausible that mutations involving FLVCR2 will have implications in CNS-related diseases, including GBM.

5.2.3. Independent overexpression of miR-125a-3p, miR-625-3p and miR-19b-5p reduces LN229 'GSC-like' cell viability in the presence of clinically relevant treatment

In order to test the functionality of the 3 selected miRNAs, the miRNA mimics for miR-125a-3p, miR-629-5p and miR-19b-3p to independently overexpress the LN229 'GSC-like' cells were used. Post-transfection, the cells were subjected to the same clinically relevant treatments: TMZ (34 μM), HDRT (60 Gy) or, combinatorial TMZ (34 uM) and HDRT (60 Gy), as described in **Chapter 4.2.5**. (See **Figures 32A** and **32B**). The results demonstrated the following: firstly, after the cells were transfected, there was no observation of any significant difference in the cell numbers in the DMSO-treatment control arm. Next, the transfected cells overexpressing each of the miRNAs were found to be more susceptible to all 3 treatment arms in the experimental setup. Overall, a similar trend was noted across all the 3 selected miRNAs, whereby higher expression of each miRNA conferred lower cell viability in the presence of treatment.

FIGURE 32: A. Cell viability assay of LN229 'GSC-like' cells transfected with miRNA mimics and treated with TMZ (34 μM), HDRT (60 Gy) or, combinatorial TMZ (34 μM) and HDRT (60 Gy).The cells were harvested 48 hours after completion of treatment. B. RT-qPCR results demonstrating transfection efficiency of the miRNA mimics used. miR-16 was used as an internal control for this experiment.

A



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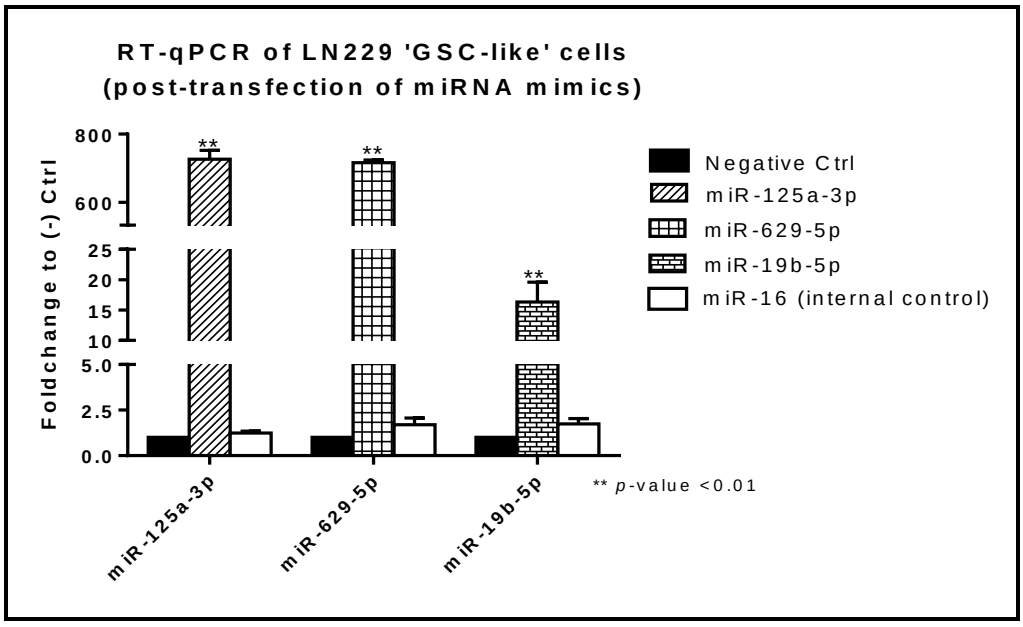
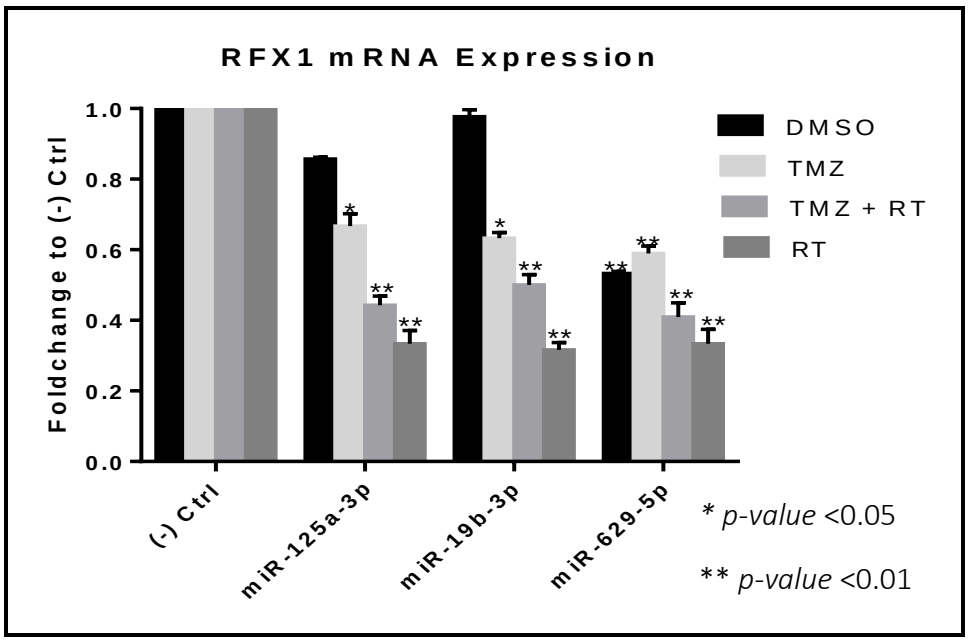


5.2.4. Independent overexpression of miR-125a-3p, miR-625-3p and miR-19b-5p in LN229 'GSC-like' cells lead to corresponding downregulation of RFX1 and FLVCR2 genes in the presence of clinically relevant treatment

Following the previous experiment (5.2.3), the next step was to seek the mRNA levels of RFX1 and FLVCR2 in LN229 'GSC-like' cells that were independently transfected with the miRNA mimics. The findings demonstrated the gene expression of RFX1 and FLVCR2 in the same cells to be reduced in the presence of clinically relevant treatment. (See **Figures 33A** and **33B**). Collectively, these results confirmed a reliable association between our 3 selected miRNAs and the 2 predicted mRNA targets, thus strengthening their intersectional miRNA-mRNA relationships.

