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MICRORNA SIGNATURES AND TRANSCRIPTIONAL REGULATORY NETWORKS IN GLIOBLASTOMA

BY
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MicroRNA Signatures and Transcriptional Regulatory Networks in Glioblastoma

Ph.D. Dissertation

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Dedicated to Linda

"If you haven't found it yet, keep looking.
Don't settle. As with all matters of the heart,
you'll know when you find it.
And like any great relationship,
it just gets better and better as the years roll on"
Steven Paul Jobs

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Møller H G, Rasmussen A P, Andersen H H, Johnsen K B, Henriksen M, Duroux M, "A systematic review of microRNA in glioblastoma multiform: micro-modulators in the mesenchymal mode of migration and invasion," *Molecular Neuropathology*, vol. 47, no. 1, pp. 131-144, 2013.

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Henriksen M, Duroux L, Pilgaard L, Agger R, Moos T, Zachar V, Duroux M, "The elusive expression of FABP7 and its association to the molecular subtype of GBM," (in preparation).

PREFACE

To further our understanding of glioblastoma (GBM) biology, a deeper insight into the mechanisms of this severe disease is necessary, though also a daunting task. Much is already discovered with respect to the genetic instability underpinning this aggressive cancer, with many key signalling pathways dysregulated providing distinguishable hallmarks. However, not everything can be explained purely by the expression of the gene. Here microRNAs (miRNAs), which are post-transcriptional regulators of gene expression, have shed light on new aspects of both gene regulation as well as post-transcriptional regulation in GBM.

This thesis has been divided into two parts composed of four papers. Part I looks at the microRNA regulatory landscape and takes the form of two papers. The first paper "*MicroRNA Expression Signatures Determine Prognosis and Survival in Glioblastoma Multiforme – A Systematic Overview*" aimed at providing an up-to-date account of the miRNA expression profiles in tumour tissue associated with prognosis and survival in GBM. Based on literature, a signature or pattern of miRNAs was derived which has a prognostic potential and the miRNAs reported to have a protective or risk-associated profile have been highlighted in relation to GBM. Finally, the studies that have reported miRNA signatures with respect to prognosis have been compared to find a common miRNA profile across the different studies. However, the coherence between these signatures was small, and correlations to clinicopathological features other than survival are seldom seen. Building on these findings, paper two "*MicroRNA Expression Signatures and their Correlation with Clinicopathological Features in Glioblastoma*" aimed to identify any significant relationship between miRNA signatures and clinicopathological data combining pathological features with miRNA and mRNA analysis. In fourteen GBM tumours a total of 61 miRNAs were shown to cluster the GBM tumours into long- and short-term-surviving patients. Many of these miRNAs were associated with differential expression in GBM, including a number of miRNAs shown to confer risk or protection with respect to clinical outcome and to modulate the mesenchymal mode of migration and invasion (MMMI). An inverse relationship between miR-125b and Nestin expression was identified

and correlated with overall survival in GBM patients, eloquently illustrating how clinicopathological findings and molecular profiling may be a relevant combination to predict patient outcome. The intriguing finding that many of the differentially expressed miRNAs contained exosome-packaging motifs in their mature sequence suggested that we must expand our view to encompass complex intercellular communication in order to identify molecular prognostic biomarkers and to increase our knowledge in the field of GBM pathogenesis.

In part II, the scope of the thesis broadens still in the field of gene regulation, looking at the expression of brain fatty acid binding protein (FABP7), a protein tightly linked to the invasive nature of GBM. Paper three, a comprehensive review "*Brain-Specific Fatty Acid Binding Protein Transcriptional Regulatory Network*" was aimed at providing an up-to-date account of transcriptional regulatory elements, factors and DNA-binding proteins of the FABP7 gene, which have been reported in the literature or extracted from public electronic databases. It was assumed that the entire set of transcription factors binding to the FABP7 gene was finite and that the tight spatio-temporal control of FABP7 expression in normal development, as well as in pathologies such as astrocytoma and other types of cancer, was in part the result of their combinations. An exhaustive list of FABP7 transcription factors, as well as a physical map of their interaction sites was established. Establishing such a map should be helpful to unravel and understand FABP7 transcriptional regulation in normal development and pathological conditions, and eventually to understand evolutionary divergence from closely related paralogues also expressed in nervous tissue (i.e. heart-type fatty acid binding protein and epidermal fatty acid binding protein). With this regulatory map in place, the expression of FABP7 was further investigated in tissue and primary cell lines and is presented in paper four "*The elusive expression of FABP7 and its association to the molecular subtype of glioblastoma*". High FABP7 expression in tumour tissue was not mirrored in patient matched primary culture, an observation previously reported by De Rosa et al. [72], which made us question the validity of such model primary cultures with respect to loss of FABP7 expression and what factors underpine this change in expression. It was hypothesed that the elusive FABP7 behaviour was due to factors known to regulate FABP7 and to the presence or absence of molecular subtypes. Here it was found that FABP7 belonged to the classical subtype of GBM, though some tumours with high FABP7 expression showed characteristics of a mesenchymal subtype. Both subtypes had characteristics that were associated with FABP7 function, which suggested that FABP7 belonged to one or the other. This, the association with both subtypes, could be multifaceted where the protein plays different functional roles linked to one or more of the GBM hallmarks that facilitate tumour progression.

ACKNOWLEDGEMENT

It was not written anywhere when I began my educational journey that I would end up having a PhD fellowship, but circumstances revealed that I in the fall of 2010 started one at the department of Health Science and Technology at Aalborg University.

The choice of doing a PhD fellowship has been a daunting task, which has challenged me and evolved me both professionally and personally. It has been tough, with long nights in the laboratory or early mornings in the rain for work. It has been quit a remarkable journey. This thesis is the culmination of all that work. Boiled down to the essentials. There have been many redirections, sideline projects, and funny investigations, which for the sake of the story have been left out, albeit still relevant for the community working with glioblastoma pathology.

It has been typed on my educational companion through the last seven years, a MacBook 2006, which has done a magnificent job. However, all of this would not have been possible if it have not been for the kindness and help of various people that I have meet along my way. First of all, a special thanks goes to my supervisor associate professor Meg Duroux for being my scientific mentor and for her moral support through this endeavour. Without her faith in me, and the courage to take me on board this would not have been possible and for that I am ever so grateful. Furthermore, I would like to acknowledge all the employees in the biomedicine group, former as well as present. The laboratory for cancer biology for providing my home for four years. The senior scientists Linda Pilgaard, Trine Fink, Vladimir Zachar, Torben Moos and Ralf Agger. The technicians, Helle, Ole, Ditte, Merete and Rikke, in the biomedicine group and Charlotte Sten. Likewise I would like to thank warmly, fellow students Kasper, Hjalte, and Joachim for their time in the laboratory and paper writing. PhD-students and colleagues, especially Chris Bath, for coffee breaks and geeky conversation, whether it was scientific or Apple related. Additionally, I would like to thank those at Aalborg University Hospital, Hybrigenics, Institute of Medicine (Göteborg, Sweeden), Systems Biology and Bioinformatics (Rostock, Germany) and Danish Cancer Society

Research Center (Copenhagen, Denmark) with whom I have had the pleasure of collaborating and helped me gain insights into new areas.

Finally, I would like to thank my family and friends for coping with me through all the ups and downs. To Kim Jensen and Anders Rosenberg for being a place to escape and keep me grounded, and to Rico Nielsen for being a solid rock of encouragement personally as well as professionally. To my parents, Jette and Palle Henriksen, for always to believe in me and to have given me the best start in life that I could ever have wanted. But most of all I would like to thank my wife Linda Tørngren Henriksen, for putting up with all the late hours, my absentmindedness, my mess of papers and book, and my lack of time orientation. I know that this has been a toll on you. This is for you. Without you in my life I would have gotten lost in the black of the night. You are my lighthouse. My guidance.

Michael Henriksen

København,

ABSTRACT

The overall objective of this thesis was to gain a better understanding of the molecular mechanisms that underpin the invasive nature of GBM. The dysregulation of key signalling pathways plays a role in tumourgenesis and relapse [160] giving rise to GBM hallmarks [134]. One of the most prominent hallmarks of GBM is the invasive or infiltrative nature which can in part be due to a population of cells called cancer stem cells (CSCs), which supports the tumour growth in a heterogeneous hypoxic niche [15]. Gene regulation, pre- and post-transcriptional (microRNA), and how this can be linked in some way to the expression of stem cell like proteins that are dysregulated has been the main focus point in this thesis and by looking at these in combination with clinicopathological features, we have learnt more about the underlying pathology of this disease and its progression.

This thesis is a compilation of several papers, which are subdivided into two parts based on theme. Part one, "*MicroRNA in Glioblastoma*", explores the role of microRNA (miRNA) in GBM and their association with survival. An overview of signatures or patterns of miRNAs, which are of prognostic potential, was established to find a common miRNA profile and to identify miRNAs associated with survival, clinical outcome and the modulation of the mesenchymal mode of migration and invasion (MMMI). A number of these were associated with differential expression in GBM patients, including miR-125b which had an inverse correlation with Nestin expression and correlated with overall survival in GBM patients, eloquently illustrating how clinicopathological findings and molecular profiling may be a relevant combination to predict patient outcome.

The second part, "*Brain Specific Fatty Acid Binding Protein in Glioblastoma*", looks extensively into the regulation of the brain fatty acid binding protein (FABP7), a highly upregulated protein in GBM and part of its underlying invasive nature. FABP7 is a stem cell marker in GBM and was early on recognised as a gene involved in GBM biology. However, the fundamental aspects of regulation within cancer are, at the moment, still elusive. The regulatory landscape of FABP7 is comprised of many aspects, with some revealed

but most still elusive. A comprehensive map of the binding of transcription factors, the primal regulator, was generated, describing the function of 27 transcription and signalling factors with reference to their effect on FABP7 expression, their role in neural development, and in GBM. A majority of these are associated with regulatory modules in GBM, confirming FABP7 as a target gene in GBM. Though FABP7 is highly upregulated in GBM, it is notoriously lacking from many GBM cell lines (e.g. U87) or primary cell cultures. It was found that FABP7 is lost upon cultivation of GBM tissue, however, for some cultures, the protein could be re-expressed upon neurosphere formation or xenografting. This elusive behaviour was hypothesised to be in part a function of the molecular subtype which led to the finding that FABP7 belongs to the classical subtype, along with Nestin, another known stem cell marker.

DANSK RESUMÉ

(Abstract in Danish)

Det overordnede formål med denne afhandling var at opnå en bedre forståelse for de molekylære mekanismer, der understøtter den invasive natur af GBM. Forstyrrelse i reguleringen af vigtige signalveje spiller en rolle i tumorgenesis og tilbagefald [160], hvilket giver anledning til kendetegnende for GBM [134]. Et af de mest fremtrædende kendetegn for GBM er den invasive eller infiltrative natur som til dels kan skyldes en population af celler kaldet cancer stamceller (CSCr), som understøtter tumorvækst i en heterogen hypoxisk niche [15]. Genregulering, før og efter transcription (microRNA), og hvordan dette kan være forbundet på en eller anden måde til ekspressionen af stamcelle lignende proteiner der ikke er korrekt reguleret, har været det vigtigste fokuspunkt i denne afhandling og ved at se på disse i kombination med klinisk-patologiske træk, har vi lært mere om den underliggende patologi af denne sygdom og dens progression.

Denne afhandling er en samling af flere artikler, der er opdelt i to dele baseret på temaer. Første del, "*MicroRNA i glioblastom*", udforsker rollen af microRNA (miRNA) i GBM og deres forbindelse til overlevelse. En oversigt over signaturer eller mønstre af miRNA, som er af prognostisk potentiale, blev etableret for at finde en fælles miRNA profil og identificere miRNA forbundet til overlevelse, kliniske udfald og modulation af mesenkymal form for migration og invasion (MMMI). En del af disse var forbundet med varierende udtryk af miRNA'er i GBM patienter, herunder miR-125b, som havde en omvendt sammenhæng med udtrykningen af Nestin og passede med overlevelse i GBM patienter samlet set, hvilket illustrerer hvordan kliniskpatologiske fund og molekylære profiler kan være en relevant kombination til at forudsige udfaldet for patienten.

Den anden del, "*Hjerne specifik fedtsyrer-bindende protein i glioblastom*", ser omfattende på reguleringen af hjernens fedtsyre-bindende protein (FABP7), et yderst opreguleret protein i GBM og en del af GBMs underliggende invasive natur. FABP7 er en stamcelle markør i GBM og blev tidligt anerkendt som et gen involveret i GBM biologi. Men de grundlæggende aspekter

af regulering inden for kræft er i øjeblikket stadig undvigende. Reguleringen af FABP7 består af mange aspekter, med nogle afsløret, men de fleste stadig uafklaret. Et omfattende kort over bindingen af transkriptionsfaktorer, de primærer regulatorer, blev genereret. Her beskrives funktionen af 27 transskriptions og signaleringsfaktorer med henvisning til deres effekt på udtrykningen af FABP7, deres rolle i udvikling af hjernen og i GBM. Et flertal af disse er forbundet med regulatoriske moduler i GBM, der bekræfter FABP7 genet som mål i GBM. Selvom FABP7 er stærkt opreguleret i GBM, er det notorisk manglende fra mange GBM cellelinier (fx U87) eller primære cellekulturer. Det blev konstateret, at FABP7 er tabt ved dyrkning af GBM væv, men for nogle kulturer kunne proteinet blive udtrykt på ny ved dannelsen af neurosfære eller et xenotransplantat. Det blev formodet at denne undvigende opførsel delvis kunne være en funktion af den molekylære undertype, som førte til den konklusion, at FABP7 tilhører den klassiske undertype, sammen med Nestin, en anden kendt stamcellemarkør.

THESIS DETAILS

Thesis Title:	MicroRNA Signatures and Transcriptional Regulatory Networks in Glioblastoma
Ph.D. Student:	Michael Tørngren Henriksen
Supervisors:	Assoc. Prof. Meg Duroux, Aalborg University

The main body of this thesis consists of the following four papers, where two are published, one is under review and one is in preparation.

[1] Henriksen M, Johnsen K B, Andersen H H, Pilgaard L, Duroux M, "MicroRNA expression signatures determine prognosis and survival in glioblastoma multiforme - a systematic review," *Molecular Neurobiology*, vol. 50, no. 3, pp. 896–913, 2014.

[2] Henriksen M, Johnsen K B, Olesen P, Pilgaard L, Duroux M, "MicroRNA expression signatures and their correlation with clinicopathological features in glioblastoma multiforme," *Neuromolecular Medicine*, vol. 16, no. 3, pp. 565–577, 2014.

[3] Henriksen M, Duroux L, Zachar V, Duroux M, "Brain-specific fatty acid binding protein transcriptional regulatory network," *BBA - Gene Regulatory Mechanisms*, (under review), 2016.

[4] Henriksen M, Duroux L, Pilgaard L, Agger R, Moos T, Zachar V, Duroux M, "The elusive expression of FABP7 and its association to the molecular subtype of GBM," (in preparation).

In addition to the main papers, contribution has been made to the following publications.

[5] Møller H G, Rasmussen A P, Andersen H H, Johnsen K B, Henriksen M, Duroux M, "A systematic review of microRNA in glioblastoma multiform: micro-modulators in the mesenchymal mode of migration and invasion," *Molecular Neuropathology*, vol. 47, no. 1, pp. 131–144, 2013.

[6] Pilgaard L, Mortensen J H, Henriksen M, Olesen P, Sørensen P, Laursen R, Vyberg M, Agger R, Zachar V, Moos T, Duroux M, "Cripto-1 expression in glioblastoma multiform," *Brain Pathology*, vol. 24, no. 4, pp. 360–370, 2014.

Conference papers and proceedings.

[7] Henriksen M, Johsen K B, Olesen P, Pilgaard L, Duroux M, "Glioblastoma miRNA signatures linked to progression free survival," 8^{th} *International MicroRNAs Europe - 2013 meeting*, Cambridge, UK, 2013.

[8] Henriksen M, Duroux L, Pilgaard L, Zachar V, Duroux M, "Down-regulation of FABP7 in glioblastoma primary cultures," *Brain tumor 2013*, Berlin, Germany, 2013.

[9] Mortensen J H, Pilgaard L, Henriksen M, Olesen P, Sørensen P, Agger R, Vyberg M, Moos T, Zachar V, Duroux M, "Cripto-1 is differentially expressed in glionlastoma and resides in the vascular niche," *Brin tumor 2013*, Berlin, Germany, 2013.

This thesis has been submitted for assessment in partial fulfilment of the PhD degree. The thesis is based on the published or submitted scientific papers, which are listed above. Parts of the papers are used directly or indirectly in the extended summary of the thesis. As part of the assessment, co-author statements have been made available to the assessment committee and are also available at the Faculty. The thesis is not in its present form acceptable for open publication but only limited and closed circulation as copyright may be ensured.