FIGURE 33: RT-qPCR results showing expression of RFX1 and FLVCR2 in LN229 'GSC-like' cells that were transfected with miRNA mimics and treated with TMZ (34 μM), HDRT (60 Gy) or, combinatorial TMZ (34 μM) and HDRT (60 Gy). A. RFX1 mRNA expression B. FLVCR2 mRNA expression

A



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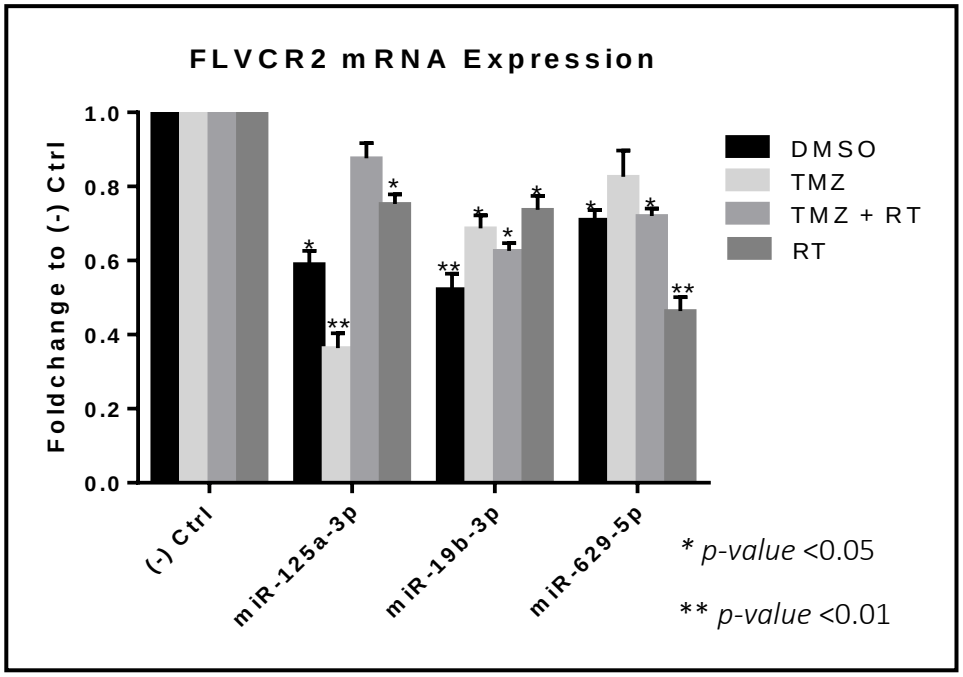


5.3. Discussion and conclusions

In this chapter, the following objectives were achieved: firstly, the demonstration of a feasible approach in the use of dysregulated miRNAs to map target mRNAs in the roles of resistance. The miRNA-derived effects of chemo- and radiation treatment on LN229 'GSC-like' cells, and established their miRNA-mRNA relationships using a miRNA transfection approach was examined. At this point in time, there remains paucity of knowledge underlying each of these miRNAs, especially in their independent, or combinatorial roles in GBM prognosis. [sl5] Following this, a well-accepted miRNA-mRNA target prediction software programme (miRror 2.0) to provide results that seek a consensus from a variety of databases was utilised. As reflected in current research consensus, many still grapple with inconsistencies among many miRNA-targets. This reflects that a large portion of false-positives remains associated with each available resource[261,262]. These inconsistent findings were encountered first-hand during the validation exercise of the 19 predicted mRNA targets, where only 2 of the genes were consistently upregulated. Nevertheless, this approach acknowledges the potential of miRNA-target prediction softwares, and anticipate their increasing importance in the near future. Moving forward, based upon the overall findings, the next step is to establish the functionality of RFX1 and FLVCR2.

CHAPTER SIX

Gene knockdown of RFX1 and FLVCR2 in LN229 ‘GSC-like’ cells confer sensitivity to clinically relevant treatment

‘Now faith is confidence in what we hope for and assurance about what we do not see.’

Hebrews 11:1; The Old Testament

Chapter 6.

GENE KNOCKDOWN OF RFX1 AND FLVCR2 IN LN229 'GSC-LIKE' CELLS CONFER SENSITIVITY TO CLINICALLY RELEVANT TREATMENTS

6.1 Introduction

Due to the complex nature of GBM, establishing key oncogenes for potential therapeutic targets has been difficult[263]. However, with the advent of better miRNA understanding, their functions in gene modulation and the input of bioinformatics, we are now more appreciative of how miRNA-mRNA mapping can contribute to the identification of therapeutic targets for GBM.

In the previous chapter (Chapter 5), based on miRNA-mRNA target prediction, the findings demonstrated consistent upregulation of RFX1 and FLVCR2 genes when miR-125a-3p, miR-629-5p and miR-19-3p were downregulated. Of interest, the opposite trend was observed when these 3 implicated miRNAs were overexpressed. Taken together, the 2 genes of interest, RFX1 and FLVCR2 show potential as oncogenes in LN229 'GSC-like' cells. As the results suggest the 3 miRNAs are capable of independently regulating RFX1 and FLVCR2 expression, the search for a possible consensus motif was trialled using 2 well-published online programmes, MBStar[264] and rna22 (version 1.0)[265]. However, based on these 2 current programmes, there was no consensus motif thus far, for these 2 transcription factors, matching to the 3 miRNAs of interest. Owing to the reality that miRNA-related biology in disease remains relatively novel, there is a definite possibility that newer,

improved versions of target prediction programmes in the near future will be able to identify such consensus motifs. [sl6] Moving forward, the roles of RFX1 and FLVCR2 were tested in LN229 'GSC-like' cells by using a siRNA knockdown approach, and the transfected cells were subjected to clinically relevant treatment. Furthermore, to ascertain if the candidates genes are clinically relevant, a bioinformatics approach was employed to assess the median expression level of each gene against patient survival, using 2 large clinical glioma databases: REMBRANDT[94]and Gravendeel[151].

6.2. Results

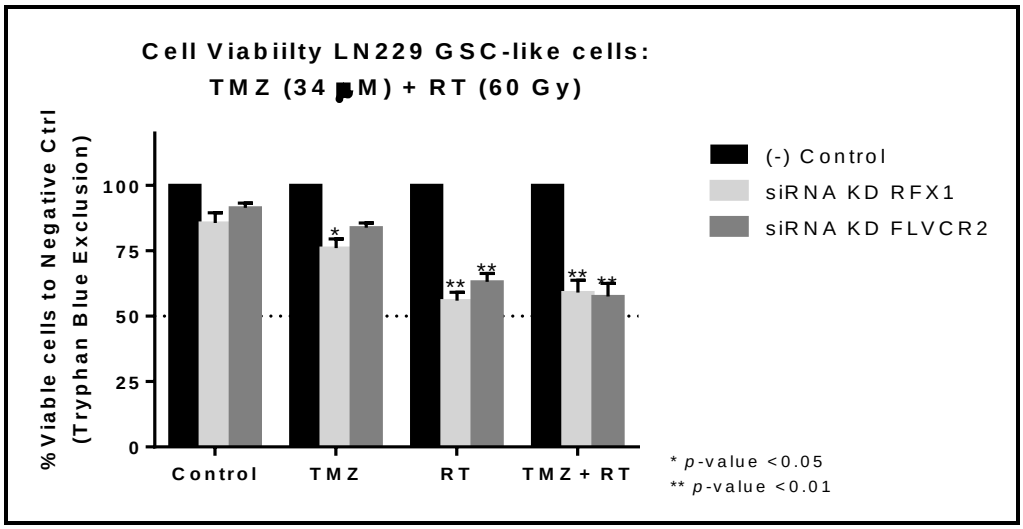
6.2.1. Independent siRNA knockdown of RFX1 and FLVCR2 in LN229 'GSC-like' cells reduce cell viability during clinically relevant treatment

In this experimental setup, the independent effects of RFX1 and FLVCR2 gene knockdown in LN229 'GSC-like' cells were investigated. The results indicated that in the presence of gene silencing, LN229 'GSC-like' cells demonstrated similar trends in cell viability reduction when subjected to clinically relevant treatment. Post-transfection, LN229 'GSC-like cells were treated with one of the following treatment arms: TMZ (34 μM) only, RT (60 Gy) only, or combined TMZ (34 μM) and RT (60 Gy). The results of individual treatments were compared to a DMSO control. (See **Figure 34A**). In order to confirm the genes of interests were silenced, RT-qPCR was performed on the harvested cells. (See **Figure 34B**). These findings help to confirm the project's previous miRNA-derived data (Chapter 5), and reinstate the potential roles of RFX1 and FLVCR2 in tumour cell survival. As the

current knowledge of both RFX1 and FLVCR2 is limited at this stage, the next step was to investigate their value in GBM disease in established patient glioma databases.

FIGURE 34: A. Cell viability assay of siRNA-transfected LN229 'GSC-like' cells that were treated with TMZ (34 μM), HDRT (60 Gy) or, combinatorial TMZ (34 μM) and HDRT (60 Gy). B. RT-qPCR results demonstrating transfection efficiency of RFX1 and FLVCR2 at transcriptional level.

A



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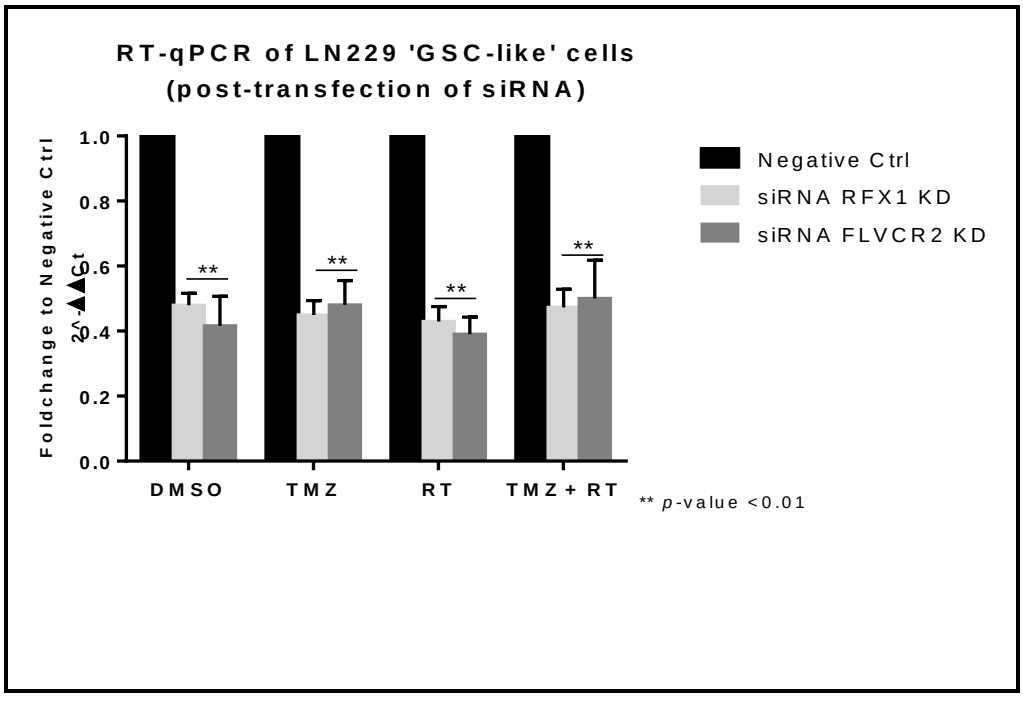
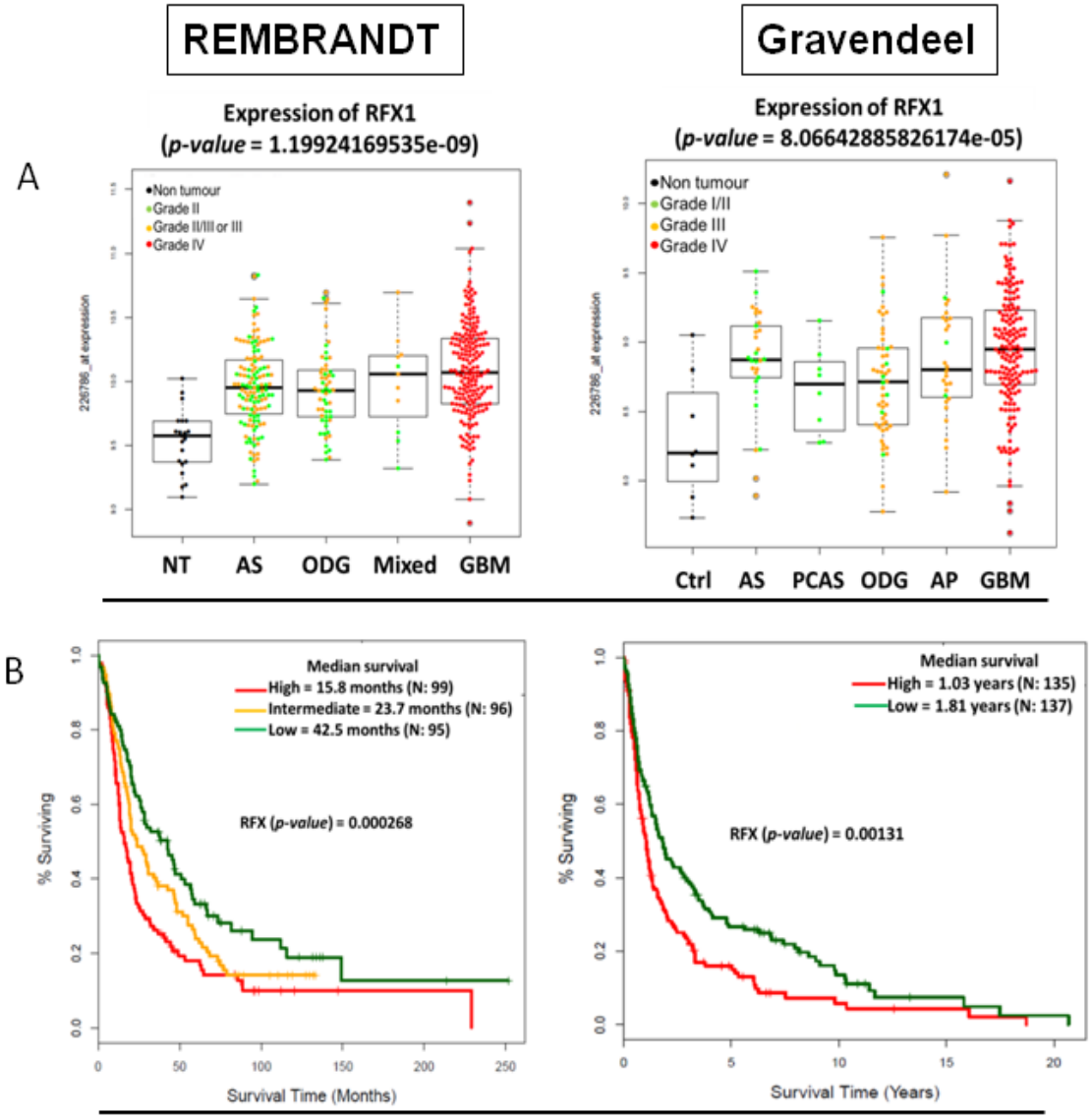