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5	
5ALA	5-aminolevulinic acid
A	
Ago2	v-akt murine thymoma viral oncogene homolog
AKT	argonaut protein 2
AP1	activator protein 1
B	
BACE1	beta-site amyloid beta precursor protein-cleaving enzyme 1
BCA	bicinchoninic acid
BMF	bcl2 modifying factor
bZIP	basic leucine zipper
C	
CaCo-2	human colon carcinoma cells
CASP	caspase
CAT	chloramphenicol acetyltransferase
CCND2	cyclin D2
$CD34^+$	CD34 positive
$CD38^-$	CD38 negative
$CD133^+$	CD133 positive
CDC25A	cell division cycle 25A
CDK	cyclin-dependent kinase
CDKN1A	cyclin-dependent kinase inhibitor 1A (p21)
CDKN1C	cyclin-dependent kinase inhibitor 1C (p57)
CDKN2A	cyclin-dependent kinase inhibitor 2A (p14)
cDNA	complementary DNA
CEA	carcinoembryonic antigen gene fmily
CEBPA	CCAAT/enhancer binding protein, alpha
CEBPB	CCAAT/enhancer binding protein, beta
CGGA	chinese glioma genome atlas
CHD5	chromodomain helicase DNA binding protein 5
CHI3L1	chitinase 3-like 1
ChIP	chromatin immunoprecipitation
M	
MDGI	mammary derived growth inhibitor
MDM2	MDM2 proteooncogene, E3 ubiquitin protein ligase
MDM4	MDM4, p53 regulator
MEIS	meis homeobox 1
MEM	menimum essential medium eagle
MERTK	MER proto-oncogene, tyrosine kinase
MET	MET proto-oncogene, receptor tyrosine kinase
MeV	multiexperiment viewer
MGMT	o-6-methylguanine-DNA methyltransferase
miRNA	microRNA
MLANA	melan-A
MMMI	mesenchymal mode of migration and invasion
MMP	matrix metalloproteinase
mRNA	messenger RNA
MS	multiple sclerosis
MSCs	mesenchymal stem cells
N	
NEFL	neurofilament, light polypeptide
NEP	neuroepithelial
NES	nestin
NF1	neurofibromin 1
NFKB	nuclear factor of kappa light polypeptide gene enhancer in B-cells
NICD	notch intercellular domain
NKX2-2	NK2 homeobox 2
O	
OLIG2	oligodendrocyte lineage transcription factor 2
OMIM	online mendelian inheritance in man
OS	overall survival
P	
p14	CDKN2A
p16	CDKN2A

ChIP-Seq	ChIP-sequencing
Chr	chromosome
CNS	central nervous system
CpG island	cytosine-phosphate-guanine island
CSC	cancer stem cells
CTCF	CCCTC-binding factor
Cx43	connexin 43

D

DAB	3,3′-diaminobenzidine
DGCR8	DiGeorge critical region 8
DLL3	delta-like 3
DMEM	Dulbecco's modification of Eagle's MEM
DNA	deoxyribonucleic acid

E

E2A	transcripton factor 3
E2F3	E2F transcription factor 3
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EGFRvIII	epidermal growth factor receptor variant III
EMT	epithelial-mesenchymal transition
ENCODE	the encyclopedia of DNA elements
ERBB2	erb-b2 receptor tyrosine kinase 2
ERBB3	erb-b2 receptor tyrosine kinase 3
ERK	extracellular signal-regulated kinases
ETS	E26 transformation-specific

F

FABP	fatty acid binding protein
FABP1	liver fatty acid binding protein
FABP2	intestinal fatty acid binding protein
FABP3	heart fatty acid binding protein
FABP4	adipocyte fatty acid binding protein
FABP5	epidermal fatty acid binding protein
FABP7	brain fatty acid binding protein
FBXO3	f-box protein 3
FFPE	formalin-fixed paraffin-embedded
FGFR3	fibroblast growth factor receptor 3
FOS	FBJ murine osteosarcoma viral oncogene homolog
FOXA	forkhead box, subgroup A
FOXO	forkhead box, subgroup O

G

G-CIMP	glioma CpG island methylator phenotype
G-CSF	colony stimulating factor
GABRA1	gamma-aminibutyric acid A receptor, alpha 1
p21	CDKN1A
p53	tumour protein p53
p57	CDKN1C
p38MAPK	MAPK14
PACT	protein activator of PKR
Pasha	partner of drosha
PAX6	paired box 6
PBS	phosphate-buffered saline
PBX1	pre-B-cell leukemia homeobox 1
PCR	polymerase chain reaction
PDCD4	programmed cell death 4 (neoplastic transformation inhibitor)
PDGFRA	platelet-derived growth factor receptor alpha
PI3K	phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
PIAS3	protein inhibitor of activated STAT3
PIC	pre-initiation complex
PFS	progression-free survival
PKNOX1	PBX/knotted 1 homeobox 1
PNS	peripheral nervous system
POU2F1	POU class 2 homeobox 1
POU3F1	POU class 3 homeobox 1
POU3F2	POU class 3 homeobox 2
POU3F3	POU class 3 homeobox 3
PPIA	cyclophilin A
PTEN	phosphatase and tensin homolog

Q

qRT-PCR	quantitative real-time polymerase chain reaction

R

RAD21	double-strand-break repair protein
Ran-GTP	guanosine triphosphate-bound ras-related nuclear protein
RANO	revised assessment in neuro-oncology
RB	retinoblastoma
RBCC	RING finger, B-box zinc-finger, coiled-coil region
RBPJ	recombination signal binding protein for immunoglobulin kappa J region
RECK	reversion inducing cysteine rish protein with kazal motifs
RELB	v-rel avian reticuloendotheliosis viral oncogene homolog B
RELN	reelin
RIPA	radioimmunoprecipitation assay
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RTK	receptor tyrosine kinase

S

SAM	significance analysis of microarray
SDS	sodium dodecyl sulfate
SLC12A5	solute carrier family 12 member 5
SMC3	structural maintenance of chromosomes 3

GABRB2	gamma-aminobutyric acid A receptor, beta 2
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GBM	Glioblastoma Multiform
GFAP	glial fibrillary acidic protein
GP130	glycoprotein 130
GRCh37	genome reference consortium human genome (build 37)
GTP	guanosine-5′-triphosphate

H

HepG2	human liver cancer cell line
HGF	heptocyte growth factor
HIF1	hypoxia inducible factor 1
hMSH2	muts protein homolog 2
hnRNPA2B1	heterogeneous nuclear ribonucleoprotein A2/B1
HOX	homeobox
HRE	hormone response element

I

IDH1	isocitrate dehydrogenase 1
iHOP	information hyperlinked over proteins
IL	interleukin
IL6	interleukin 6
IL8	interleukin 8
IL10	interleukin 10

K

Ki67	marker of proliferation ki67 (MKI67)
KPBS	phosphate-buffered saline with potassium
KRAB	krûppel associated box

L

LOH	loss of heterozygocity

M

MAD	median absolute deviation
MAPK14	mitogen-activated protein kinase 14
MAZ	myc-associated zinc finger protein
MBP	myelin basic protein
MCP	macrophage chemo-attractant protein
SNCG	synuclein gamma
SOX2	SRY-box 2
SPSS	statistical package for the social sciences
STAG2	stromal antigen 2
STAT3	signal transducer and activator of transcription 3 (acute-phase response factor)
SYT1	synaptotagmin 1

T

TAZ	tafizzin
TBP	TATA-box binding protein
TCGA	the cancer genome atlas
TF	transcription factor
TGFB	transforming growth factor beta 1
TIMP3	TIMP metallopeptidase inhibitor 3
TLR2/4	toll-like receptor 2/4
TMZ	temozolomide
TNFRSF1A	tumour necrosis factor receptor superfamily member 1A
TP53	tumour protein p53
TRADD	TNFRSF1A-associated via death domain
TRBP	tar RNA binding protein
TRIM28	tripartite motif containing 28

U

UTR	untranslated region

V

VEGF	vascular endothelial growth factor
VAK	volume, age, karnofsky performance score
VEGFA	vascular endothelial growth factor A

W

WHO	world health organisation

Y

YWHAZ	tyrosine3/tryptophan 5-monooxygenase activation protein

General Introduction

1

Despite advances in our knowledge about glioblastoma (GBM) pathology, clinical challenges still lie ahead with respect to treatment of GBM due to high prevalence, poor prognosis and frequent tumour relapse [77, 108, 159, 246, 297, 298]. This is in part due to the invasive nature, a hallmark of GBM, which makes it a difficult task at tumour resection. Early treatment and proper characterisation of the tumour is therefore of critical impact [191].

This chapter gives a general introduction to GBM pathology and ends with the aims of the thesis. There is a chapter on methodology, which describes the methods outlined in the papers. Following this, the thesis is divided into two parts, each of which with an introduction relevant for the chapter and papers presented. The thesis is closed by a general discussion and conclusion.

1 Glioblastoma Pathology

Cancer can arise in all types of tissue. Although it is composed of a heterogeneous group of diseases, these diseases all share a common feature; abnormal cells growing past their natural limitations. Hanahan and Weinberg proposed six hallmarks of cancer in 2001, which encapsulate the fundamental basics of malignant transformation [133]. These basic hallmarks are defined as: (1) self-sufficiency in growth signals, (2) insensitivity to anti-growth signals, (3) tissue invasion and metastasis, (4) limitless replicative potential, (5) sustained angiogenesis and (6) evading apoptosis. During the last decade, a better understanding of these hallmarks has been enabled through remarkable progress in cancer research, which has led to modifications and expansion of the original concept [134].

The number of genetic alterations needed for tumourigenesis is still unknown [379]; however, it is evident that several disruptions to the cellular circuitry are required for carcinogenesis. These genetic alterations can happen either in the form of activation or inactivation of specific genes contributing to the carcinogenic process [379]. Glioma, similar to other cancers, spawns from an accumulation of genetic alterations, such as epigenetic modifications, mutations, amplification and modification of the gene function, which change the signalling pathways leading to the cancer phenotype [134]. Of all central nervous system (CNS) malignancies, approximately 30 % are attributed to glioma, while 80 % of primary brain tumours alone are of glioma origin [121].

Malignant glioma are tumours of the neoplastic glial cells and can, according to the World Health Organisation (WHO), be classified as astrocytoma, oligodendroglioma, mixed oligoastrocytoma and ependymoma, which are based on histological similarities [252]. Additional stratification based on histopathological criteria, specified by nuclear atypia, mitotic index, microvascular proliferation and necrosis, can further subclassify the gliomas into low grade (WHO grades 1 and 2) or high grade (WHO grades 3 and 4) [252].

The four grades of astrocytomas are; pilocytic astrocytoma (grade 1), diffuse astrocytoma (grade 2), anaplastic astrocytoma (grade 3) and glioblastoma (GBM) (grade 4). Of primary brain tumours, GBM is the most common and accounts for 15 %, while 55 % of all gliomas are GBMs [121]. Although the number of malignant glioma cases per year is fairly low, it is considered one of the most aggressive forms of human cancer [434].

2 Biology of Glioblastoma

One of the enabling characteristics of cancer is genomic instability [379]. The ability of gliomas to circumvent the receptor-driven pathways to reduce the dependence on ligand activation facilitates their inappropriate cell division, survival and motility.

These genetic alterations have been supported by the Cancer Genome Atlas (TCGA), a comprehensive study of GBM initiated in 2008 with reports on alterations such as copy number, acquired DNA sequence, gene expression patterns and DNA methylation [274]. Akin to previous literature, these analyses verified three major pathways involved in GBM: (1) the tumour protein p53 (TP53) pathway, (2) the retinoblastoma (RB) pathway and (3) receptor tyrosine kinase (RTK) signalling, see figure 1.1.

The mutation of TP53 was one of the first genetic alterations associated

with glioma [290]. TP53 encodes p53, a transcription factor involved in cell cycle arrest [131, 416], which upon DNA damage is activated leading to transcriptionally regulation of potential effector genes [154, 231]. Although mutation in the TP53 gene is relatively rare in primary GBM, loss of TP53 function is observed in approximately 50 % of primary and in over 75 % of secondary GBM [461].

The RB pathway control proliferation through the binding of RB with transcription factors in the E2F family, essential for inactivation of genes involved in cell cycle progression [354]. Perturbations of the RB pathway is found in more than 50 % of primary gliomas [25, 137, 274, 344, 402], however, on its own the RB pathway is incapable of facilitating sufficient cell cycle control required for cellular transformation, pointing to other pathways important for the prevention of gliomagenesis [11, 156, 157, 165, 337, 372, 403, 404, 451].

Activation of receptor tyrosine kinases (RTKs), such as Epidermal growth factor receptor (EGFR) and Platelet-derived growth factor receptor alpha (PDGFRA), seems to be the most important among the many mechanisms at play in GBM. EGFR is affected by the most common genetic alteration in primary glioma at chromosome 7p12 92, with EGFR gene amplification occurring in 40 to 50 % of GBM [85, 244, 245, 252, 440]. The most commonly described event in primary GBM is encoding of a truncated form of EGFR missing exon 2 to 7 generating EGFR variant III (EGFRvIII) [86, 226, 436], facilitating constant activation of downstream pathways. This is further enhanced by the loss of Phosphatase and tensin homolog (PTEN), a major tumour suppressor shown to have a significant role in high grade glioma, found to be inactivated in 50 % of high-grade gliomas [202, 298, 398]. Amplification has also been found to happen at chromosome 12q13-15 affecting both the RB and TP53 pathway [97, 172, 183, 252] and at chromosome 4q12 affection several RTKs [352].

In addition to alterations in the three core pathways of GBM, Isocitrate dehydrogenase 1 (IDH1) mutation, O-6-methylguanine-DNA methyltransferase (MGMT) promoter methylation and loss of heterozygosity (LOH) are reported. IDH1 is involved in energy metabolism [133] and mutation of this gene is largely associated with secondary GBM, where it is an early and frequent genetic alteration [14, 133, 296, 312, 371, 382, 421], while only occasionally found in primary GBM [14, 133, 218]. It has though been found that mutation of IDH1 is an independent and positive prognostic marker [133, 218, 225, 421]. In regard to prognostic markers for GBM, the methylation status of MGMT has been found to be one of the most important. MGMT, a DNA repair enzyme that facilitates removal of DNA adducts [387], has been found to have a CpG (cytosine-phosphate-guanine) island situated at its tran-

scriptional start site [277]. Methylation of the CpG island has been found to transcriptionally silence MGMT transcription [147, 433]. Due to the toxic nature of DNA modifications and the property of temozolomide, an alkylating agent that is the current standard of care for GBM patients, the expression of MGMT correlates with sensitivity to temozolomide [143]. The methylation of MGMT thus positively correlates with better outcome for patients receiving temozolomide.

With the application of genome-wide analysis, common pathways mutated in GBM have been uncovered, with several of the characteristics of GBM represented in molecular subclasses identified within this disease.

3 Glioblastoma Molecular Subtypes

Identification of molecular subclasses through genome-wide mRNA expression analysis [120] has fundamentally changed the understanding of GBM and emphasised that glioma, which represents few histopathological WHO grade cases, is a molecular heterogeneous disease [34, 106, 169, 242, 294, 316, 407].

Through the TCGA, Verhaak et al. [407] characterised four subtypes: Proneural, Neural, Classical and Mesenchymal, which were associated with distinct genetic alterations, such as copy number alterations and genetic mutations, table 1.1.

3.1 Characteristics of the Proneural Molecular Subtype

The proneural subtype was found to, especially, have alterations of PDGFRA and point mutations in IDH1 [35, 122, 407]. Amplification of PDGFRA was observed in all GBMs, though to a higher degree in the proneural subtype. IDH1 mutations, on the other hand, were almost exclusively present in this subtype. Mutations in the IDH1 gene has been associated with young patients [312, 453] corresponding with the proneural subtype having the largest percentage of young patients [293, 316, 407]. Both PDGFRA alterations, IDH1 mutation and younger age have all previously been shown to be prevalent in secondary GBM which also is enriched in the proneural subtype [10, 108, 201, 431, 453]. Secondary GBM has also previously been shown, in comparison to primary GBM, to have prevalence for promoter methylation, especially in the RB and MGMT gene [288, 297]. Methylation of cytosines generally occurs in conjunction with CpG di-nucleotide repeats, or CpG islands [58]. Hypermethylation of CpG islands within promoters are a general trait of cancers and widely reported in glioma [93, 198, 293]. Recently, a sub-

set of GBMs have been identified to have a glioma-CpG island methylator phenotype (G-CIMP), displayed as a concentration of CpG island hypermethylations at a large number of loci, which represent 30 % of the proneural subtype [293]. In addition, mutation in or LOH of TP53 was more frequent in the proneural subtype, a characteristic of GBM [274], while paired loss of chromosome 10 and gain of chromosome 7, another GBM trademark, was less prevalent in the proneural subtype. The paired chromosome 10 loss and chromosome 7 gain was, however, present in all of the classical subtype [407].

3.2 Characteristics of the Classical Molecular Subtype

This subtype also showed amplification of EGFR along with point or vIII EGFR mutations together with loss of PTEN and Cyclin-dependent kinase inhibitor 2A (CDKN2A), however, it also has a recognisable absence of TP53 mutation. Classical tumours exhibiting EGFR amplification was associated with CDKN2A alterations which occurred nearly exclusively together with alteration of the RB pathway suggesting that the RB pathway in these tumours is almost solely affected through the CDKN2A deletion. The classical subtype was also characterised by high expression of the neural precursor and stem cell marker Nestin (NES), together with the Notch and Sonic hedgehog signalling pathways [407].

3.3 Characteristics of the Neural Molecular Subtype

The neural subtype is the only subtype with no real characteristics and to date no unique molecular alterations. It was defined based on the expression of neural markers such as Neurofilament, light polypeptide (NEFL), Gamma-aminobutyric acid A receptor, alpha 1 (GABRA1), Synaptotagmin 1 (SYT1) and Solute carrier family 12 member 5 (SLC12A5) and classified together with normal brain tissue samples [407].

3.4 Characteristics of the Mesenchymal Molecular Subtype

Lastly, Verhaak et al. [407] identified a subtype with mesenchymal character. This subtype exhibited mutations and/or loss of Neurofobromin 1 (NF1) and co-mutation with PTEN; both related to the V-akt murine thymoma viral oncogene homolog (AKT) pathway (A pathway which is involved in survival, migration, cell cycle progression and metabolism, promoted by the phosphorylation of AKT [80, 185, 317, 412]). The mesenchymal subtype was represented by expression of mesenchymal markers such as Chitinase 3-like 1 (CHI3L1) and MET proto-oncogene, receptor tyrosine kinase (MET)

[316, 407]. Increased activity of both mesenchymal and astrocytic markers (CD44 and MER proto-oncogene, tyrosine kinase (MERTK)) is suggestive of an epithelial-to-mesenchymal transition (EMT), which has been associated with trans- and dedifferentiated tumours [393]. Furthermore, increased expression of genes in the tumour necrosis factor super family pathway and Nuclear factor of kappa light polypeptide gene enhancer in B-cells (NF-κB) pathway, like Tumor necrosis factor receptor superfamily member 1A (TNFRSF1A), v-rel avian reticuloendotheliosis viral oncogene homolog B (RELB) and TNFRSF1A-associated via death domain (TRADD), is probably due to a higher overall necrosis and associated inflammatory response in the mesenchymal class [407].