6.2.2. Higher expression of RFX1 and FLVCR2 demonstrate poorer overall survival in patient glioma databases (with assistance of Edwin Sandanaraj, Senior Research Fellow in bioformatics at NNI)

Regulatory factor X1 (RFX1)

In both REMBRANDT[94] and Gravendeel[151] databases, RFX1 demonstrated similar trends in its gene expression and overall survival. For gene expression, RFX1 was observed to be significantly elevated in the higher grade gliomas, especially GBM (See **Figure 35A**). Also, it was noted that patients with higher RFX1 expression had poorer overall survival. (See **Figure 33B**). However, when data from each database was subjected to multivariate Cox-regression analysis, the results were varied. RFX1 gene expression was analysed against 2 known prognostic factors in glioma tumours: age and WHO grading. In the REMBRANDT dataset, RFX1 showed that it is an independent prognostic factor after accounting for age and histological grading. In contrast, in the Gravendeel analysis, RFX1 showed up as an alternative prognostic indicator instead. (See **Figure 35C**). Nevertheless, putting it all together, our results suggest that presence of RFX1 portends poorer prognosis in glioma patients.

FIGURE 35: A. Gene expression of RFX1 in REMBRANDT and Gravendeel in relation to glioma histological subtype. (NT = Non-tumour, AS = Astrocytoma, ODG = Oligodendroglioma, GBM = Glioblastoma, PCAS = Pilocyticastrocytoma, AP = Anaplastic Astrocytoma) B. Survival curves of glioma patients correlated with variable gene expression of RFX1. C. Multivariate analysis using Cox regression for RFX1 independently against age and WHO grading.



C

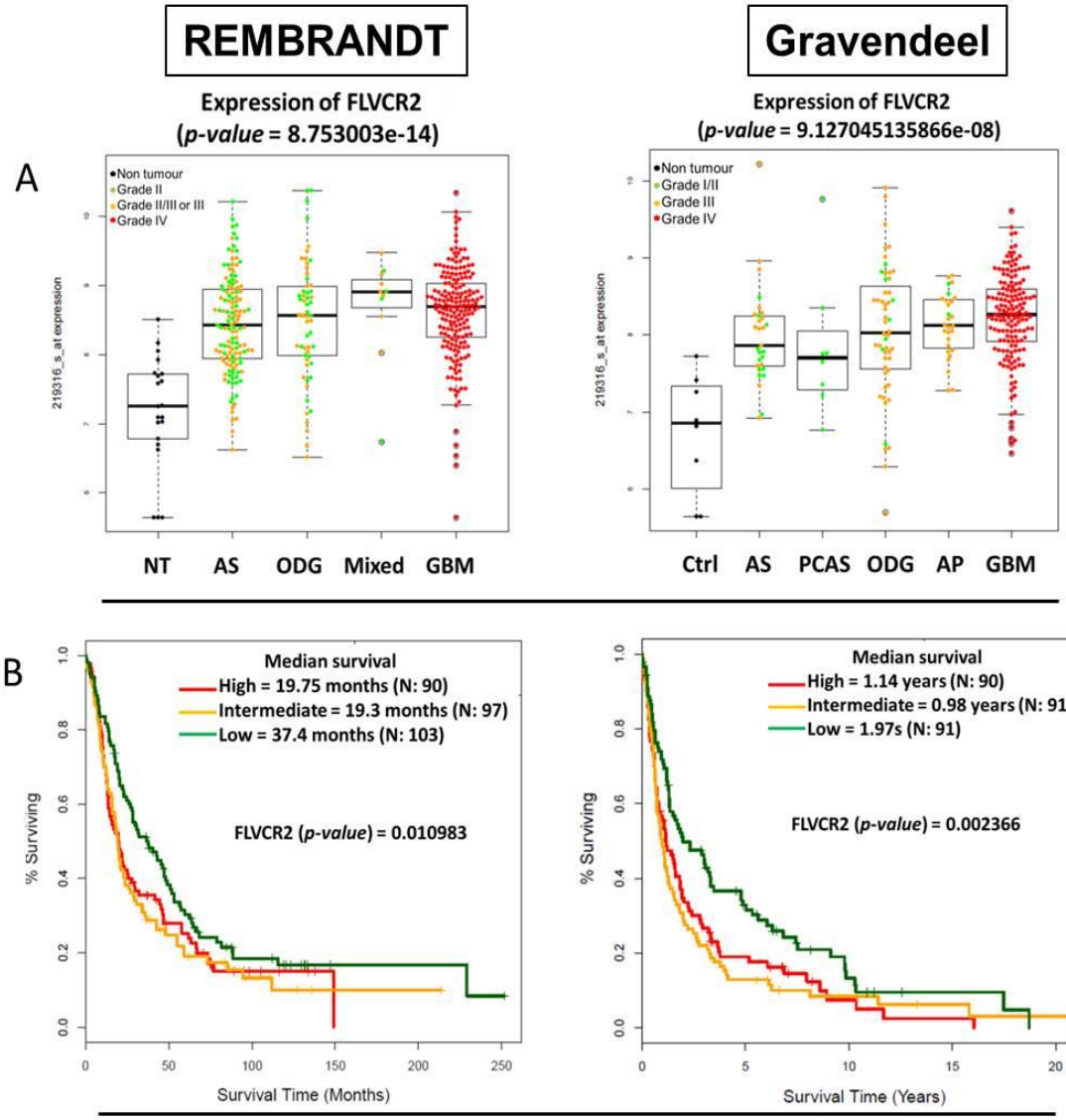
Covariates (RFX1 reference: High expression GRADE reference: II)	HR	se (coef)	95% CI	Z	Pr(>|z|)	Statistical Significance
RFX1: Intermediate	0.78891	0.15777	0.5791-1.075	-1.503	0.13287	
RFX1: Low	0.65061	0.16555	0.4703-0.9	-2.596	0.00942	**
Age	1.06199	0.023	1.0152-1.111	2.615	0.00894	**
GRADE: II/III	0.89447	0.41314	0.398-2.01	-0.27	0.78721	
GRADE: III	1.60851	0.21886	1.0474-2.47	2.172	0.02988	*
GRADE: IV	3.31277	0.19407	2.2646-4.846	6.172	6.74E-10	***

Covariates (RFX1 reference: High expression GRADE reference: II)	HR	se (coef)	95% CI	Z	Pr(>|z|)	Statistical Significance
RFX1: Intermediate	1.09069	0.16369	0.7913-1.503	0.53	0.596	
RFX1: Low	0.79968	0.16543	0.5782-1.106	-1.351	0.177	
Age	1.04001	0.00543	1.029-1.051	7.222	5.1E-13	***
GRADE: III	1.40941	0.27973	0.8146-2.439	1.227	0.22	
GRADE: IV	3.89508	0.27593	2.268-6.689	4.928	8.3E-07	***

Feline leukaemia virus subgroup C cellular receptor family, member 2 (FLVCR2)

In the case of FLVCR2, its gene expression, overall survival and multivariate Cox-regression analysis showed similar trends in both REMBRANDT and Gravendeel. The gene of expression of FLVCR2 was observed to higher in GBM, in comparison to non-tumour controls and low grade gliomas. (See **Figure 36A**). Next, we also noted that glioma patients with high and intermediate expression of FLVCR2 have statistically significant poorer overall survival, in contrast to patients who had lower expression. (See **Figure 36B**). The multivariate Cox-regression model in both glioma databases showed that FLVCR2 is an alternative prognostic factor after accounting for age and histological grading. As previously for RFX1, our analysis implies that the expression of FLVCR2 is correlative with poorer prognosis for glioma patients.

FIGURE 36: A. Gene expression of FLVCR2 in REMBRANDT and Gravendeel in relation to glioma histological subtype. (NT = Non-tumour, AS = Astrocytoma, ODG = Oligodendroglioma, GBM = Glioblastoma, PCAS = Pilocytic Astrocytoma, AP = Anaplastic Astrocytoma) B. Survival curves of glioma patients correlated with variable gene expression of FLVCR2. C. Multivariate analysis using Cox regression for FLVCR2 independently against age and WHO grading.



C

Covariates (FLVCR2: reference: High expression GRADE reference: II)	HR	se (coef)	95% CI	Z	Pr(>|z|)	Statistical Significance
FLVCR2: Intermediate	0.93925	0.1628	0.6827-1.292	-0.385	0.70027	
FLVCR2: Low	0.75719	0.16529	0.5477-1.047	-1.683	0.09243	
Age	1.06941	0.02301	1.0222-1.119	2.916	0.00354	**
GRADE: II/III	0.9106	0.41393	0.4046-2.05	-0.226	0.82101	
GRADE: III	1.74613	0.21648	1.1424-2.669	2.575	0.01003	*
GRADE: IV	3.40575	0.19385	2.3292-4.98	6.322	2.59E-10	***

Covariates (FLVCR2 reference: High expression GRADE reference: II)	HR	se (coef)	95% CI	Z	Pr(>|z|)	Statistical Significance
FLVCR2: Intermediate	1.08731	0.15712	0.7991-1.479	0.533	0.5942	
FLVCR2: Low	0.75289	0.16418	0.5457-1.039	-1.729	0.0838	
Age	1.04049	0.00544	1.0295-1.052	7.302	2.8E-13	***
GRADE: III	1.36293	0.27866	0.7894-2.353	1.111	0.2665	
GRADE: IV	3.79728	0.27268	2.2252-6.48	4.893	9.9E-07	***

6.3. Discussion and conclusions

Although current (albeit limited) literature suggests the role of RFX1 as a tumour suppressor, these results indicate otherwise, especially in the context of clinically relevant treatment. More importantly, the oncogenicity of RFX1 and FLVCR2 is reinforced in 2 independent patient glioma databases, reinforcing the potential of their translation relevance. This aspect of the project highlights the importance of biomedical informatics and tools for validating specific genomic queries in GBM[94]. In this aspect, it was observed that high expression of RFX1 and FLVCR2 independently stratified patient survival. Patients with poor prognosis correlated with high gene expression levels, while patients with favourable prognosis demonstrated lower gene expression patterns. The collective clinical data not only augments the initial *in vitro* results, but also provides evidence that both genes are important prognostic indicators of glioma disease progression and survival outcome.