4 Glioma and Stemness

One of the most problematic features of GBM is the recurrent nature of cancer. The fact that a small population of cancer cells with limitless replicative potential can reconstitute a glioma tumour suggests that gliomas arise from stem or progenitor cells [11, 472, 479]. The concept of a subpopulation of tumour-initiating cells has profoundly changed the view on solid tumours and is another example on the heterogeneity of cancer. The first indication of a subpopulation of tumour-initiating cells within cancer was reported for acute myeloid leukaemia with the characteristics of CD34+/CD38- [31]. The presence of tumour initiating cells, first described by several groups [144, 172, 363], was identified by Singh et al. [364] who identified a population of CD133+ cells in GBM. However, later studies have questioned the aspect of CD133+ as the tumour-initiating cells in the brain [19, 20, 52, 296, 421] thus, the search for novel markers [220], with a range of possible markers for brain cancer stem cells has been, such as Nestin (NES) [23], sex determining region Y-box 2 (SOX2) [112] and activated NOTCH [95], along with brain fatty acid binding protein (FABP7), a protein tightly linked to the invasive nature of GBM. FABP7 is highly upregulated in GBM cancer stem cells [248] and was early on recognised as a gene involved in GBM biology and cell migration [280].

The notion that these tumour-initiating cells, drive carcinogenesis with the capacity to self-renew has lead to the hypothesis of CSCs [76, 219, 389]. This hierarchical model is based on the subset of cells, forming a minority of the tumour mass, as the critical subpopulation of GBM. An alternative model has a less hierarchical structure, in which the majority of cells have a low but equal possibility for functioning as tumour-initiating cells [409]. In recent years, the interrelation between CSC and the tumour microenvironment has been shown to be capable of influencing cell fate in GBM [18, 141, 151].

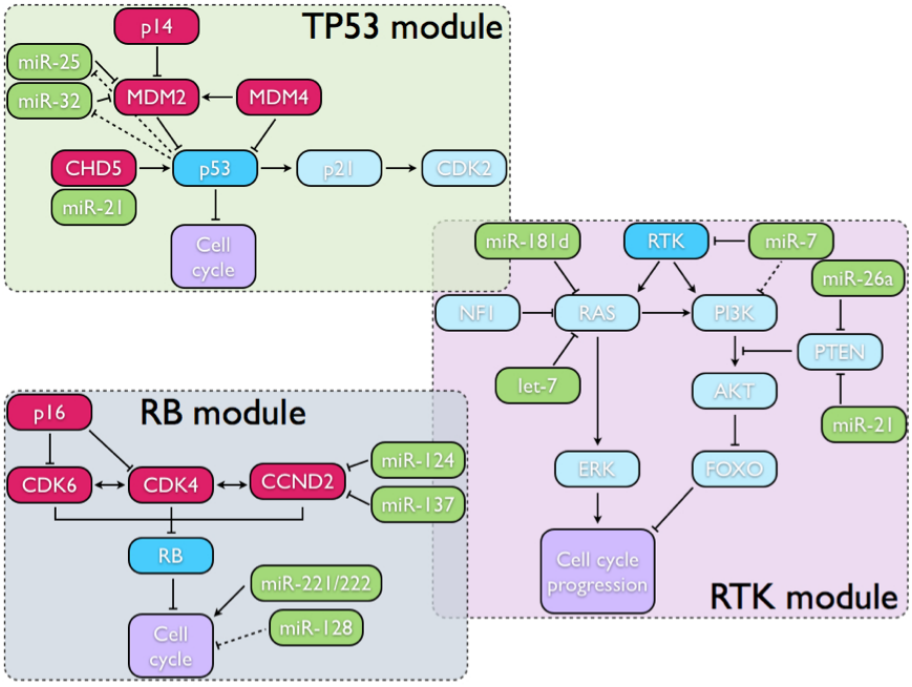

Fig. 1.1: The three core pathways found in GBM. The dark blue indicates the core protein(s) of the module, red indicate proteins affecting the expression of the core protein(s) upstream, light blue indicate proteins downstream of the core protein(s), and green indicate microRNAs. Modified from [274] to include microRNAs [444]. Abbreviations: AKT = v-akt murine thymoma viral oncogene homolog 1 ; CCND2 = cyclin D2 ; CDK2 = cyclin-dependent kinase 2 ; CDK4 = cyclin-dependent kinase 4 ; CDK6 = cyclin-dependent kinase 6 ; CHD5 = chromodomain helicase DNA binding protein 5 ; ERK = mitogen-activated proein kinase 1 (MAPK1) ; FOXO = forkhead box O ; MDM2 = MDM2 proto-oncogene, E3 ubiquitin protein ligase ; MDM4 = MDM4, p53 regulator ; NF1 = neurofibromin 1 ; p14 = cyclin-dependent kinase inhibitor 2A (CDKN2A) ; p16 = cyclin-dependent kinase inhibitor 2A (CDKN2A) ; p21 = cyclin-dependent kinase inhibitor 1A (CDKN1A) ; p53 = tumor protein 53 ; Pi3K = phosphatidylinositol-4,5-bisphosphate 3-kinase ; PTEN = phosphatase and tensin homolog ; RB = retinoblastoma.

Table 1.1: Overview of the molecular heterogeneity of the molecular subtypes. * Molecular markers specified by Verhaak et al. [407]. Abbreviations: AKT2 = v-akt murine thymoma viral oncogene homolog ; CASP1/4/5/8 = caspase 1/4/5/8 ; CDKN2A = cyclin-dependent kinase inhibitor 2A ; CEBPB = CCAAT/enhancer binding protein, beta ; CHI3L1 = chitinase 3-like 1 ; Chr = chromosome ; CpG = cytosine-phosphate-guanine ; DLL3 = delta-like 3 ; ERBB3 = erb-b2 receptor tyrosine kinase 3 ; EGFR = epidermal growth factor receptor ; EGFRvIII = EGFR variant III ; FBXO3 = F-box protein 3 ; FGFR3 = fibroblast growth factor receptor 3 ; GABRB2 = gamma-aminobutyric acid A receptor, beta 2 ; GBM = glioblastoma ; IDH = isocitrate dehydrogenase ; LOH = loss of heterozygocity ; MBP = myelin basic protein ; NES = nestin; NF1 = neurofibromin 1; NKX2-2 = NK2 homeobox 2 ; OLIG2 = oligodendrocyte lineage transcription factor 2 ; PDGFRA = platelet-derived growth factor receptor alpha ; PTEN = phosphatase and tensin homolog ; RELB = v-rel avian reticuloendotheliosis viral oncogene homolog B ; TAZ = tafazzin ; TLR2/4 = toll-like receptor 2 ; TNFRSF1A = tumor necrosis factor receptor superfamily member 1A ; TP53 = tumour protein p53 ; TRADD = TNFRSF1A-associated via death domain ; SNCG = synuclein gamma ; SOX2 = SRY-box 2 ; STAT3 = signal transducer and activator of transcription 3 (acute-phase response factor)

Subtype	Proneural	Classical	Neural	Mesenchymal
Amplification	PDGFRA	EGFR, NES, Notch, Sonic Hedgehog		
Loss		PTEN, CDKN2A		NF1
Mutation	IDH			PTEN
Signature mutation	LOH TP53	EGFRvIII		NF1
Genomic features	CpG-islands	Chr10 loss, Chr 7 gain		
Proteomic features				CD44, TRADD, RELB, TNFRSF1A
Transcriptional regulators				STAT3, CEBPB, TAZ
Clinical features	Young patients, Secondary GBM			Necrosis, Inflammatory infiltrates
Markers*	DLL3, NKX2-2, SOX2, ERBB3, OLIG2	FGFR3, PDGFRA, EGFR, AKT2, NES	FBXO3, GABRB2, SNCG, MBP	CASP1/4/5/8, ILR4, CHI3L1, TRADD, TLR2/4, RELB

5 The Tumour Microenvironment

The tumour microenvironment constitutes the supporting network for tumour growth GBM is composed of a multitude of astrocytes, endothelial cells and cells of the immune and inflammatory system [40, 41], along with blood vessels [103–105], signalling molecules [318] and the extracellular matrix [28, 84]. In addition to the genetic alteration in determining cancer aetiology, the autocrine and paracrine signalling influences on the tumour milieu is perhaps of equal importance.

For example, cell-to-cell communication is paramount, and investigations into the mechanisms of EGFR overexpression in GBM revealed that EGFRvIII overexpression facilitated secretion of two cytokines, namely Interleukin 6 (IL6) and leukaemia inhibitor factor, which lead to activation of EGFR in non EGFRvIII cell through phosphorylation of Glycoprotein 130 (GP130) [174], illustrating the signalling between subpopulations in GBM. The intercommunication is also in play when normal cells are recruited to the tumour centre and during phenotypic changes [134, 187, 199], leading to the secretion of growth factors, which facilitate and maintain neoplastic transformation [24] or drive angiogenesis [187].

5.1 The Angiogenic Niche

Angiogenesis is crucial for tumour progression and malignant gliomas are among the most angiogenic of tumours to alleviate the hypoxic conditions as a consequence of rapid growth [88, 448]. The priming of endothelial cells may arise from the release of secreted factors such as vascular endothelial growth factor (VEGF) [335], from both tumour cells and endothelial cells [99], while differentiation of CSC into endothelial cells has also been shown to be a contributing factor [336]. Endothelial cells also expand the population of CSCs in the perivascular niche by secreting nitric oxide [47], while VEGF has been shown to be secreted by CSCs to support their local vascular niche [13], establishing a CSC niche. This is enhanced by the release of Notch ligands from endothelial cells essential for CSC maintenance [96, 478]. The hypoxic nature might not only facilitate angiogenesis, but also increase the attainment of stem-like characteristics within the tumour [140] which can lead to tumour cells migrating away from the hypoxic niche [71]. The ability for CSCs to migrate and infiltrate has lead to the suggestion that the invasive edge might be where a population of CSCs reside [219].

5.2 The Hypoxic Niche

The hypoxic environment within the tumour is involved in the inflammatory response [287] with Hypoxia inducible factor 1 (HIF1) and NF-κB (NF-κB) activation facilitating release of cytokines capable of attracting inflammatory cells, which in return secrete VEGF [87]. Within the brain, microglia is the primary immune effector and they contribute to a large fraction of tumours [12, 124, 283, 284, 432]. The recruitment of microglia to the glioma microenvironment is facilitated by secretion of several factors, such as macrophage chemoattractant protein 1 and 3 (MCP1, MCP3), Colony stimulating factor (G-CSF) and Heptocyte growth factor (HGF) [196, 228, 270, 301, 380]. Microglia beside secreting VEGF, and thereby stimulating angiogenesis, also releases epidermal growth factor (EGF) and proteases affecting tumour proliferation and invasion, respectively [12, 69, 101, 129, 152]. Although gliomas are capable of immunosuppression of microglia through secretion of for example Transforming growth factor beta (TGFB), Interleukin 10 (IL10) or prostaglandin E2 [12, 102, 166, 195, 204, 209, 302, 417], reports suggest inhibition of growth by microglia is also present [111, 271]. Immunosuppression within the CNS can also be facilitated by astrocytes [204]. They are activated glial cells, a process known as reactive gliosis, which occur upon injury of the CNS generating a scar-like structure around the affected region crucial for the confinement of inflammation within the demarcated tissue [369, 370].

5.3 Cell-to-Cell Communication: The Extracellular Communication Niche

The communication interplay between the microenvironment and cancer cells is complex and the question whether cancer cells activate the microenvironment or vice versa might partly be answered by the information carried by extracellular vesicles such as exosomes [193]. They are a class of nanoparticles that are secreted by the majority of cell types [74, 177, 214, 263, 314, 319, 330, 348, 481], and are able to transport biological active cargo between cells, including proteins, lipids, mRNA and miRNA [4]. They are 30 to 120 nm in diameter and have a complex surface structure composed of special lipids and transmembranous proteins, which facilitate specific interaction and fusion with the recipient cells but also help avoiding uptake by the reticuloendothelial system [413].

The interplay between cancer cells and microenvironment through secretion of extracellular vesicles, exosomes, might facilitate the transfer of oncogenes (e.g. β-catenin [43], Carcinoembryonic antigen gene family (CEA), Erb-b2 receptor tyrosine kinase 2 (ERBB2), Melan-A (MLANA) [5] and LMP-1 [408]) and onco-microRNAs [203, 300, 390] (e.g. let-7, miR-1, miR-15, miR-16

and miR-375) with the latter being a more persistent and long-lasting modulation of the recipient cells.

6 Aims of thesis

The overall objective of this thesis was to gain a better understanding of the molecular mechanisms that underpin the invasive nature of GBM. The dysregulation of key signalling pathways plays a role in tumorgenesis and relapse [160] giving rise to GBM hallmarks [134]. One of the most prominent hallmarks of GBM is the invasive or infitrative nature which can in part be due to a population of cells called cancer stem cells, which supports the tumour growth in a heterogenous hypoxic niche [15]. Gene regulation, pre- and post-transcriptional (microRNA), and how this can be linked in some way to the expression of stem cell like proteins that are dysregulated has been the main focus point in this thesis.

This has lead to the following aims.

[1] To present a comprehensive overview of all the available literature on the expression profiles and function of microRNA in GBM.

[2] To present a comprehensive overview of all the available literature, evaluating miRNA signatures as a function of prognosis and survival in GBM.

[3] To combine clinicopathological findings with molecular profiling of GBM tumours and to identify connections between these features that may have the potential of predicting survival and illuminate aspects of GBM pathogenesis.

[4] To provide an up-to-date account of transcriptional regulatory elements, factors and DNA-binding proteins of the FABP7 gene, which is reported in the literature or extracted from public electronic databases.

[5] To investigate the expression of FABP7 in GBM and to correlate it to the expression of known regulatory elements or the molecular subtype.

Methodology

2

The methods applied in the work of this thesis are described in detail for the specific paper. This section is meant as an overview of the methodological procedures. Some of the methods are similar for all papers (e.g., culture conditions), while others are specific for a certain paper (like, miRNA array). In this section some additional comments will be presented.

1 Collection of Tumour Specimens

The basis for the research papers (Chapter 5 and 8 was the investigation into the regulatome of surgically resected GBM specimens. These were collected from the Department of Neurosurgery at Aalborg University Hospital from November 2010 to June 2012. The study was reviewed and approved by the regional Committee on Biomedical Research Ethics (N-20100069) in Northern Jutland, Denmark prior to execution of the experiments, and all patients provided informed consent for the use of tumour samples. The diagnoses were confirmed by immunohistochemistry against Nestin (NES), Marker of Proliferation Ki67 (Ki67) and Glial Fibrillary Acidic Protein (GFAP) using the routine methodology at the Institute of Pathology, Aalborg University Hospital, and all relevant clinical information was extracted from patient journals. Though 47 patients were initially enrolled in the study, fewer tumour samples were included for further analysis due to inclusion criteria.

For the investigation of the clinicopathological link to microRNA signatures fourteen patients fulfilled the inclusion criteria of the study: diagnosed with primary GBM, first-time resection, and before having received any sort of treatment. These stringent inclusion criteria were chosen because determination of a prognostic miRNA signature should be possible after first tumour

resection. Median age of the patients was 61 years (range 27–85), and the gender ratio (men/women) was around 1:2 (five men: nine women). Surgery consisted of resection with (71 %) or without (29 %) tumour visualisation by 5-aminolevulinic acid (5ALA)-induced fluorescence. Due to limited amounts of tissue, not all samples were applicable for downstream analysis. All samples are listed in table 2.1

Table 2.1: Overview of patient samples.

Tumou	Status	Recurrence	OS	Gender	Age	5'ALA	Diagnosis	Nestin	Ki67	Cluster	Molecular subtype
C4	0	0	112	F	53	No	GBM	+	+++	Long	C
C9	1	0	69	M	74	No	GBM	+	NA	Short	M
C11	0	1	75	F	65	Yes	GBM	+	+++	Long	P
C12	1	0	20	F	60	Yes	GBM	+	+++	Short	C
C13	0	1	74	F	54	Yes	GBM	+	+++	Short	M/P
C15	1	1	207	M	61	Yes	GBM	NA	NA	Long	C
C16	1	1	48	M	43	Yes	GBM	+++	+++	Short	C/M
C17	1	0	14	F	79	Yes	GBM	+++	++	Short	N
C18	1	0	22	F	75	No	GBM	+++	+++	Short	N
C21	0	1	51	F	61	Yes	GBM	+	++	Long	N
C22	0	1	49	M	40	Yes	GBM	+++	+++	Long	N
C24	0	0	44	F	27	Yes	GBM	+	+++	Long	M
C25	1	0	10	M	75	No	GBM	+++	+++	Short	M
C27	1	0	5	F	85	Yes	GBM	+++	++	Long	M

Status: 0 signify alive and 1 deceased; Recurrence: 0 signify no recurrence and 1 recurrence; Gender: F = female, M = male; GBM = glioblastoma; Molecular subtype: C = classical; P = proneural; M = Mesencumal; N = neural

In order to consolidate the finding in the tissue cohort, two public databases were access. 146 glioma samples corresponding to newly diagnosed glioma cases with a minimum of 80 % tumour nuclei and a maximum of 50 % necrosis were obtained from TCGA on May 2014 (https://tcga-data.nci.nih.gov/tcga/, [274]). For a detailed description of the data see the TCGA Data Primer, available at https://wiki.nci.nih.gov/display/TCGA/TCGA+Data+Primer, as well as supplementary methods from McLendon et al. [274]. The TCGA data set for expression had already been processed and normalised (level 3). Gene expression profiles of 219 glioma samples were obtained from the Rembrandt database on May 2014 (https://caintegrator.nci.ni-h.gov/rembrandt/home.do/, [289]).