CHAPTER SEVEN

General discussion and future work proposal

'Doctor, I don't'know why I am still here'.

A glioblastoma patient, Singapore

(Quote said at the time of last outpatient appointment when imaging showed no tumour recurrence since diagnosis after 3 years)

Chapter 7.

GENERAL DISCUSSION AND FUTURE WORK PROPOSAL

7.1. Summary of project

To recap the aims of the project:

Hypothesis

1. There is a subpopulation of GBM cells that is resistant to standard chemo-radiation therapy.
2. Therapeutically-resistant GBM cells are a molecularly distinct subpopulation whose properties are regulated by miRNAs

Objectives

3. To demonstrate a subpopulation of GBM cells is resistant to chemo-radiation therapy.
4. To examine whether different miRNA expression underlying GBM heterogeneity contributes towards resistance in chemo-radiation therapy.

This project explored the hypothesis for therapeutic resistance in GBM using an *in vitro* approach. Based on the acknowledgement that elusiveness to treatment is due to tumoral heterogeneity in GBM tumours, appropriate cellular models were developed to simulate implicated subpopulations in the presence of clinically relevant treatment. In the temozolomide-resistant LN229 glioblastoma models, the significance of MGMT in chemoresistance and

discovered that MGMT-positive cells were able to maintain the cancer hallmarks of proliferation, migration and invasion as compared to the temozolomide-sensitive, MGMT-negative cells were uncovered. However, in the presence of higher doses of IR, these cells succumbed to treatment. Following that, the impact of clinically relevant therapeutics on a LN229 'GSC-like' glioblastoma population that expressed pluripotency markers and displayed tumorigenic properties were explored. Not only did this particular population demonstrate therapeutic resistance and continued cell viability, they were able to demonstrate distinct miRNA changes in the presence of treatment. In this cluster, 3 miRNAs in particular, miR-125a-3p, miR-629-5p and miR-19b-3p demonstrated statistically significant downregulation in comparison to the control cells. Independent overexpression of these 3 miRNAs in LN229 'GSC-like' cells showed reduced cell viability in the presence of the same clinically relevant treatment.

Next, the 3 miRNAs, miR-125a-3p, miR-629-5p and miR-19b-3p, were mapped to mRNA to using mirRor 2.0, a predictive miRNA-mRNA target analysis database. The resulting list of predict mRNA targets were counter-checked against mRNA microarray data from the same cell populations. In order to ensure the biological certainty of the target genes, each mRNA was individually validated with RT-qPCR. From the predicted mRNA list, RFX1 and FLVCR2 were found to significantly upregulated in the presence of these 3 downregulated miRNAs. In order confirm their functional meaning, independent knockdown of RFX1 and FLVCR2 was achieved using siRNA knockdown in LN229 'GSC-like' cells. These cells were then

subjected to the initial clinically relevant treatment set-up. Our observations indicate that when RFX1 and FLVCR2 were silenced, the LN229 'GSC-like' cells became less viable during treatment. More significantly, the oncogenic roles of RFX1 and FLVCR2 were further re-established in 2 independent patient glioma databases, REMBRANDT and Gravendeel. In both databases, individual higher expression of RFX1 and FLVCR2 conferred poorer survival in GBM patients.

In summary, this project was able to establish a molecularly-distinct subpopulation that was therapeutically resistant against current GBM treatment regimen. A miRNA-based approach to look for mRNA targets of interest, especially in the context of resistance was utilized. The 3 miRNAs, miR-125a-3p, miR-629-5p and miR-19b-3p, were firstly, independently validated using miRNA mimics in LN229 'GSC-like' cells, and subsequently mapped to potential mRNA targets using prediction miRNA-mRNA analysis. After experimentally validating these mRNA targets, RFX1 and FLVCR2 were found to be of functional relevance (*in vitro*) and clinical significance for survival stratification (in patient glioma databases). Overall, the project demonstrated diverse interplays of current knowledge and key concepts underlying GBM therapeutic resistance—all integral parts of a common aim to better understand this devastating disease.

7.2. Future work: proposed directions for a disease understanding in therapeutically resistant glioblastomas

Owing to the wide scope and complexities of the disease, certain pitfalls faced in the scientific execution of this project are readily acknowledged: the approach was broad-based and the overall findings were of moderate depth. Hence, the subsequent discussion proposes an outline of the future work to address these issues.

7.2.1. Significance of RFX1 and FLVCR2 in glioblastoma

RFX1 and FLVCR2 are relatively new in GBM. At this point in time, there is little known of their exact contributions to the disease, especially for FLVCR2. Although there is some recent literature on RFX1 in GBM, the understanding of its role in this tumour remains preliminary. Based on the preliminary results of the 3 miRNAs and the 2 associated mRNAs, the project proposes to include the following imperative experiments: firstly, since all 3 miRNAs (miR-125a-3p, miR-625-3p and miR-19b-5p) are downregulated, it will be interesting to observe if cell viability is even more significantly reduced when all 3 miRNAs mimetics are present in LN229 'GSC-like' cells. In addition, the use of dual silencing of both RFX1 and FLVCR2 in the same cell lines may demonstrate a synergistic loss of more cell viability as well in the presence of administered treatments.

However, despite the promising results in discovering 2 potential genes of interest in therapeutic resistance, the following factors should also be

highlighted. Firstly, in this project, we used a human glioblastoma cell line (LN229) for the purposes of elucidating GBM's underlying biology. Following this, although studies have shown that some discrete features of glioma biology are conserved in established glioma cell lines, we now accept that they possess significant differences from those found in primary tumours[266].

Moving forward, it will therefore, be imperative to establish the roles of RFX1 and FLVCR2 in patient-derived glioma-propagating cells both *in vitro* and *in vivo*, in order to decipher their mechanisms underlying therapeutic resistance. Here, the relevance of using glioma-propagating cells directly from primary tumours is important: there is now evidence these tumour cells re-capitulate the genomics and gene expression profiles better than those from established cell lines[266]. In addition, one is also aware that patient-derived glioma-propagating cells are able to form orthotopic tumours in xenograft mouse models[267], reflecting a good simulation of brain tumour development in its appropriate microenvironment. Here, the next proposal is to study the knockdown effect of RFX1 and FLVCR2 in such glioma-propagating cells *in vivo*, followed by in-depth surveillance of possible resistance pathways to elucidate their mechanisms. Hence, under these circumstances, the project can look forward to the functionality of RFX1 and FLVCR2 to be suitably tested.

7.2.2. Potential applications of a miRNA-centred approach in glioblastoma management

Malignant gliomas are often viewed as a manifestation of multiple inter-connected tumour networks controlled by both intra- and extra-cellular events[114]. In pre-miRNA era, there was much difficulty in establishing the key connections underlying GBM biology. However, one is now aware that miRNA-mediated mechanisms can greatly enlighten researchers on the previously-deemed elusive layer of regulation in these networks. As a continuation of our project findings, the project proposes to study the mechanisms underlying therapeutic resistance in the 3 implicated miRNAs (miR-125a-3p, miR-629-5p and miR-19b-3p) in patient-derived glioma-propagating cells both *in vitro* and *in vivo*, especially in the context of RFX1 and FLVCR2. In this situation, the hypothesis is that these 3 miRNAs (miR-125a-3p, miR-629-5p and miR-19b-3p) are likely targeting a common tumour suppressive pathway in GBM cells. As the mechanisms underlying the project's miRNA-mRNA findings are scarce at this stage, as a starting point, one shall rely on the known pro-survival pathways in GBM, such as PI3K-AKT (Phosphatidylinositol-3-Kinase and Protein Kinase B) and MAPK-ERK (Mitogen Activated Protein Kinase and Extracellular signal regulated Kinase) pathways to guide our study. Furthermore, the project's findings also emphasize that there is still much to be done in the bioinformatics aspect of current miRNA-mRNA prediction programmes, as reflected in our results. Predicted targets still need to conscientiously validated by the user. Nevertheless, in the near future, we can anticipate miRNA research will continue to bridge the gap between bench-to-bedside in a timely fashion.

7.3. Conclusions

In conclusion, this project explored key concepts underlying therapeutic resistance and used a miRNA-centred investigation in attempts to uncover possible gene targets associated with GBM cell survival in the face of clinically relevant treatment. Overall, it hopes to have laid the groundwork for a feasible approach to understanding this problem.

As clearly demonstrated by the clinical reality and the project findings, the elusive nature of GBM urgently calls for efforts to implement strategies to improve current treatment and identify molecular markers to provide insight into the disease[53]. To emphasize this, a local study by Tham *et al* on 62 patients with malignant gliomas over a period of 10 years reported no significant benefit of combinatorial TMZ with RT, as compared to RT alone[268]. At this point in time, this has been the only clinical study published, involving malignant glioma patients in Singapore. A key point noted by the authors was that the use of molecular biomarkers was not a routine part of the diagnostic assessment. Their results concluded that the availability of such modalities would have helped to stratify GBM patients, in order to select patients who can benefit from concomitant TMZ and RT[268].

Clinicians and scientists are constantly struggling to undermine the biology of GBM. In this disease, the challenges are multiple: the biology, where itsinnate heterogeneity is a source of mechanistic elusiveness; the therapeutics, where risks of CNS and systemic complications are morbid; and

most of all, the human effects, where patients and their families walk in the path of helplessness. To bring forward improvements in GBM management, Hippocrates' aphorism still holds today[269]:

"Life is short,

The art of medicine long.

Time is fleeting,

Experience fallible.

Decisions difficult.

The physician must not only be prepared to do what is right himself, but also make the patient, and everyone cooperate."

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SUPPLEMENTARY DATA

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Cell ID Report

Prepared by:	Sarah Low Hong Hui
Date Prepared:	30 April 2014

1 Sample Information

Sample Order	1
Sample ID	LN229
Additional ID Comments	NA
Sample Type	Cell Pellet
Estimated Cells in Pellet	1 x 10^6
DNA volume (µl)	NA
DNA concentration (ng/µl)	NA
DNA quality (A260/280)	NA
Volume Solution Provided (µl)	NA
Operator (Processing)	Tan Sili
Date Cell Pellet Received	2014-04-15
Date DNA Received/Extracted	2014-04-25

DNA volume (µl)	50
DNA concentration (ng/µl)	193.43
DNA quality (A260/280)	1.95
Volume DNA Used (µl)	2.00
Date GenePrint Solution expire	2015-06-22
GenePrint Solution Used (µl)	10

DNA Extraction: DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany)

DNA Quantification: Nanodrop ND1000 (Wilmington, DE)

2 Run Information

Operator (CE)	Sarah Low Hong Hui
Run Date	2014-04-30
Run File Name	20140430_cellID_sharonlow
Run Loading Number	A11

Cell ID Analysis: Geneprint 10 system (Promega, Madison, WI)

Capillary Electrophoresis: ABI 3130xl Genetic Analyser (Life Technologies, Foster City, CA)

3 Run Results

Locus	**Positive**	**LN229**
TH01	6, 9.3	9.3
D21S11	29, 31.2	29,30
D5S818	12, 12	11,12
D13S317	9, 11	10,11
D7S820	8, 11	8,11
D16S539	9, 13	12
CSF1PO	12, 12	12
Amelogenin	X, Y	X
vWA	16, 19	16,19
TPOX	11, 11	8
In Reference Database	Yes	Yes
Closest Reference	2800M Control DNA	LN229
Reference Source	Promega	DSMZ
Percent Match	100%	100%
Percent Match Status	Pass	Pass
Interpretation	Run Passed Quality Control	Cell line is LN229

Analysis: Gene mapper V4.0 software. (Life Technologies, Foster City, CA)

Numbers indicate the allele designations for each locus.

Electrophoragram is added below under Appendix for your reference

Percent Match according to ICLAC Match Criteria Worksheet v1.1 (http://standards.atcc.org)

Designation of “Pass” Status is based on having a Percent Match of ≥80% to the Reference.