2 Tumour Tissue Processing

For the investigation into the regulation of FABP7 (Chapter 8), primary cell cultures were generated in order to investigate the gene expression. Primary cells were generated from glioma tissue by homogenising the tissue in a Medimachine (BD, Franklin Lakes, NJ, USA). Briefly, the tissue was rinsed in PBS, weighted and divided into smaller pieces. These small clumps of tissue

were processed piece by piece in a Medimachine for 1 minute together with Accutase (Millipore, Billerica, MA, USA). When all tissue was processed, the homogenised suspension was filtered through a 100 μm nylon cell strainer (BD, Franklin Lakes, NJ, USA). The cell suspension was centrifuged, and the red blood cells were lysed using an erythrocytes lysis buffer containing 155mM NH4Cl, 5.7mM K_2HPO_4 in 0.1mM EDTA-Na. Cells were counted and 5000 cells/cm^2 were seeded in Collagen Type I (BD, Franklin Lakes, NJ, USA) coated culture flasks.

3 Culture Conditions

For the investigation into the regulation of FABP7 (Chapter 8), immortalised cell lines were utilised in order to investigate the gene expression. The cell lines U251 and U87 were grown in Dulbecco's modification of Eagle's MEM (DMEM) (Lonza, Basel, Switzerland) supplemented with 10 % (v/v) foetal calf serum (Invitrogen, Carlsbad, CA, USA) and 1 % (v/v) penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA). Primary cell cultures were grown in DMEM-F12 (Lonza, Basel, Switzerland) supplemented with 10 % (v/v) foetal calf serum (Invitrogen, Carlsbad, CA, USA) and 1 % (v/v) penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA). Cells were cultured at 37 °C in a humidified atmosphere containing 5 % CO_2.

4 RNA Isolation and Quantification

Total RNA was isolated from fresh GBM tumour samples using the Aurum Total RNA Isolation kit (Bio-Rad, CA, USA) according to the manufacturer's instruction. Except for the first few initial steps, which are specific for the given starting material, the procedure share a common protocol and all centrifugations steps are performed at maximum speed at room temperature. Briefly, for cultured adherent cells, they were detached from the flask and transferred to a 2 ml capped microcentrifuge tube and centrifuged for 2 minutes. The supernatant were discarded and 350 μl lysis solution (supplemented with 1 % β-mercaptoethanol) were added to each tube. The solution was pipetted up and down several times in order to thoroughly lyse the cells. 350 μl of 70 % ethanol was then added to each tube and mixed thoroughly. For tissue, a small chunk was added to 700 μl lysis solution and the solution was homogenised for 30 to 60 seconds and thereafter centrifuged for 3 minutes. The supernatant was transferred to a new tube and 700 μl of 70 % ethanol was added and mixed thoroughly. After lysis, no matter the starting material, the lysate was added to a RNA binding column and centrifuged

for 30 seconds. The flow through was discarded and 700 μl low stringency wash buffer was added. The RNA binding columns were centrifuged for 30 seconds and the flow through discarded. 80 μl diluted DNase I was added to the membrane stack at the bottom of the column and incubated at room temperature for 30 minutes in order to allow digestion. 700 μl high stringency wash buffer was added and centrifuged for 30 seconds and the flow through discarded. 700 μl low stringency wash buffer was added and the columns were centrifuged for 1 minute and the flow through discarded. The RNA binding columns were centrifuged for an additional 2 minutes to allow for removal of residual wash buffer. Finally, 60 μl of the elute solution was added onto the membrane stack and allowed to incubate for 1 minute at room temperature. Thereafter, the RNA was eluted by centrifugation for 2 minutes. RNA concentration and quality were assessed by spectrophotometry using a NanoDrop (NanoDrop products, Thermo Scientific, DE, USA).

5 Quantitative Real-Time Polymerase Chain Reaction Analysis

To assess the expression of specific genes in regard to the regulation of FABP7 (Chapter 8), quantitative real-time polymerase chain reaction (qRT-PCR) was applied. This platform semi-quantify the relative expression levels through the aid of standard curves produced by a four-fold serial dilution derived from a pool of all cDNA samples. It uses a SYBRGreen dye, which binds double stranded DNA in a sequence independent manner. This therefore requires primers to be designed to be highly specific. All primers (Table 2.2) were designed using the open source software primer 3 with a relative high melting temperature (60 to 69 °C), to be approximately 20 base pairs of length with a GC content of minimum 45 % and produced by TAG Copenhagen (Copenhagen, Denmark) and all primer pair candidates were put through a primer optimisation to find the optimal annealing temperature.

Table 2.2: List of primers.

Gene	Forward	Reverse	Temperature
EGFR	GCAGCTGCCAAAAGTGTGAT	CAGATGATTTTGGTCAGTTTCTGGC	63°
FABP7	GGCGTGGGCTTTGCCACTAGG	TGTGCTGAGAGTCCTGATGACCAC	65°
NFIA	TGTCACAGACACCAATAGCTGCA	CTTTGTGGAGCTCGTAGAGGAT	60°
NFIB	GAATCCTCCAGGTTACCTTG	GTTATGGGCGTTCTGGATAC	60°
NFIC	TGCGTGAGCGAGATGCAGAG	TGGAAGTCGGTCGTGTCCAG	60°
NFIX	CCCAACGGGCACTTAAGTTT	GCAACAGGAGTCTGTGATAC	60°
PAX6	CCTGGAGAAAGAGTTTGAGAGA	AGAAAACCATACCTGTATTCTTGC	60°

For the qRT-PCR, 250 ng of RNA was reverse transcribed using the iScript cDNA synthesis kit (BioRad, Hercules, CA, USA) following the manufacturer's recommendations. PCR amplification was performed using 25 μl 1:50 dilution of cDNA as template. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis was done on a My-Cycler real-time PCR system (Bio-Rad, Hercules, CA, USA). The final PCR reaction mix included a final volume of 25 μl containing 0.188 μM of each primer, 12.5 μl cDNA using the SYBR Green PCR supermix (Bio-Rad, Hercules, CA, USA). The thermal cycling protocol involved initial denaturation at 95 °C for 3 minutes and was followed by 50 cycles of denaturation at 95 °C for 15 seconds and primer annealing and elongation for 30 seconds at a predetermined optimal temperature (Table 2.2). To test for specificity of the product, a melt curve function of the IQ5 Optical System Software 2.1 (Bio-Rad, Hercules, CA, USA) was applied. Furthermore, PCR runs producing standard curves with a correlation coefficient $R^2 > 0.98$ were preferred. For normalisation purposes, the geometric mean of two reference genes, cyclophilin A (PPIA) and tyrosine 3/Tryptophan 5-monooxygenase activation protein (YWHAZ), was used [100, 206].

6 Microarray and miRNA Arrays

Gene expression across all tumour samples included was done through microarray. This was done in an effort to assess the gene expression profile across multiple samples to evaluate the correlation between FABP7 and its transcription factors. HumanHT-12 V3 Expression BeadChip (Illumina, CA, USA) arrays were performed at AROS Applied Biotechnology, Aarhus, Denmark to investigate the expression of 34602 unique mRNA transcripts in the fourteen GBM tumour samples. The arrays were quantile normalised and evaluated with R using the Bioconducter [116] R-package lumi for beadarrays [81], and the expression of relevant target genes of differentially expressed miRNAs was determined [82]. In short, quantile normalisation is the effort of making the distribution of probe intensities equal across multiple arrays. This is based on the notion that two data vectors will produce a straight diagonal line in a quantile-quantile plot if they are equal. This is extended to n dimension in which case if all n data vectors are plotted in n dimensions it will give a straight line described by the unit vector $[\frac{1}{\sqrt{n}}, ..., \frac{1}{\sqrt{n}}]$. By projecting the points of the n dimensional quantile plot onto the diagonal it would make a set of data that has the same distribution. Let $\vec{q}_k = [q_{k1}, ..., q_{kn}]$ for $k = 1, ..., p$ be vector of the kth quantile for all n arrays and $\vec{d} = [\frac{1}{\sqrt{n}}, ..., \frac{1}{\sqrt{n}}]]$ be the unit diagonal, then the projection of $\vec{q}$ onto $\vec{d}$ is described by

$$\texttt{proj}_{\vec{d}}\,\vec{q}_k = \left[\frac{1}{n}\sum_{j=1}^{n} q_{kj}, \ldots, \frac{1}{n}\sum_{j=1}^{n} q_{kj}\right].$$

The equation is the equivalent of substituting the data in the original dataset with the value of the mean quantile, thereby generating arrays with the same distribution. The normalised data was clustered in order to find any similarities between the samples. Agglomerative hierarchical clustering was performed using Multiexperiment Viewer (MeV) from the TM suite of micro array tools [341], by using a Eucledian complete linking algorithm.

In order to investigate the expression of microRNA in the tumour samples generated, microRNA expression profiling was performed using the TaqMan Low Density Array Cards A and B (Life Technologies, NY, USA). These analyses were performed in collaboration with AROS Applied Biotechnology, Aarhus, Denmark. The cards contained assays for 750 mature human miRNAs present in the Sanger miRBase v19.0. The Ct value of an endogenous control gene (MammU6) was used for normalisation, and a cut off of 40 was set as threshold for the Ct value. In order to define miRNA patterns, hierarchical clustering identified clusters based on expressional similarity. First, the miRNAs with most varying levels of expression were identified based on median absolute deviation (MAD) with a cut off at the first quartile. MAD is a robust statistic and a measure of dispersion in univariate samples, which is more resilient to outliers than the standard deviation. A similarity matrix was then generated containing all pairwise similarities between samples. Finally, the Euclidean distance and complete linkage was applied to compute the similarities.

7 Western Blotting Analysis

Although gene analysis have revealed the level of transcription, that does not entail translation, wherefore western blotting was employed in order to get an idea of the protein expression level of FABP7. Cytoplasmic and nuclear extracts were prepared using Radioimmunoprecipitation assay (RIPA) buffer containing 0.1 % (v/v) Sodium dodecyl sulfate (SDS), 50 mM Tris-HCl (pH 7.4), 1 % (v/v) Igepal, 0.25 % (w/v) Sodium deoxycholate, 1 mM Ethylenediaminetetraacetic acid (EDTA), 150 mM Sodium chloride (NaCl), and one Complete Mini Protease Inhibitor Cocktail tablet (Roche Diagnostics, Indianapolis, IN, USA) per 10 ml total lysis solution. Protein concentrations were determined using the bicinchoninic acid assay (BCA) Protein Assay Reagent (Pierce; Thermo Science, Wilmington, DE, USA). 50 μg of total

protein extracts were heat denatured, electrophoresed through a 12 % (w/v) polyacrylamide-SDS gel and transferred onto a nitrocellulose membrane using the iBlot transfer apparatus (Invitrogen). Membranes were blocked using Phosphate-buffered saline (PBS) containing 5 % skimmed milk (Fluka; Sigma-Aldrich, St. Louis, MO, USA) and 0.5 % tween-20 (Sigma-Aldrich, St. Louis, MO, USA) and incubated at 4 °C overnight with 1 μg/ml rabbit polyclonal FABP7 antibody or mouse monoclonal β-actin antibody (1:5000) (Sigma-Aldrich, St. Louis, MO, USA; A1978). The FABP7 antibody was generated after immunisation by full-length recombinant protein in rabbits and isolating the serum (Eurogentec, Seraing, Belgium). The serum was affinity purified over a protein-A sepharose column (Pierce; Thermo Science, Wilmington, DE, USA). Membranes were then incubated at room temperature for half an hour with horseradish peroxidase-(HRP)-conjugated secondary polyclonal antibody either goat anti-rabbit (1:10000) (Santa Cruz Biotechnology, Inc., Dallas, Texas, USA; sc-2004) or rabbit anti-mouse (1:10000) (DAKO, Glostrup, Denmark; P0260). The antibody-antigen complex was visualised using enhanced chemiluminescence (Amersham ECL Plus; GE Healthcare, Chalfont St. Giles, UK). Signal acquisition was accomplished using a Kodak Image Station 4000 mm Pro (Carestream Health Denmark, Brøndby, Denmark). ImageJ (National Institutes of Health, Bethesda, MD, USA) was used to quantify the intensity of western blot bands, normalising to β-actin level.

8 Immunohistochemistry

The investigation of expression was also conducted in xenografted tumours. These were done to get a better understanding of the expression of FABP7 in a tumour microenvironment. An intracranial tumour xenograft model was established using the GBM stem cell line, C16 (generated in-house from resected GBM tumour tissue) as previously described. After tumour establishment for 21 days, the brains were removed and immediately immersed into 3.7 % paraformaldehyde for fixation. After 24 hours, the fixative was removed, and the brains were washed three times in Phosphate-buffered saline with potassium (8 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4, 0.2 g KH_2PO_4, pH 7.4-7.6 (KPBS)) followed by immersion into a 30 % sucrose solution. The brains remained immersed in sucrose for 48 hours. Immunohistochemical staining was performed on 40 μm sections of the brains. Briefly, all sections were blocked for 30 minutes in incubation buffer (3 % swine serum and 0.3 % Triton X-100 in KPBS) and incubated overnight with a primary antibody against FABP7 (Hycult Biotech, HP9029). After this incubation, the sections were washed and incubated with a biotinylated secondary antibody, followed by incubation with a peroxidase-conjugated avidin–biotin enzyme complex

(Vectastain Elite ABC System, Vector Labs, CA, USA) before reaction with 3,3'-diaminobenzidine (DAB). Afterwards, sections were mounted on slides and analysed by bright field microscopy.

9 Bioinformatics

In order to get an overview of the field of microRNA in relation to glioblastoma, survival, prognosis and progression (Chapter 4) a comprehensive literature search was conducted. As described in Henriksen et al. [145],

> 'a Medline database search on *"microRNA, glioblastoma, survival, prognosis and progression"* (typed: "(microRNA OR miRNA) AND (glioblastoma OR glioma) AND (survival OR prognosis OR progression)") was performed (date of last search entry: November 26, 2013). The results contained a total of 270 papers; 125 of these were chosen based on title and abstract content. Of the remaining, 100 papers were cell culture studies and 45 were reviews or review-like and were therefore excluded. A total of 125 papers were reviewed for miRNA expression level in GBM correlated to survival and/or progression, 25 involved database studies, and 35 contained studies on GBM tissue (not database-derived). The miRNA profiles, often presented in the form of signatures, were extracted from the papers. This review summarises the studies investigating miRNAs in GBM and explores their correlation to clinical outcome and highlights the functional characteristics of the miRNAs linked to protection (i.e., longer survival) or risk (i.e., shorter survival). The miRNAs that are included in the signature of more than one study and involved in the progression of GBM have been identified, and their functional role, if known, is discussed.' (Henriksen et al. [145])

In order to link microRNAs to clinicopathological features (Chapter 5) text data mining of functional characteristics of miRNA target interactions was downloaded through miRTarBase (accessed January 25, 2014) [162]. Targets for selected miRNAs were examined for correlation with the miRNA clustering (Mann–Whitney U test; $p < 0.05$), where only targets differentially expressed were considered. These were further investigated for connection to GBM through various literature databases using combinations of keywords including: *"gene name of target," "gene nomenclature of target," "glioblastoma multiforme," "GBM"* and *"glioma."* Gene nomenclature was used according to the HUGO Gene Nomenclature Committee at the European Bioinformatics Institute (`www.genenames.org`). Furthermore, a systematic review of all

known mRNA targets of miRNAs in GBM was consulted [282].

A number of previous studies have provided miRNA signatures associated with survival or progression in GBM and reported that individual miRNAs of these signatures could be regarded as either protective or risk-associated, most often based on determination of the hazard ratio (reviewed in Henriksen et al. [145]). In addition, the miRNAs were analysed for any modulating properties on the MMMI [282, 474]. The miRNAs determined in this study were analysed with respect to miRNAs known to be in these two categories, and dysregulation of known validated targets mRNAs were analysed in the microarray analysis data. The presence of exosome-packaging-associated motifs (EXO-motif) was investigated in all differentially expressed miRNAs by analysing all mature sequences for their content of four individual EXO-motifs (GGAG, UGAG, CCCU and UCCU), as recently reported in Villarroya-Beltri et al. [411].

In order to gain a complete overview of the literature with respect to regulation of FABP7 (Chapter 7), a comprehensive compilation of the literature on FABP7 promoter and transcriptional regulation was conducted, integrating data from man and rodents. To this end, iHOP (`http://www.ihop-net.org/`; [153]), OMIM (`http://www.omim.org/`; [273]) and UniProt (`http://www.uniprot.org/`; [392]) databases were instrumental. This literature review was complemented with a survey of a dedicated meta-database with author-curated information the Transcription Factor Encyclopaedia [460] and from results produced by The Encyclopedia of DNA Elements (ENCODE, `http://www.genome.gov/10005107`; `http://genome.ucsc.edu/ENCODE/`).

ENCODE is a project launched and coordinated by the National Human Genome Research Institute which is aimed at identifying the functional elements in the human genome, using various human cell lines and state-of-the-art analytical methods like chromatin immunoprecipation (ChIP) followed by DNA sequencing (ChIP-Seq) in the case of TF binding sites [391]. At the time of our survey, 56 cell lines and 161 transcription factors were included in ENCODE. Investigation of the human FABP7 gene using UCSC Genome Browser (http://genome.ucsc.edu/, [192]) on genome assembly version GRCh37/hg19, revealed several additional DNA binding proteins. Here, we will only discuss TFs identified by ENCODE showing multiple interaction sites or with a signal higher than 0.5 (an index between 0 and 1 corresponding to the relative level of enrichment for the TF with respect to the highest signal monitored in the cell line analysed). TFs which have been shown experimentally to regulate FABP7 transcription will be presented first, and TFs discovered by the ENCODE project will be presented afterwards as data showing their regulatory effect on FABP7 transcription is currently not available. However, the functions of these new TFs will be discussed in the context

of brain biology, development and neoplastic pathology, as they might shed additional light on FABP7 regulation.

10 Statistics

Prior to clustering, the most differentially expressed miRNAs across all tumour samples were identified using the MAD. The non-parametric Mann–Whitney U test in TM4 MultiExperiment Viewer, determined statistical significance of aberrantly expressed miRNAs between the two clusters. Survival analyses were based on Kaplan–Meier plot with univariate testing performed by means of the log-rank test and Fisher's Exact test using GraphPad Prism 5. A p value <0.05 was considered as significant.

Statistical significance of differences in expression between two groups was determined by the non-parametric Mann-Whitney U test using Multiexperiment Viewer (MeV) from the TM suite of micro array tools [341], while for three groups Kruskal-Wallis was performed in SPSS (version 22). Pearson correlation was used to correlate expressions between two genes and Fisher's Exact test was used to correlated expression of transcription factor and cofactor with expression of FABP7, both using SPSS (version 22). A p value of < 0.05 was considered as significant.

PART I
MicroRNA in Glioblastoma

3

Over a decade ago, the human genome-sequencing consortium [217] released a final draft of the human genome. This sparked the characterisation of the human transcriptome in resent years due to novel techniques, which has uncovered an unforeseen finding. Opposed to previously believed, a considerable wider part of the human genome is ubiquitously transcribed with up to 70 % being transcribed while only 2 % is translate into proteins [22, 39, 54, 89, 186]. While genetic alteration within the coding sequences has been demonstrated to affect cell development and carcinogenesis, expanding our understanding of this severe disease, emerging evidence reveal that alterations within the non-coding sequences likewise contributes to carcinogenesis [272].

1 The Essential of MicroRNA

A family of these non-coding RNA molecules are microRNAs (miRNAs), which are small, approximately 22 nucleotides in length, single-stranded and endogenous molecules. They are considered interfering, due to their ability to bind within the 3′ untranslated regions (3′UTRs) of mRNA transcripts leading to a lowered post-transcriptional gene expression [17, 213]. A single miRNA is able to target several different gene transcripts since perfect complementarity between the miRNA and its mRNA target is not required [303, 394]. They are considered as micro modulators of various processes in the normal cellular homeostasis, thus their dysregulation have been linked to several pathological conditions, like autoimmune, cardiovascular and neurodegenerative diseases, along with most cancer types [1, 37, 267, 410, 477]. Furthermore, there has recently been a great scientific effort in comparing

aberrant expression of miRNAs in pathological specimens or biofluids to non-pathological samples [423]. These efforts have provided interesting perspectives in terms of novel diagnostic and prognostic approaches based on miRNAs, and is the first step in uncovering the role of individual miRNAs in the context of different diseases, eventually leading the way for the development of novel miRNA-based therapies or miRNA-based biomarkers of disease.

2 The Biogenesis of MicroRNA

The biogenesis of miRNA is initiated by the transcription of miRNA genes by RNA polymerase II/III, giving rise to the primary transcript, pri-miRNA, which is characterised by polyadenylation and capping (Figure 3.1). Due to intrastrand base-pairing, the pri-miRNA transcript folds into a hairpin loop structure [438]. The loop structure of the pri-miRNA is next endonucleolytically cleaved inside the cell nucleus by a micro-processor complex formed by the RNase III enzyme Drosha and the DiGeorge critical region 8 (DGCR8), also known as Pasha (Partner of Drosha) in *D. melanogaster* og *C. elegans*. The resulting pre-miRNA is transported out of the cell nucleus by Exportin-5 in a Ran-GTP dependent manner [458]. Once in the cell cytoplasm, the RNAse III enzyme known as Dicer cleaves the pre-miRNA aided by the double-stranded RNA-binding domain proteins Tar RNA binding protein (TRBP) and protein activator of PKR (PACT) and the core component Argonaut protein 2 (Ago2) leading to a miRNA duplex, of which only one strand (called the guide-strand) is incorporated into the RNA-induced silencing complex (RISC), the cytoplasmic effector machine of miRNA. The other strand, termed passenger-strand, is subsequently degraded, though in some cases the passenger-strand is incorporated into a RISC of its own thus remaining functional. The choice of which strand that is incorporated into the RISC is based on the asymmetry in the miRNA duplex [347] or the thermodynamic stability of the two strands, where the strand with least thermodynamic stability in its 5′ end will be incorporated into the RISC [194]. Post-transcriptional mRNA-silencing is achieved mostly via imperfect complementarity binding between the miRNA strand with associated RISC accessory proteins, and the target mRNA, resulting in translational inhibition [437]. Interestingly, miRNAs are also secreted into the extracellular space and plasma via lipoproteins, exosomes and micro vesicles, hereby participating in the intercellular communication. This is important in relation to the nature of sampling material, because miRNA patterns of circulating micro particles might be a useful diagnostic and/or prognostic marker of on-going pathological processes [53, 376].

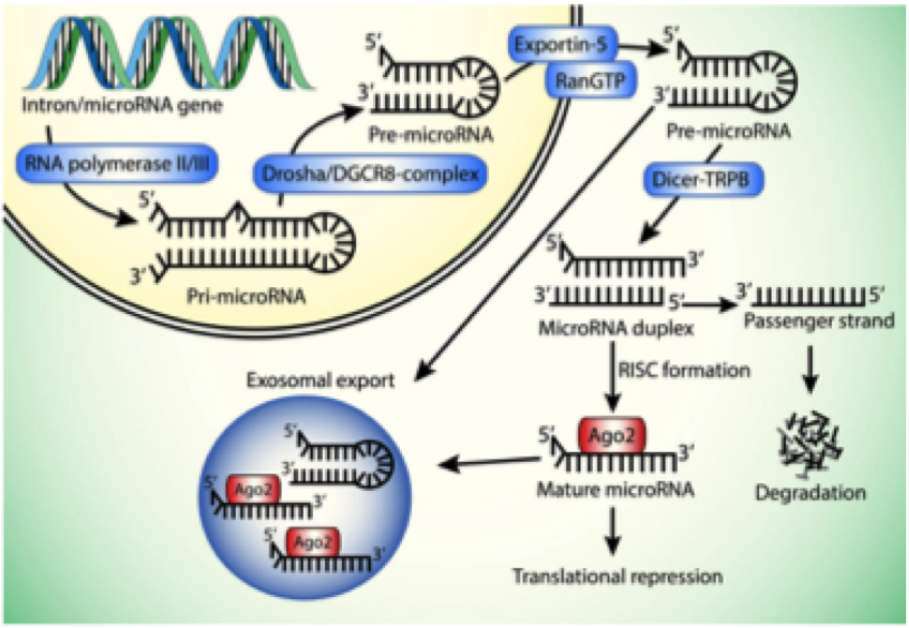

Fig. 3.1: The biogenesis of miRNA requires RNA polymerase II/III for the transcription of pri-miRNA. The pri-miRNA product is then cleaved by the Drosha-DGCR8 complex into pre-miRNA. The pre-miRNA is exported to the cytoplasm by Exportin-5 in the presence of Ran-GTP co-factor. Once in the cytoplasm, the pre-miRNA is cleaved by the Dicer-TRBP complex into a miRNA duplex, which is unwound into two products: a guide strand bound to Ago2, which is incorporated into the RISC, and a passenger strand, which is degraded. Finally, the miRNA binds to its target mRNAs resulting in mRNA target cleavage, translational repression, or mRNA decay. A more novel fate of the miRNAs is the selective secretion via microvesicles or exosomes. Ran = Ras-related nuclear protein ; GTP = guanosine-5'-triphosphate ; TRBP = TAR (HIV-1) RNA binding protein ; Ago2 = Argonaute protein 2 ; RISC = RNA-induced silencing complex. From Henriksen et al. [145].

3 The Expression of MicroRNA in Glioblastoma Multiforme

As mentioned elsewhere:

> '[Presently, nearly every analysis of tumour tissue] ... by miRNA profiling has ... [revealed] a distinct miRNA ... [profile in comparison] to normal tissue [254], ... [which] can be further associated with prognostic factors and disease progression [38, 384, 454]. ... The [vast] number of studies pertaining to miRNA expression and functional characterisation [in GBM] has grown and miRNA signatures are refining ... [the] classification

[of GBM], differentiating between the different grades and stages, providing key regulatory links to disrupted signalling pathways such as those facilitating cell growth ... [, which] has lead to a more in depth understanding about GBM pathology [197].

[Previous studies have shown that expression of miRNA] in tumour samples seem lower, and this could be a function of cellular differentiation status [254, 415]. [Based on our systematic literature review [282]] it appears that the most common dysregulation of miRNA in GBM is observed to be overexpression. ... For example, miR-17, miR-21, miR-93, miR-221 and miR-222 have been intensively investigated with respect to both their expression and functionality, but the functional properties of the vast majority remain completely unknown.

The most extensively investigated miRNA is miR-21, which is consistently reported to be overexpressed in GBM in a grade-specific manner [44, 48, 57, 60, 62, 79, 109, 114, 126, 168, 211, 212, 215, 221, 234, 239, 240, 268, 310, 328, 333, 334, 343, 346, 355, 361, 447, 462, 473, 475, 476]. At least for GBM, miR-21 appears to be the major anti-apoptotic and pro-survival factor that is linked to shorter progression-free survival [46, 215, 325].

Expression ... [profiles] of miRNA in patient tissue and investigation of their putative function using in vitro primary cultures and in vivo studies have provided an insight not only into the genes that are regulated by respective miRNA, but also the pathways that are disrupted, many of which are hallmarks of GBM biology (reviewed by Lakomy et al. [215]). The pattern of miRNA expression, whether it is up- or downregulated, is now becoming a recognised tool in addition to gene expression profiling to stratify GBM patients into different groups [197]. Here, the miRNA cohorts are smaller and miRNA signatures pertaining to overall or progression-free survival are starting to evolve, albeit they are still very much dependent on the individual patient history, tumour size, age and treatment regimen.' (Henriksen et al. [145])

4 Overall and Progression-Free Survival as Clinical Endpoints in Glioblastoma Multiforme

As previously described:

'in the literature, both overall survival (OS) and progression-free

survival (PFS) are widely used end points to assess the predictive factor of a given miRNA signature; however, the two terms do not provide equal information [73, 323, 331]. When evaluating a treatment response, OS is used as a measure of the end result including the complete disease history and possible other factors affecting the lifespan. The PFS is more specific in its measure of the effect of a specific treatment in the form of tumour control or radiographic response. Reviewing the literature and trying to draw conclusions are therefore challenging when both OS and PFS are applied [56]. The response assessment criteria for GBM has been developed over the course of several decades as a result of technology advances in imaging and expanded knowledge on tumour biology. Before 1990, the Levin and WHO Oncology Response Criteria, which primarily was based on contrast-enhanced computer tomography, was the standard assessment methods [230]. These were substituted by the standardised McDonald Criteria, which took into account that contrast enhancement could be affected by clinical factors such as the use of corticosteroids [266]. The McDonald Criteria incorporated the clinical assessment (neurology status) of the patient in the designation of response to therapy as being a complete response, partial response, or stable or progressive disease. With the arrival of magnetic resonance imaging and new therapeutic options, the response assessment criteria was developed further and standardised with regard to all aspects of imaging, timing, and evaluation techniques. Especially the introduction of bevacizumab, a monoclonal antibody targeting VEGF-A and a resulting increased risk of pseudoprogression interpreted as disease progression stimulated the modification and lead to the Revised Assessment in Neuro-Oncology (RANO) Criteria in 2010 [33, 55, 107, 171]. Because of this development over the last twenty years, caution should be taken in the comparison of particular PFS data.' (Henriksen et al. [145])

4

MicroRNA Expression Signatures Determine Prognosis and Survival in Glioblastoma Multiforme - A Systematic Overview

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Determination of disease prognosis is most often based on histological classification combined with information on patient age and tumour size and location. These factors have all been defined as indicators of patient survival and treatment outcome, but due to the sustained poor overall survival of GBM patients, new arrays of prognostic indicators have been requested to aid in the clinical decision making [246]. In recent years, several molecular biomarkers have been characterised including chromosomal aberrations, methylation status of the methyl guanine methyl transferase (MGMT) promoter, mutations in important genes (isocitrate dehydrogenase 1 (IDH1), EGFR, and p53), and dysregulation of miRNAs [49]. LOH in chromosomes 9p and 10q is associated with decreased survival, while co-deletion of 1p and 19q correlates with better treatment response and longer survival [49]. Hypermethylation of the MGMT promoter leads to lower expression levels of MGMT, which sensitizes GBM tumours to chemotherapeutic treatment and thus is associated with a significantly better patient outcome [94, 142]. Improvement of the disease condition is also observed in patients with mutation in the IDH1 gene, which is most often found in secondary GBM. Furthermore, the expressional profile of specific microRNA signatures also correlates with overall survival, time to progression and treatment success [215, 429, 453].

Based on studies derived from clinical data in databases and independent tissue cohorts where smaller samples sizes were investigated, miRNA associated to longer survival (protective) and miRNA with shorter survival (risk-associated) have been identified and their signatures based on different prognostic attributes are described.

Here, these studies are stratified into those based on publicly available databases and those conducted on independent tissue cohorts. These data sets have been extensively reviewed and combined to derive a signature or pattern of miRNAs, which has a prognostic potential. The miRNAs reported to have a protective or risk-associated profile have been highlighted in relation to GBM. Finally, the studies that have reported a miRNA signature with respect to prognosis have been compared to find common miRNA profile across the different studies.

1 Prognostic MicroRNA Signatures in Glioblastoma Multiforme

1.1 MicroRNA Signatures Derived from Database Mining

A total of 25 studies were based on database entries. For the individual studies, the database accessed, cohort size, cohort factor and normalisation methodology along with miRNA signature were documented (Table 4.1). The majority of the studies used TCGA (cancergenome.nih.gov). However, four studies used the Chinese Glioma Genome Atlas (CGGA) (www.cgga.org.cn), which uses the Illumina Human v2.0 miRNA Expression BeadChip microarray platform [265, 388, 429, 445]. The study by Ma et al. [265] evaluated two large cohorts of data, the CGGA with 198 samples containing low-grade gliomas and 91 GBMs and an additional cohort of 128 samples, with low-grade gliomas and 68 GBMs to validate the array data. High expression of miR-196b was conferring poor prognosis when stratifying the patients into high miR-196b expression and low miR-196b expression groups. Following a similar experimental setup, Wu et al. [445] looked at 91 GBM patients taken from the array data and validated their findings in a cohort of 60 GBM patients. Here, they focused on miR-328, showing that a low level of expression was conferring poor prognosis. The TCGA dataset has also been used for developing a new method for predicting the outcome based on miRNA expression; however, only one of the studies provided the miRNA identified [241].

While a few studies gave rise to a defined multiple-miRNA signature, eight of the studies looked at a single miRNA. For example, two studies evaluated the functionally well-characterised miRNA, miR-10b. Gabriely et al. [110] showed that miR-10b was expressed in GBM tissue while not present in normal brain tissues. Using TCGA data, they investigated the association between the expression of miR-10b and clinical outcome and found that miR-10b correlated with survival although with stratified conditions, the association was insignificant. When the correlation with survival for miR-10b was assessed together with miR-10a, however, the association with survival was significant regardless of stratification; hence, high levels of miR-10 conferred poor survival. [128] also found a correlation between high levels of miR-10b and poor survival by analysing the TCGA data and further reported on a functional role of miR-10b in GBM stem cells.