4 References

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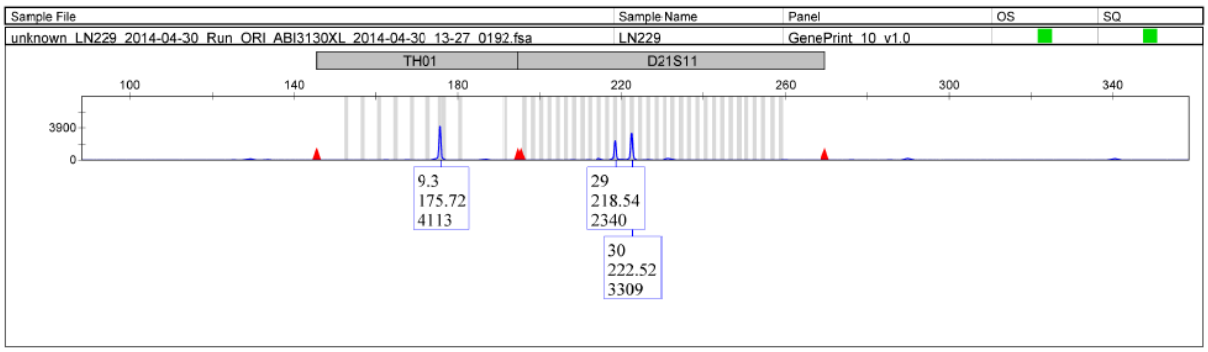
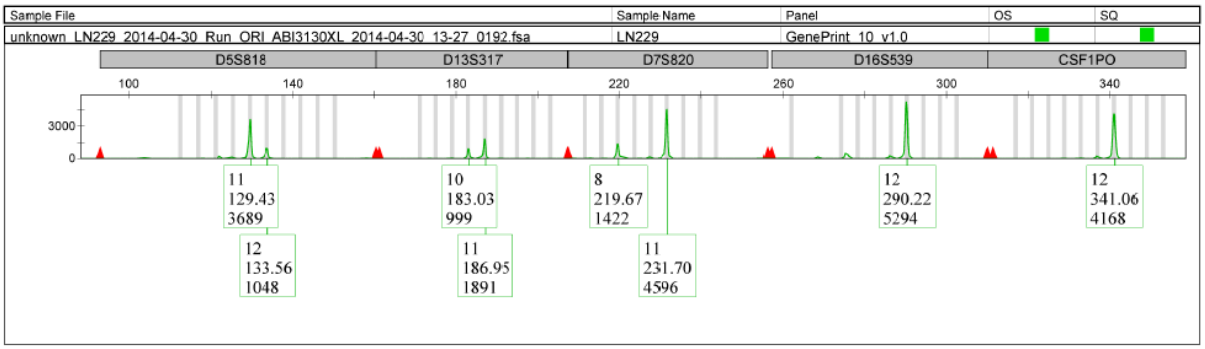
5 Appendix

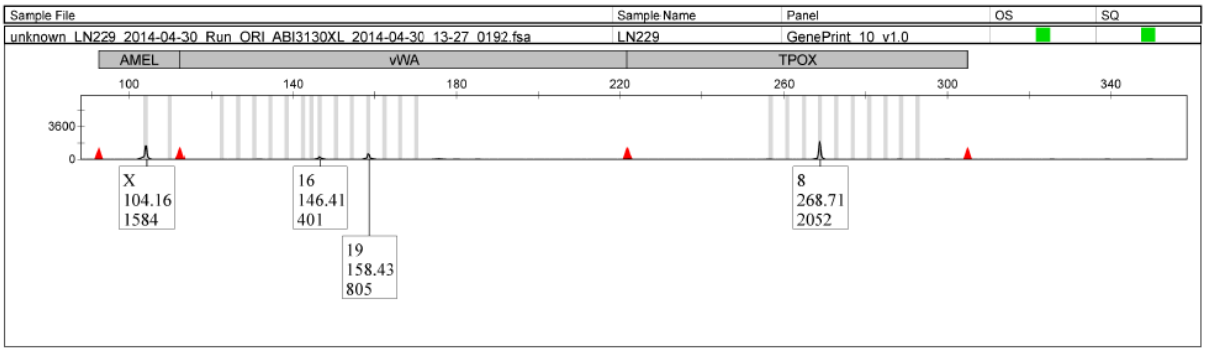
First number in the box represents the allele call that is reflected in the results table above.

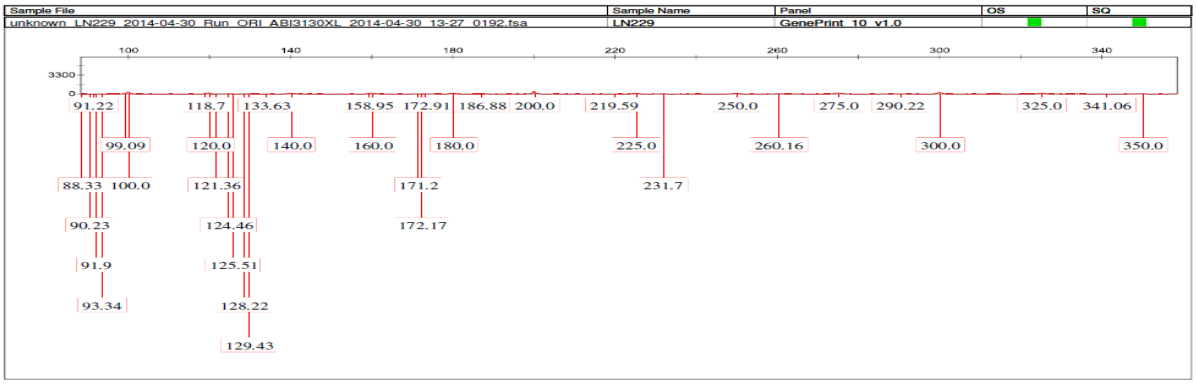
Second number in the box represents the size of the repeat.

Sample: LN229








Sample File
unknown_LN229_2014-04-30_Run_ORI_ABI3130XL_2014-04-30_13-27_0192.fsa
Sample Name
LN229
Panel
GenePrint_10_v1.0
OS
SQ
100
140
180
220
260
300
340
3300
0
91.22
99.09
118.7
133.63
120.0
140.0
158.95
172.91
186.88
200.0
160.0
180.0
219.59
250.0
275.0
290.22
225.0
260.16
300.0
325.0
341.06
350.0
88.33
100.0
121.36
171.2
231.7
90.23
124.46
172.17
91.9
125.51
93.34
128.22
129.43

Sample: Positive Control (2800M)