Since the majority of the studies use the TCGA dataset in analysing the expression of miRNAs in GBM, the platform for generating the data was the same. The only differences seen were in the downstream analysis, other clinically prognostic factors, and the type of filtering applied. Expression analysis was conducted on Agilent 8 $\times$ 15 K Human miRNA microarrays, with data available at four levels. The first level is the raw non-normalised data from the array (level 1), and the second level (level 2) is the processed normalised signal. The third level (level 3) is the segmented data, assemblies of the pro-

Table 4.1: Studies performed on dataset obtained from public databases. For each study, the cohort size, the database utilized, the date of accession, and the cohort factor of investigation are stated. The type of normalization used and the levels of data are described; level 1 is the raw data, level 2 or 3 are normalized data from TCGA. Finally, the number of miRNAs investigated is reported. VAK = Volume, Age, Karnofsky performance score; MGMT = methyl guanine methyl transferase; TMZ = temozolomide; EGFR = epidermal growth factor receptor; IDH1 = isocitrate dehydrogenase 1; FOS = FBJ murine osteosarcoma viral oncogene homolog; GBM = glioblastoma multiforme

Reference	Cohort size	Database	Accessed	Cohort factor	Normalisation	Validation	No. of miRNAs studied
Bozdag et al. [32]	385	TCGA	Jul, 2011	Age-specific signature	Level 3		19
Dai et al. [66]	465	TCGA				In vitro tissue	1
Delfino et al. [73]	253	TCGA	Dec, 2009	miRNA biomarkers of glioblastoma survival	Quantile normalised, collapsed within microRNA, and log2 transformed		45
Gabriely et al. [110]	261	TCGA			Level 2	In vitro tissue	1
Genovese et al. [115]	290	TCGA			Level 3	In vitro xenografting	8
Guessous et al. [128]		TCGA				In vitro tissue	1
Haapa-Paananen et al. [130]	308	TCGA			Level 3	Used to validate signature found in cell culture	8
Hua et al. [163]	580	TCGA		Antagonistic activity on cell proliferation and "stemness"			12
Kim et al. [197]	261	TCGA		Classification	Level 3 - Mean centered, and the STD was normalised to 1 per array		121
Lee et al. [224]	491	TCGA	Sep, 2011		Level 3	In vitro tissue	1
Li et al. [241]	371	TCGA		Method development		Tissue	5
Ma et al. [265]	198	CGGA		Assess prognostic value		Tissue	1
Qiu et al. [321]	480	TCGA			Level 3	In vitro tissue	1

Qiu et al. [322]	480	TCGA	Jul, 2013	Signature for GBM survival	Level 3		6
Srinivasan et al. [374]	222	TCGA	Jul, 2010	Signature for GBM survival	Level 1 - Quantile normalised and log2 transformed		10
Suzuki et al. [383]	478	TCGA	Mar, 2012	Method development	Level 3 - Mean centered, and the STD was normalised to 1 per array	Divided into a training set and a testing set	
Tao et al. [388]	220	CGGA		FOS expression	Illumina BeadStudio Data Analysis Software	Tissue, In vitro xenografting	2
Wang et al. [429]	170	TCGA		IDH1 mutation signature	Level 3		23
Wang et al. [428]	198	CGGA				In vitro tissue	1
Wu et al. [445]	198	CGGA		Grade specific miRNAs		Tissue	1
Xiao et al. [452]	378	TCGA		miRNA-mRNA modules			11
Yin et al. [459]	188	TCGA		EGFR amplification		In vitro tissue	1
Zhang et al. [468]	424	TCGA	Feb, 2011	TMZ and MGMT	Level 3	In vitro tissue	9
Zhang et al. [469]	345	TCGA		Signature for GBM survival		Tissue	5
Zinn et al. [480]	255	TCGA	Oct, 2011	VAK classification	Level 2 - Multi array algoritm	No	8

cessed data from single samples, and grouped by probed loci to form larger contiguous regions. The fourth level (level 4) is the summary, a quantified association across classes of samples and associations based on molecular abnormalities, sample characteristics and/or clinical variability. Not all studies state the level of data they use, but most use the third level. Only one study, looking at a ten-miRNA signature, used level 1 and quantile-normalised the expression data. Here, they segregated patients in to high- and low-risk groups and identified seven miRNAs associated with high risk of disease progression and three miRNAs that were found to be protective [374]. A more elaborate study by Delfino et al. [73] used quantile-normalised data (although it does not specify the level of the data set analysed) and identified 45 miRNAs in the TCGA data across race, gender, recurrence, and therapy linked to survival. Using level 2 data with multiarray algorithm normalisation, Zinn et al. [480] looked at 78 patients and included a Volume (tumour volume), Age, and Karnofsky Performance Score (VAK) classification to dichotomize the patients into VAK A (good prognosis) and VAK B (poor prognosis). A total of five miRNAs were associated with short-term survival (miR-566, miR-505, miR-345, miR-484, and miR-92b), and three miRNAs were associated with long-term survival (miR-511, miR-369-3p, and miR-655).

Though the normalisation was not standard for all studies along with the variation in cohort size, most of them used normalised data from the TCGA database and therefore had the same material. The cohort size, however, ranges from 170 to 580 (mean = 329 $\pm$ 121) and is a function of the cohort factors that are investigated. For example, factors such as age, grade, MGMT methylation, chemotherapy regimen, IDH1 mutation, or grade subclassification are just some examples of where the investigation is based on prior knowledge of clinical data correlated with expression and survival [32, 197, 429, 445, 468]. In addition to the TCGA, a number of the studies have used independent GBM tissue validation cohorts for identifying differentially expressed miRNAs with respect to cohort factors [265, 445, 469].

2 MicroRNA Signatures Derived from Independent Tissue Cohorts

The studies performed on independent sample sets can generally be characterised as being validation of database findings, validation of literature findings, or novel array-based determination of miRNA profiles of clinical interest in GBM (Table 4.2). A total of 35 studies identified miRNA signatures associated to survival, and many of these have used tissues to validate signatures previously found in datasets described in the last section and con-

tained in Table 4.1. The majority of studies (n = 30) use PCR-based methods when validating miRNA expression, while several studies use different types of arrays. The PCR-based methods require normalisation, and most of the studies use RNU6B, though there are a few studies that use others, such as hsa-miR-16 or RNU5A [326, 473].

The starting material used in the studies was either tissue or formalin-fixed paraffin-embedded (FFPE) tissue with a variable cohort size (min = 12, max = 253, mean = 91 $\pm$ 59). With regard to sample preparation, de Biase et al. [70] have shown that there is no difference in the miRNA expression obtained from tissue and FFPE tissue, and some studies also use both types to validate their findings. Twenty-five studies focused on single-miRNA candidates, while the remainder focused on expression profiles of several miRNAs (min = 1, max = 30, mean = 3 $\pm$ 5).

While most of the studies focus on smaller miRNAs signatures, Niyazi et al. [291] present a larger cohort of miRNA as a putative survival signature. They used a top-down approach, where they filtered the miRNAs based on the variance in expression across the samples and chose the 30 most dysregulated miRNAs. These miRNAs were used to stratify the samples into two patterns, which correlated with short- and long-term survival. This approach was also applied in several database studies, limiting the number of miRNAs down to a specific signature [241, 468, 469, 473]. Others looked at pre-selected miRNAs already linked to GBM pathogenesis in the literature [173, 326, 463]. Zhang et al. [463] found that miR-221 and miR-222 expression was significantly increased in high-grade gliomas compared with low grade, positively correlated with degree of glioma infiltration. This corresponded well to the fact that overexpression of miR-221 and miR-222 increased cell invasion [215]. In addition, Quintavalle et al. [326]showed that miR-221 and miR-222 were upregulated in GBM patients and that they target MGMT mRNA thereby inducing greater temozolomide-mediated cell death.

Of all the studies, twelve of them utilised both databases and independent tissue cohorts. They all link an expression of one or more miRNAs to survival; however, some studies categorise a given miRNA to be protective or risk-associated. Three studies used hazard ratio to assess whether a specific miRNA was protective or risk-associated, while Wang et al. [429] used a Significance Analysis of Microarray (SAM) and Li et al. [241] used the Cox-regression coefficient to designate the miRNAs [241, 374, 429, 468]. Interestingly, large variations can be found in the choice of control tissues across the individual studies, ranging from purchased RNA from normal brains to tissues from epilepsy patients or patients with cerebral trauma. Such differences in control tissues might also be a factor in the incoherency between the miRNA signatures found in the different studies (Table 4.2). In addition,

Table 4.2: Studies performed on independent tissue cohorts. For each study, the cohort factor of investigation, the cohort size, the methodology and the choice of control tissue are stated. The method of normalisation, whether it is validated, and the number of miRNAs reported in the study are described. TMZ = temozolomide; MGMT = methyl guanine methyl transferase; EGFR = epidermal growth factor receptor; RT-qPCR = real-time quantitative polymerase chain reaction; GBM = glioblastoma multiforme; AVM = arteriovenous malformation

Reference	Cohort factor	Cohort size	Method	Control	Normalisation	Validation	No. of miRNAs studied
Chang et al. [45]		128	RT-qPCR	10 × non-neoplastic brain tissue	RNU6B		1
Chen et al. [50]		43	In situ hybridisation RT-qPCR	Normal brain tissue		*In vitro*	1
Dai et al. [66]		19	RT-qPCR	3 × severe traumatic brain injury		In vitro	1
Gabriely et al. [110]			RT-qPCR	Normal brain tissue		In vitro xenografting	1
Gao et al. [113]		151	RT-qPCR	15 × severe traumatic brain injury	RNU6B		1
Guan et al. [126]	Grade specific miRNAs	92	PCR array (TaqMan Human miRNA array v1.0 (PE Applied Biosystems)) RT-qPCR	1 × epilepsy and 1 × no tumour	RNU44 and RNU48		1
Guessous et al. [128]		20	RT-qPCR	5 × normal brain tissue	RNU6B	In vitro	1
He et al. [139]		112	RT-qPCR	10 × non-neoplastic brain tissue from decompressive craniectomy after brain injury	RNU6B		1
Hermansen et al. [149]		193	In situ hybridisation	Not described	RNU6B		1

Hou et al. [161]		102	RT-qPCR	20 × non-neoplastic brain tissue from de-compressive craniectomy after suffering brain injury	RNU6B		1
Ilhan-Mutlu et al. [173]	Progression - compare paired primary and secondary	15	RT-qPCR	3 × epilepsy	RNU6B		7
Jiang et al. [179]		253	RT-qPCR and In situ hybridisation	3 × died in traffic accident	RNU6B		1
Jiang et al. [178]		166	RT-qPCR	10 × non-neoplastic brain tissue from de-compressive craniectomy after brain injury	RNU6B		1
Lakomy et al. [215]	Methylation and TMZ	38	RT-qPCR	6 × normal AVM and commercial RNA from adult brain			8
Lee et al. [224]			RT-qPCR	Non-neoplastic brain tissue	RNU6B	In vitro	1
Li et al. [241]	Method develop-ment	160	Numan v2.0 miRNA ex-pression BeadChip	Not decribed	Log transform		5
Li et al. [235]		128	RT-qPCR	Paired adjacent non-neoplastic brain tissue	RNU6B		1
Lu et al. [255]		108	RT-qPCR	20 × dead from traffic accident	RNU6B		1
Lu et al. [256]		108	RT-qPCR	20 × dead from traffic accident	RNU6B		1
Ma et al. [265]	Assess prognostic value	128	RT-qPCR	Not decribed			1

Niyazi et al. [291]	Signature for GBM survival	35	Biochip "Geniom Biochio MPEA homo sapiens" (Febit)	Not described			30
Qiu et al. [321]		25	RT-qPCR	14 × non-neoplastic brain tissue	RNU6B	In vitro	1
Quintavalle et al. [326]	MGMT and common classification	34	RT-qPCR	Not described	RNU5A, β-actin		2
Speranza et al. [373]	NEDD expression		RT-qPCR	Not described	RNU6B	Proliferation and invasion assay, transfection	1
Sun et al. [381]		168	RT-qPCR, Taqman miRNA array	21 × cerebral trauma samples	RNU6B		1
Tao et al. [388]	FOS expression	50	In situ hybridisation	3 × normal brain	RNU6B	In vitro xenografting	2
Wang et al. [424]		108	RT-qPCR	20 × normal controls no pathological lesions	RNU6B		1
Wang et al. [428]		30	RT-qPCR	Severe traumatic brain injury		In vitro xenografting	1
Wu et al. [445]	Grade-specific miRNAs	100	RT-qPCR	Not described	RNU6B	Validation set	1
Wu et al. [446]		128	RT-qPCR, Taqman miRNA array	10 × cerebral trauma samples	RNU6B		1
Yin et al. [459]	EGFR amplification	55	RT-qPCR, SNP-chip analysis	Not described	RNU48	Transfection, proliferation, migration, luciferase assay	1
Zhang et al. [463]		50, 22	In situ hybridisation, RT-qPCR	Not described	RNU6B	Transwell assay, wound healing assay, transfection, xenografting	2
Zhang et al. [468]	TMZ and MGMT	82	Illumina Human v2.0 miRNA Expression BeadChip	Not described			9

Zhang et al. [469]	Signature for GBM survival	117	Illumina Human v2.0 miRNA Expression BeadChip	Not described	Average expression	Validation set	5
Zhi et al. [473]	Signature for GBM survival	124	RT-qPCR	60 × normal adjacent tissue	hsa-miR-16	Split into training and validation set	3

only few studies specify their use of the terms OS and PFS, which also makes direct comparison difficult.

3 MicroRNA Reported to be Protective or Risk-Associated

3.1 Clinically Protective MicroRNAs

In the group of the protective miRNAs (n = 22), only two miRNAs, miR-544 and miR-1227, have not been described previously in relation to miRNA alterations in GBM pathogenesis. Eleven of the protective miRNAs were significantly increased in studies comparing GBM specimens to normal brain tissue, while only six were significantly downregulated. Surprisingly, three of these miRNAs are well described as miRNAs with an oncogenic potential and have several validated targets considered to be tumour suppressor genes. This includes the extensively investigated miR-17-5p, which in vitro has been shown to increase angiogenesis and growth when overexpressed and decrease viability and proliferation when inhibited, making it unlikely that this miRNA, at least solely, should be considered protective [75]. As to miR-19a, miR-19b, and miR-106a, there is currently an inconsistency in the literature regarding their role in GBM development. The functional data available on miR-106a shows that overexpression by transfection of GBM cell lines causes a significant decrease in proliferation and an increase in apoptosis, likely mediated by the suppression of E2F1, supporting the notion of it being tumour suppressive [455]. More in line with what would be expected, miRNAs with previously investigated tumour suppressive capabilities are present on the list whereby miR-128a and miR-181d are most notable. miR-128 has been investigated in thirteen studies demonstrating its wide range of oncogenic mRNA targets and its ability to inhibit angiogenesis and proliferation and even to significantly decrease total tumour volume in vivo [57, 63, 119, 212, 215, 221, 234, 328, 358, 414, 447, 462, 470, 475]. Similarly, although, less extensively investigated is miR-181d, which has been shown to target the oncogenes Bcl-2 and K-Ras whereby apoptosis is increased and proliferation decreased. miR-181d transfection is demonstrated to decrease in vivo tumour size and has been shown to increase the susceptibility to the chemotherapeutic agent, temozolomide [207, 427].

3.2 Risk-Associated MicroRNAs

Within the cohort of miRNAs described as risk-associated (n = 22), nine have not been previously associated with miRNA modulation in GBM. Of the thirteen miRNAs mentioned in the literature, ten are overexpressed in GBM specimen, three are underexpressed, and four have been functionally characterised. miR-34a is well studied in numerous GBM cell lines and shown to increase cell differentiation and decrease total tumour volume in a xenograft mouse model of GBM [127, 459]. The less investigated miR-146b is similarly known to decrease in vitro invasiveness, migration, proliferation, and tumour volume in mice [189, 449]. Both miR-34a and miR-146b are, in terms of isolated functional characteristics, not associated with risk of GBM progression (Table 4.3). The oncogenic miRNAs, miR-221 and miR-222, clinically associated with risk, have been studied in relation to a diverse list of cancers including GBM. They inhibit a number of common gene targets such as PUMA and P57 both involved in apoptosis. When overexpressed in vitro, both miR-221 and miR-222 potentiate classic cancer hallmarks, i.e., proliferation, angiogenesis, and invasion. In vivo studies have revealed that miR-221 or miR-222 overexpression is associated with increased tumour growth, a situation that can be reversed with administration of corresponding antagomirs [275, 464].

Out of 44 miRNAs reported to be protective or risk-associated, only eight were not previously described as significantly modulated in GBM samples. This demonstrates a relatively broad coverage in terms of the miRNAs investigated purely to assess miRNA modulation in GBM pathogenesis without correlating the data to clinical outcome (Table 4.3). No general patterns apply to these cohorts of protective and risk- associated miRNAs, as such, several miRNAs, which are described as oncogenic from a functional standpoint, are present within the cohort of protective miRNAs and vice versa. This comparison between in vitro functionality and clinical implication of GBM-related miRNAs illustrates that although a specific miRNA may have a specific set of functional characteristics when artificially over or underexpressed in isolated in vitro models, this is not necessarily a good indicator for the multifactorial clinical progression of GBM. For more elaborate details of the functional characterisation of miRNAs involved in GBM, please refer to [282].

4 MicroRNAs Included in Several Signatures

Based on the multiple-miRNA signatures identified from both tissue and database studies pertaining to survival, the miRNAs found in multiple studies were identified (Table 4.4). Most of the miRNA found in signatures are

Table 4.3: miRNAs reported to be protective (pale green) or risky-associated (pale magenta). MicroRNAs described as either protective (pale green) or risk-associated (pale magenta) compared with their corresponding functional characteristics. The terms overexpressed and underexpressed refers to miRNA expression data comparing GBM samples to normal brain tissue. Disputed expression signifies that different studies present contradictory results. The numbered brackets are numbers of studies supporting the observation.

miRNA	Reference	Function in GBM	Validated targets	Reference
hsa-miR-9	[424]	Overexpressed (5), oncogenic properties	CAMTA1	Huse et al. [168], Lages et al. [212], Malzkorn et al. [268], Rao et al. [328], Schraivogel et al. [345]
hsa-miR-17-5p	[374, 424]	Overexpressed (9), oncogenic properties	CAMTA1, CTGF, POLD2, PTEN, TGFβ-RII	Dews et al. [75], Ernst et al. [92], Lages et al. [212], Lavon et al. [221], Malzkorn et al. [268], Rao et al. [328], Schraivogel et al. [345], Wuchty et al. [447]
hsa-miR-19a	[424]	Disputed expression in GBM (6)	CTGF	Dews et al. [75], Ernst et al. [92], Lavon et al. [221], Malzkorn et al. [268], Rao et al. [328], Wuchty et al. [447]
hsa-miR-19b	[424]	Disputed expression in GBM (4)		Ernst et al. [92], Lavon et al. [221], Rao et al. [328], Wuchty et al. [447]
hsa-miR-20a	[374, 424]	Overexpressed (5), oncogenic properties	TGF*beta*-RII, CTGF	Dews et al. [75], Lages et al. [212], Lavon et al. [221], Malzkorn et al. [268], Wuchty et al. [447]
hsa-miR-99a	[424]	Overexpressed (2), oncogenic properties		Rao et al. [328], Wuchty et al. [447]
hsa-miR-106a	[374, 424]	Disputed expression in GBM (5)	E2F1	Lavon et al. [221], Rao et al. [328], Yang et al. [455], Zhi et al. [473**?**]

hsa-miR-128a	[424]	Underexpressed (13), tumour suppressive	WEE1, p70S6K1, Msi1, E2F3a, Bmi-1, EGFR, PDGFRα	Ciafre et al. [57], Cui et al. [63], Godlewski et al. [119], Lakomy et al. [215], Lavon et al. [221], Li et al. [234], Rao et al. [328], Shi et al. [358], Vo et al. [414], Wuchty et al. [447], Zhang et al. [462**? ? ?**]
hsa-miR-128b	[424]	Underexpressed (7)	WEE1	Ciafre et al. [57], Lavon et al. [221], Li et al. [234], Skalsky and Cullen [365], Wuchty et al. [447], Zhang et al. [462**?**]
hsa-miR-139	[395, 424]	Underexpressed (5)		Lages et al. [212], Lavon et al. [221], Silber et al. [361], Skalsky and Cullen [365], Wuchty et al. [447]
hsa-miR-181d	Zhang et al. [468, 471]	Underexpressed (1), tumour suppressive	Bcl-2, K-Ras	Kreth et al. [207], Wang et al. [427]
hsa-miR-183	[424]	Underexpressed (2)		Huse et al. [168], Jiang et al. [179], Lavon et al. [221], Wu et al. [441], Wuchty et al. [447]
hsa-miR-217	[424]	Overexpressed (2)		Lavon et al. [221], Wuchty et al. [447]
hsa-miR-301	[424]	Overexpressed (2)		Lavon et al. [221], Wuchty et al. [447]
hsa-miR-324-5p	[424]	Overexpressed (1)		Li et al. [234]
hsa-miR-328	Wu et al. [445]	Underexpressed (2)		Lavon et al. [221], Malzkorn et al. [268]
hsa-miR-374	[424]	Overexpressed (1)		Lages et al. [212], Rao et al. [328]
hsa-miR-497	[424]	Overexpressed (1)		Wuchty et al. [447]
hsa-miR-524-5p	Li et al. [241], Zhang et al. [471]	Overexpressed (1)		Wuchty et al. [447]
hsa-miR-544	Li et al. [241]	Overexpressed (1)		Wuchty et al. [447]
hsa-miR-628-5p	Li et al. [241]			Rao et al. [328]
hsa-miR-1227	Zhang et al. [471]	No studies		
hsa-miR-15a	Tian et al. [395]	Overexpressed (4)		Malzkorn et al. [268], Rao et al. [328], Wuchty et al. [447], Zhang et al. [462]

hsa-miR-31	Srinivasan et al. [374]	No studies		
hsa-miR-34a	[424]	Underexpressed (5), tumour suppressive	SIRT1, c-Met, Notch1/2, PDGFRA, Msi1	Guessous et al. [127], Li et al. [238], Luan et al. [259], Silber et al. [362], Vo et al. [414]
hsa-miR-34b	[424]	No studies		
hsa-miR-146b	Srinivasan et al. [374]	Underexpressed (5), tumour suppressive		Katakowski et al. [189], Xia et al. [449]
hsa-miR-148a	[424][374]	Overexpressed (1)		Huse et al. [168]
hsa-miR-155	[424]	Overexpressed (6)		Dong et al. [79], Lages et al. [212], Lavon et al. [221], Meng et al. [276], Rao et al. [328], Wuchty et al. [447]
hsa-miR-193a	[374]	Overexpressed (1)		Lavon et al. [221], Wuchty et al. [447]
hsa-miR-200b	[374]	Overexpressed (2)		Wuchty et al. [447]
hsa-miR-221	[374, 424]	Overexpressed (11), oncogenic properties	P27, Akt, PUMA, P57, PTPμ, Cx43, TIMP3, MGMT	Conti et al. [60], He et al. [138], Lu et al. [258], Medina et al. [275], Quintavalle et al. [324], Zhang et al. [462, 464, 466**? ? ?**]
hsa-miR-222	[374, 424]	Overexpressed (9), oncogenic properties	P27, Akt, PUMA, P57, PTPμ, Cx43, TIMP3, MGMT	Ciafre et al. [57], Medina et al. [275], Quintavalle et al. [324], Wang et al. [426], Zhang et al. [462, 464, 466**? ?**]
hsa-miR-297	Zhang et al. [469]	No studies		
hsa-miR-299-3p	Zhang et al. [469]	Underexpressed (1)		Lavon et al. [221]
hsa-miR-346	Zhang et al. [469]	No studies		
hsa-miR-518b	Zhang et al. [469]	Overexpressed (1)		Wuchty et al. [447]
hsa-miR-541*	Zhang et al. [469]	No studies		
hsa-miR-551a	Zhang et al. [469]	No studies		
hsa-miR-566	Zhang et al. [469]	Overexpressed (1)		Wuchty et al. [447]
hsa-miR-661	Zhang et al. [469]	Overexpressed (1)		Wuchty et al. [447]
hsa-miR-768-3p	[424]	Overexpressed (1)		Wuchty et al. [447]
hsa-miR-936	Zhang et al. [469]	No studies		
hsa-miR-1238	Zhang et al. [469]	No studies		

Table 4.4: miRNA signatures correlating with survival in GBM. Overview of the miRNA signatures reported in database studies and independent tissue cohort studies correlated with survival or progression of GBM. The miRNAs marked in bold were detected in two or more studies.

Reference	microRNA
Bozdag et al. [32]	Ebv-miR-BART1-5p, Ebv-miR-BHRF1-2, Hcmv-miR-UL70-5p, hsa-miR-142-3p, hsa-miR-142-5p, hsa-miR-147, hsa-miR-223, hsa-miR-302c, hsa-miR-325, hsa-miR-422b, hsa-miR-453, hsa-miR-507, hsa-miR-552, hsa-miR-558, hsa-miR-620, hsa-miR-649, **hsa-miR-661**
Hua et al. [163]	**hsa-miR-19a**, hsa-miR-93, **hsa-miR-221**, **hsa-miR-222**
Lakomy et al. [215]	**hsa-miR-21**, **hsa-miR-128a**, hsa-miR-181c, **hsa-miR-195**, hsa-miR-196a, hsa-miR-196b, **hsa-miR-221**, **hsa-miR-222**
Li et al. [241]	hsa-miR-15a, hsa-miR-139-5p, **hsa-miR-524-5p**, hsa-miR-544, hsa-miR-628-5p
Niyazi et al. [291]	hsa-let-7a, hsa-let-7f, hsa-let-7g, hsa-let-7i, hsa-miR-26a*, hsa-miR-29b, hsa-miR-30b, hsa-miR-124, hsa-miR-129-3p, hsa-miR-136, **hsa-miR-195**, **hsa-miR-210**, hsa-miR-374b, hsa-miR-409-3p, hsa-miR-487b, hsa-miR-539, hsa-miR-555, hsa-miR-578, hsa-miR-590-3p, hsa-miR-595, hsa-miR-720, hsa-miR-1260, hsa-miR-1282, hsa-miR-1286, hsa-miR-1305, hsa-miR-2113, hsa-miR-3065-3p, hsa-miR-3132, hsa-miR-3163, hsa-miR-4286
Qiu et al. [322]	hsa-miR-130a, **hsa-miR-155**, **hsa-miR-210**, hsa-miR-323, hsa-miR-326, hsa-miR-329
Srinivasan et al. [374]	**hsa-miR-17-5p**, **hsa-miR-20a**, hsa-miR-31, **hsa-miR-106a**, hsa-miR-146b, **hsa-miR-148a**, hsa-miR-193a, hsa-miR-200b, **hsa-miR-221**, **hsa-miR-222**
Wang et al. [429]	hsa-miR-9, **hsa-miR-17-5p**, **hsa-miR-19a**, hsa-miR-19b, **hsa-miR-20a**, hsa-miR-34a, hsa-miR-34b, hsa-miR-99a, hsa-miR-106a, **hsa-miR-128a**, hsa-miR-128b, hsa-miR-139, **hsa-miR-148a**, **hsa-miR-155**, hsa-miR-183, hsa-miR-217, **hsa-miR-221**, **hsa-miR-222**, hsa-miR-301, hsa-miR-324-5p, hsa-miR-374, hsa-miR-497, hsa-miR-768-3p
Zhang et al. [468]	**hsa-miR-181d**, hsa-miR-297, hsa-miR-299-3p, hsa-miR-346, hsa-miR-541*, hsa-miR-551a, **hsa-miR-661**, hsa-miR-936, hsa-miR-1238
Zhang et al. [469]	**hsa-miR-181d**, **hsa-miR-566**, **hsa-miR-524-5p**, hsa-miR-518b, hsa-miR-1227
Zhi et al. [473]	**hsa-miR-21**, **hsa-miR-106a**, hsa-miR-181b
Zinn et al. [480]	hsa-miR-92b, hsa-miR-345, hsa-miR-369-3p, hsa-miR-484, hsa-miR-505, hsa-miR-511, **hsa-miR-566**, hsa-miR-655

specific for the given study. Thirteen of the miRNAs were identified in more than one study; however, no miRNAs were identified in more than three studies. Through miRNA array analysis, Niyazi et al. found a 30-miRNA signature in an independent cohort, which divided the samples into short- and long-term survival [291]. Furthermore, Zhang et al. and Srinivasan et al. used similar methods and the same database, but the overlap between these studies was poor [374, 469].

It is striking that the database studies do not reveal better coherency; however, this could be attributed to the cohort factors studied or the filtering of the miRNAs during the analysis. The tissue studies represent independent cohorts; however, many of the database studies have validated their results in independent cohorts, whereby the cohort factors could be the prime source for the lack of overlap. Additionally, Ilhan-Mutlu et al. [173] chose to inves-

tigate seven well-characterised miRNAs (miR-10b, miR-21, miR-181b, miR-181c, miR-195, miR-221, miR-222) and found that none of them correlated with survival contradicting other studies [374, 424]. Therefore, the thirteen miRNAs identified in more than one signature could be more applicable in their prediction of survival and of great interest in relation to GBM prognosis.

4.1 Functional Analysis of the MicroRNAs Included in Several Signatures

The majority of the thirteen miRNAs included in more than one signature have been functionally characterised in GBM and associated with the expression of validated target genes (Table 4.5). The most well-characterised miRNA in GBM is miR-21, which functions as an oncogenic miRNA. miR-21 has numerous validated target genes that it represses in GBM and therefore it is interesting that this miRNA is included in two signatures. The target genes of miR-21 include genes associated with proliferation (e.g., PTEN and PDCD4), invasiveness (e.g. TIMP3 and RECK), and susceptibility to chemo and radiation therapy (e.g., hMSH2), factors, which are all characteristics of GBM tumours [46, 109, 114, 475]. The same characteristics of GBM tumour growth are also modulated by miR-221 and miR-222, both of which appear in three signatures. Being less well characterised than miR-21, miR-221 and miR-222 still have several validated target genes including some important tumour suppressor genes such as p27, p57, TIMP3, and Cx43 [135, 258, 275, 463].

In addition to the oncogenic miRNAs identified in more than one signature, different tumour suppressor miRNAs were also found in several signatures. miR-195 has validated target genes, including some cyclins and E2F3, which are associated with cell proliferation [167, 467]. Hence, a low expression of this miRNA should in theory correlate with a favourable clinical outcome, which is in fact reflected in the clinical data [215]. Another interesting miRNA shown in more than one signature is miR-136, which has very little functional characterisation, but the current validated target genes include the important oncogene, Bcl-2 [457]. Several of the thirteen miRNAs (miR-155, miR-17-5p, miR-181b, miR-195, miR-20a, miR- 21, miR-221, and miR-222) are known to modulate the MMMI, which is an important characteristic of GBM cells [282, 474]. Three of the miRNAs identified in more than one signature have no functional characterisation and could possibly reveal numerous relevant target genes to substantiate the importance of the thirteen miRNAs in future determination of patient prognosis.

Table 4.5: Functional characteristics of miRNAs found in several signatures. MiRNAs found in several signatures and their functional characteristics. Each miRNA is noted along with their validated targets, their functional role, and how many signatures they appear in. Regarding the functional role, 1 designate the functional role of the miRNA when it is overexpressed and 2 the functional role when it is inhibited in vitro or in vivo.

microRNA	Validated targets	Functional role when, 1: Overexpressed, 2: Inhibited	No. of signatures included	Reference to functional studies
hsa-miR-106a	E2F1, SLC2A3	1: Proliferation↓, Apoptosis↑	2	Munoz et al. [286], Yang et al. [455]
hsa-miR-136	AEG-1, Bcl-2	1: Apoptosis↑	2	Yang et al. [457]
hsa-miR-148a	No validated targets	No functional analysis performed	2	
hsa-miR-155	GABRA-1, FOXO3a	1: Proliferation↑, Apoptosis↓, Invasion↑	3	D'Urso et al. [83], Ling et al. [247]
hsa-miR-17-5p	POLD2, TGFβ-RII, CTGF, CAMTA1, PTEN	1: Angiogenesis↑, Growth↑, Invasion↑, Migration↑, Chemosensitivity↓ 2: Viability↓, Apoptosis↑, Proliferation↓	2	Dews et al. [75], Ernst et al. [92], Li and Yang [236], Malzkorn et al. [268], Schraivogel et al. [345]
hsa-miR-181b	FOS, MEK1, IGF-1R	1: Xenograft growth↓, Chemosensitivity↑, Invasion↓, Proliferation↓, Migration↓	2	Shi et al. [359], Tao et al. [388], Wang et al. [422]
hsa-miR-195	E2F3, CCND3, Cyclin D1, Cyclin E1	1: Invasion↓, Proliferation↓, Xenograft growth↓	3	Yang et al. [457], Zhang et al. [467]
hsa-miR-20a	TGFβ-RII, CTGF	1:Angiogenesis↑, Growth↑ 2: Viability↓, Proliferation↓	2	Dews et al. [75], Ernst et al. [92]
hsa-miR-21	RECK, TIMP3, APAF1, ANP32A, SMARCA4, Caspases, PTEN, Cdc25A, HNRPK, TAp63, Spry2, LRRFIP1, PDCD4, hMSH2	1:Invasiveness↑, Radiosensitivity↓ 2: Invasiveness↓, Apoptosis↑, Viability↓, Proliferation↓, *In vivo* tumour volume↓, Chemosensitivity↑, Radiosensitivity↑	2	Chan et al. [44], Chao et al. [46], Ciafre et al. [57], Corsten et al. [62], Gabriely et al. [109], Gaur et al. [114], Han et al. [132], Kwak et al. [211], Li et al. [239, 240], Papagiannakopoulos et al. [310], Ren et al. [333, 334], Schramedei et al. [346], Shi et al. [355], Zhou et al. [476?]
hsa-miR-210	No validated targets	No functional analysis performed	2	
hsa-miR-221	P27, Akt, PUMA, P57, PTPμ, Cx43, TIMP3, MGMT	1: Proliferation↑, Invasiveness↑, *In vivo* tumour volume↑, Apoptosis↓, Migration↑ 2: Proliferation↓, Apoptosis↑, *in vivo* tumour volume↓, Radiosensitivity↑	3	Hao et al. [135], Li et al. [237], Lu et al. [258], Lukiw et al. [262], Medina et al. [275], Quintavalle et al. [324, 326], Zhang et al. [463, 464]

hsa-miR-222	P27, Akt, PUMA, P57, PTPμ, Cx43, TIMP3, MGMT	1: Proliferation↑, Invasiveness↑, *In vivo* tumour volume↑, Apoptosis↓, Migration↑ 2: Proliferation↓, Apoptosis↑, *In vivo* tumour volume↓, Radiosensitivity↑	3	Hao et al. [135], Li et al. [237], Lu et al. [258], Medina et al. [275], Quintavalle et al. [324, 326], Zhang et al. [463, 464?]
hsa-miR-566	No validated targets	No functional analysis performed	2	

5 Perspectives on MicroRNA-Based Therapies for the Treatment of Glioblastoma Multiforme

Given the fact that several miRNA signatures associated with OS or PFS have been identified and that these miRNAs have functional characteristics with importance in GBM progression, a therapeutic concept taking advantage of such correlations seems inherent. The use of miRNA-based therapies in the treatment of GBM is still in its primary phases with exciting basic research being published frequently [396].

Approaches for utilizing miRNAs in such treatment regimens includes both inhibition of oncogenic miRNAs (e.g., miR-21) or overexpression of tumour suppressor miRNAs (e.g., miR-146b) with different types of carriers to facilitate delivery directly to the tumour tissue [125, 190]. Systemic administration of a liposome-encapsulated tumour suppressor miRNA, miR-7, led to a significant tumour size reduction in a xenograft mouse model of GBM. In addition, several key oncogenes were downregulated upon the tumour suppressor miRNA delivery [425]. Another more sophisticated type of lipid-based delivery was exploited by Griveau et al. [125] where locked nucleic acid miRNA inhibitors against miR-21 conferred increased radiosensitivity in U87MG cells. miRNA carriers have also been generated with polymer- based technology, using poly(amido amine) to encapsulate miR-7 for delivery to U251 cells, which resulted in a higher transfection efficiency than liposomal delivery [251].

Of particular interest in solving the problems with efficient drug delivery to the brain, both in malignancies and neurodegenerative diseases is the use of exosomes as drug carriers [227]. Exosomes are endogenous vesicular structures with a diameter ranging from 40 to 120 nm produced by all cells in the body [4]. They are characterised by expression of specific proteins in the membrane (especially tetraspanins) and their ability to deliver proteins, mRNA and miRNAs [413]. The delivered mRNAs and miRNAs are fully functional and can be translated into protein or inhibit mRNA targets in the recipient cells [188, 406].

The potential of exosomes to deliver functional RNAs to cells was utilised by Alvarez-Erviti et al. [3], who provided interesting evidence as to how exosomes might be used to deliver drugs across the ever troubling blood-brain barrier. Immature dendritic cells were transfected to produce exosomes that expressed a neuron-specific targeting peptide on their surfaces to facilitate specific delivery of the exosome cargo. These exosomes successfully delivered both GAPDH- and BACE1-siRNA across the blood-brain barrier resulting in specific gene silencing in the neuronal tissue. Using a somewhat similar approach, Ohno et al. [299] showed that exosomes targeted to EGFR could deliver the tumour suppressor miRNA, let-7a, to a xenograft breast cancer model after intravenous administration. Furthermore, let-7a suppressed the growth of the tumour underscoring the relevance of using exosomal delivery in malignant diseases.

Evidence is now emerging showing that exosomal delivery of interfering RNAs could be relevant in the treatment of GBM. GBM cell lines were shown to be resistant to treatment with anti-miRs against the oncogenic miRNA, miR-9, described in Table 4.3. However, if these GBM cells were co-cultured with anti-miR-transfected mesenchymal stem cells (MSCs) or cultured in the presence of anti-miR-transfected MSC-exosomes, miR-9 was significantly downregulated. This decrease in miR-9 expression made the GBM cells more susceptible to treatment with the chemotherapeutic drug, temozolomide [286]. [190] also produced exosomes in MSCs, which were transfected with a miR-146b expression vector. The resulting miR-146b-containing exosomes were injected into xenograft GBM tumours, leading to a significant reduction in tumour volume compared to vehicle-treated controls. Interestingly, it has previously been shown that miR-146b negatively correlates with survival in GBM [374]. The use of exosomes in the treatment of GBM may have a great potential and should be substantiated with more evidence including choice of relevant miRNA cargo and direct targeting of GBM cells to facilitate intravenous administration.

6 Concluding Remarks

This review presents the studies investigating the expression of specific miRNAs or miRNA signatures with respect to their correlation to clinical progression of GBM. A large part of the studies utilise data from the same databases (TCGA or CGGA), but they do not necessarily reveal the same results. This is because the extracted data and the filtering based on clinical information differ across individual studies, which makes comparison difficult (Table 4.1). The studies using individual tissue cohorts also reveal different miRNA signatures with only some consistency between them. Such varying results

may be caused by several factors, including miRNAs investigated, type of array platform utilised, cohort size and especially the choice of control tissue. Comparing miRNA expression data to control tissue obtained from another type of diseased brain (i.e., epilepsy) might be problematic because it may induce variations in the miRNA expression data compared to studies using non-diseased normal brain tissue.

Furthermore, imperfect description of terminology with regard to OS and PFS may also add complexity to the comparison of the different miRNA signatures. Several studies report some miRNAs to have a protective or risk-associated profile with respect to their correlation with clinical outcome in GBM. Interestingly, several of these miRNAs have validated functions in vitro and in vivo, which are opposite to the way that they should mediate either protection or risk. Therefore, the in vitro and in vivo studies available for numerous miRNAs are not necessarily good indicators for the multifactorial clinical progression of GBM (Table 4.3). However, many of the miRNAs reported to be either protective or risk-associated or the miRNAs included in several signatures do in fact have validated targets and functional characteristics, which are in line with their correlation to clinical progression or survival of GBM (Tables 4.3, 4.4, and 4.5). Having been associated with disease progression or survival in several studies, these miRNAs may be valuable for future determination of patient prognosis and could possibly serve as targets for miRNA-based therapies, which hold a great potential in the treatment of this severe malignant disease.

5

MicroRNA Expression Signatures and Their Correlation with Clinicopathological Features in Glioblastoma Multiforme

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The increased interest devoted to the development of miRNA-based prognostic signatures to aid in the clinical decision-making has revealed a large number of available miRNA signatures correlating with disease outcome and survival, but the level of coherency between the different signatures was low, see Chapter 4 [145]. Interestingly, the great use of TCGA data did not reveal any particular coherency across the studies, possibly due to the different types of data that can be extracted from this database. The type of control tissue varied between the studies and was often derived from other diseased brains. Interestingly, several of the miRNAs found to be associated with either risk or protection have multiple targets validated in model cell culture systems opposite to what would be expected from such an association. This could point to the fact that these in vitro evaluations may not be good indicators for the multifactorial clinical progression of GBM. Some miRNAs were present in several signatures, suggesting that they could be of interest both in prognosis and as therapeutic targets [145]. However, a combination of molecular profiling (e.g., with miRNA signatures) and clinicopathological findings is seldom seen.

This present study aims to combine clinicopathological findings with molecular profiling of GBM tumours and to identify connections between these features that may have the potential of predicting survival and illuminate aspects of GBM pathogenesis.

1 Results

1.1 Clinical Data

Tissue from fourteen GBM tumours isolated from patients was examined (Appendix A). Median overall survival of the patient cohort was 57 weeks, ranging from 5 to 207 weeks. Median age was 61 years (range 27–85). The gender ratio (men/women) was 1:2 (five men/ nine women). Surgery consisted of resection with (79 %) or without (21 %) tumour visualisation by 5ALA-induced fluorescence. Six tumour samples exhibited increased positivity of the neural stem cell marker, NES, while nine tumour samples had high expression of the proliferation indicator, Ki67, determined at the time of pathological diagnosis. All clinical features were analysed with the univariate log-rank test to identify any correlations to patient survival. These analyses revealed that age [p = 0.0497, Hazard ratio = 0.2230 (95 % CI 0.04982–0.9980)], NES expression [p = 0.0169, Hazard ratio = 7.799 (95 % CI 1.445–42.09)] and tumour recurrence [p = 0.025, Hazard ratio = 0.176 (95 % CI 0.038–0.804)] were all predictors of patient outcome, while no correlations could be found between any other clinical feature and patient survival (Table 5.1).

Table 5.1: Univariate analysis using the log-rank test.

Factor	*p* value	Hazard ratio	95% CI
Gender	0.839	0.854	0.186-3.926
Age	0.035	0.198	0.044-0.889
5ALA	0.365	2.563	0.335-19.63
Nestin	0.008	10.30	1.845-57.47
Recurrence	0.025	0.176	0.038-0.804
Ki67	0.175	0.202	0.020-2.041

1.2 MicroRNA Expression Profile Determines Patients Survival

The fourteen GBM tumour samples were tested by the TaqMan Low Density Array Cards to analyse the expression of 750 mature human miRNAs across two cards (A and B). Based on MAD, miRNAs were filtered with a threshold set at the first quartile calculated for all miRNAs with a MAD above zero. This resulted in 287 miRNAs, which had different expression levels across the fourteen tumour samples. These 287 miRNAs were used to make a hierarchical clustering of the samples, resulting in two distinct clusters (Figure 5.1) The two clusters were analysed by Kaplan–Meier estimation and univariate analysis for their correlation with respect clinical data such as overall survival. This analysis showed that the miRNA pattern clustering defined two groups of patients based on the clinical data, which corresponded to either long-term or short-term survival [p = 0.030, Hazard ratio = 5.235 (95 % CI 1.170–23.43)] (Figure 5.2).

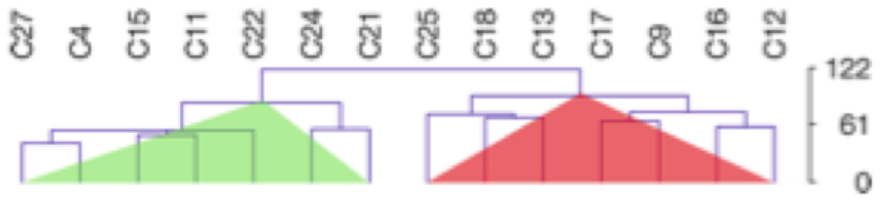

Fig. 5.1: Dendrogram presenting the hierarchical clustering of the tumour samples based on the expression profile of 287 miRNAs. The clustering was made based on the median absolute deviation, Euclidean distance and complete linking. Tumor numeration: C = tumour sample coding

Comparing the miRNA patterns between the two clusters defined a total of 161 miRNAs (cluster 1 vs. cluster 2), which had significantly different expression levels with a twofold change as a cut off. These upregulated miRNAs of cluster 1, indicative of long-term survival, were grouped based on current evidence of their aberrant expression profile in GBM tissue compared to normal brain tissue [282]. The grouping showed that miRNAs upregulated in tumour samples from patients with long-term survival could be associated

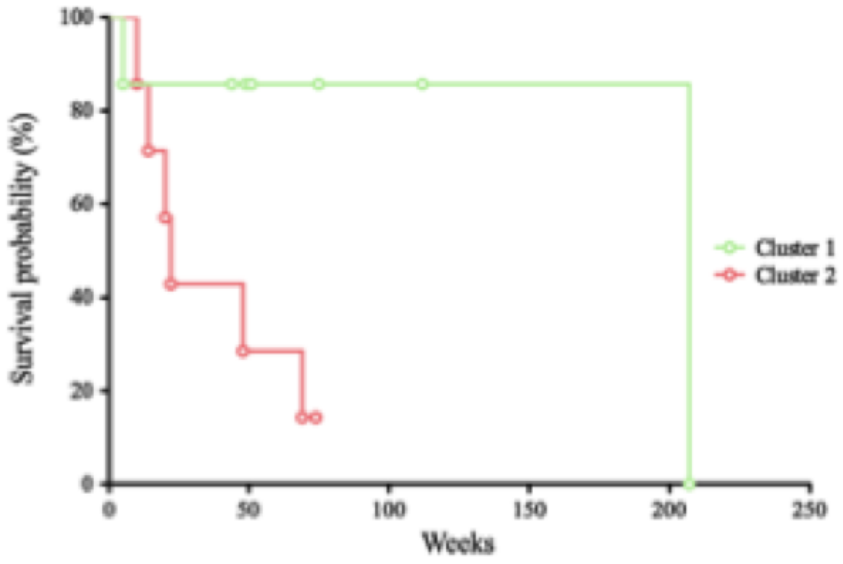

Fig. 5.2: Kaplan–Meier survival diagram for cluster 1 (n = 7) or cluster 2 (n = 7) based on hierarchical clustering of the tumour samples miRNA expression profile. Univariate analysis showed that the patients in cluster 1 had significantly longer survival than those in cluster 2 (log-rank test, p = 0.030)

both with up- and downregulation in GBM. A small number of miRNAs had a disputed role in GBM pathogenesis, while almost a third of all the miRNAs identified in this experiment had no reported dysregulation in GBM. In addition, both validated oncogenic and tumour suppressor miRNAs were found between these long-term survival-associated miRNAs (Figure 5.3; Appendix A Tables A.1-A.4).

1.3 MicroRAs Dysregulated Between Long- and Short-Term Surviving Patients are Reported to Confer Risk or Protection or Modulate the Mesenchymal Mode of Migration and Invasion in GBM

Several of the miRNAs found to be dysregulated between short- and long-term surviving patients were previously reported to be involved in the tumourigenesis and progression of GBM. Some of these miRNAs have been reported to confer either risk or protection with regard to GBM patient survival, as was recently reviewed [145]. Thirteen of the miRNAs with increased expression between the two clusters were previously found to confer either

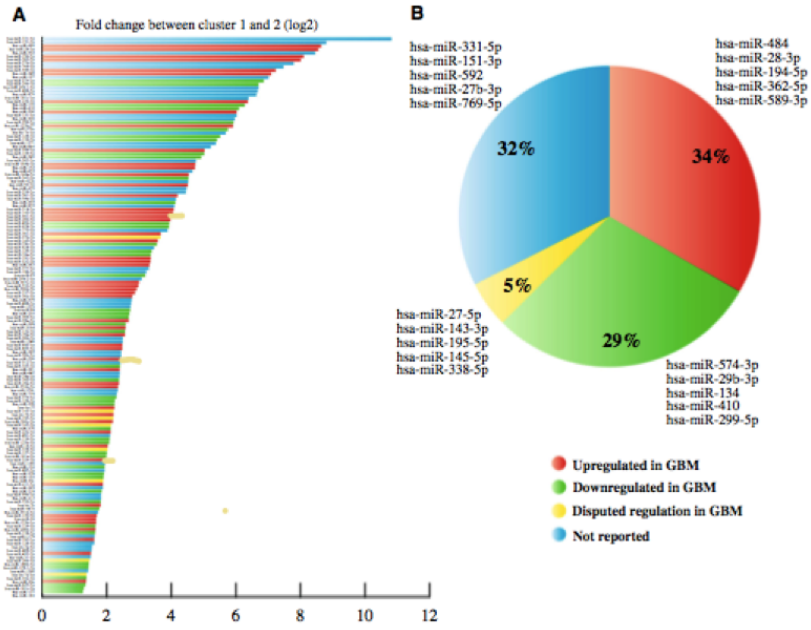

Fig. 5.3: Graphical representation of the 161 differentially expressed miRNAs between cluster 1 and 2. a The fold change between cluster 1 and 2 with each miRNA color-coded corresponding to previously reported dysregulations in GBM as upregulated (red), downregulated (green), disputed (yellow) and not reported (blue). b Pie chart depicting the distribution of miRNAs within the four color-coded groups.

risk or protection. For each of these miRNAs, functionally verified targets were identified through miRTarBase, and the expression of these targets was analysed using microarray gene expression analysis. Target genes with significantly different expression between the two clusters were identified and correlated to miRNAs with these genes proposed as their functional targets (presented in Table 5.2). The same analysis was performed to identify sixteen miRNAs known to regulate the MMMI (Table 5.3) [282]. In total, three of these genes (E2F3, CTGF, Akt2) associated with the miRNAs conferring risk or protection or modulate the MMMI have been shown previously to be important validated targets of miRNAs responsible for disease progression in GBM (highlighted in bold).

Table 5.2: MicroRNAs reported to be protective or risk-associated, and those targets that have been functionally characterised and differentially expressed between cluster 1 and 2.

microRNA	Proposed function	Target genes
hsa-miR-20a	Protective	ARFGEF2, BACH1, **E2F3**, GATA6, IL17RC, IRF2, PLEKHM3, PPARG, RBL1, RBL2, RPL18A
hsa-miR-99a	Protective	GNAL, KIRREL, PPP5C, RNF187, TUBG1
hsa-miR-128	Protective	CDKN1B, **E2F3**, ETS1, FAB126B, FAM83G, GCN1L1, HERC4, HSP90AA1, OGFOD1, RFC1, SELE, SERTAD4, SKIV2L, TATDN2, TOP3A
hsa-miR-139-3p	Protective	
hsa-miR-139-5p	Protective	
hsa-miR-328	Protective	
hsa-miR-374b	Protective	MRPL19, RANBP6
hsa-miR-628-5p	Protective	
hsa-miR-31	Risky	CASR, CXCL12, DKK1, ETS1, NFATC2IP, PPIL2, SELE, TXNDC5
hsa-miR-34a	Risky	ACSL4, ATXN2L, CDKN2A, CDON, CFL1, **E2F3**, EHD1, EIF4G1, EMP1, FOXP1, GRM7, HIST1H1D, HIST1H2BO, HIST1H4L, MAP3K9, OGFOD1, PPP1R10, PPP3R1, PUM2, STAT1, TATDN2, TRAIP, VAMP2, VPS37B, XRN1, ZAP70
hsa-miR-146b-5p	Risky	AKT3
hsa-miR-200b	Risky	CREB1, **E2F3**, ETS1, FLT1, FN1
hsa-miR-222	Risky	CDKN1B, ETS1, FAB126B, FAM83G, GCN1L1, HERC4, HSP90AA1, OGFOD1, RFC1, SELE, SERTAD4, SKIV2L, TATDN2, TOP3A

Table 5.3: MicroRNAs linked to MMMI, and those targets that have been functionally characterised and differentially expressed between cluster 1 and 2.

microRNA	Proposed function	Target genes
hsa-miR-26b	Tumour suppressor	ABCF1, ACVR1B, ASTN2, ATF3, ATXN2L, BCL11B, BNC1, BRDT, C22orf29, CATSPERB, CDIPT, CEND1, CNOT4, CNPY3, CXCL6, DDX60, DHX30, DRD3, DSC1, DYNC2LI1, EEF1A1, EFNA2, EGR3, EIF4A1, ENTPD7, EXOC6B, FANCC, FBLN5, FN1, FOXE1, GKN1, GNB5, GPR107, HIST1H1D, IBSP, IL17RC, IL1RL1, INPP5B, INSR, ISOC2, KCNQ2, KIAA1107, KREMEN2, KRTAP5-7, LAMA3, LRPAP1, MAFG, MAGEA9, MED22, METTL9, NACC2, NOP2, NSF, NXPH4, ODAM, ODF2, OTC, PEX5L, PHLDA2, PHLPP2, PNOC, PODXL, POU4F1, PPP3R1, PSMD5, PAB11FIP1, RAB3A, RAD54L2, RNF187, RORC, RSAD2, SCP2, SIPA1, SLC17A5, SLC35C1, SLC6A9, SV2B, SYNE1, TAGLN, TAZ, TGFB1I1, UPF3B, ZNF35
hsa-miR-29b	Tumour suppressor	BMP1, CDC42, COL1A1, COL3A1, CTNNBIP1
hsa-miR-34a	Tumour suppressor	ACSL4, ATXN2L, CDKN2A, CDON, CFL1, E2F3, EHD1, EIF4G1, EMP1, FOXP1, GRM7, HIST1H1D, HIST1H2BO, HIST1H4L, MAP3K9, OGFOD1, PPP1R10, PPP3R1, PUM2, STAT1, TATDN2, TRAIP, VAMP2, VPS37B, XRN1, ZAP70
hsa-miR-124	Tumour suppressor	
hsa-miR-125a-5p	Tumour suppressor	ERBB2, MMP11, NIN, PDPK1, PREPL, PUM2, SRGAP2
hsa-miR-137	Tumour suppressor	CDC42, CTBP1, NCOA2
hsa-miR-184	Tumour suppressor	**AKT2**

hsa-miR-195	Tumour suppressor	**E2F3**, BCL2L11, CDC42
hsa-miR-218	Tumour suppressor	
hsa-miR-326	Tumour suppressor	ATXN2L, CD9, ERBB2
hsa-miR-27b	Oncogenic	CTSB, EYA4, NPEPPS, PPARG, RAPGEF1
hsa-miR-93	Oncogenic	CDC42, DHX30, EEF1A1, EIF4A1, HYOU1, MAFG, PAMR1, WASH3P, ZNF703
hsa-miR-143	Oncogenic	COL1A1
hsa-miR-145	Oncogenic	**CTGF**, ERG, NEDD9, PODXL, STAT1, TPRG1
hsa-miR-222	Oncogenic	CDKN1B, ETS1, FAB126B, FAM83G, GCN1L1, HERC4, HSP90AA1, OGFOD1, RFC1, SELE, SERTAD4, SKIV2L, TATDN2, TOP3A
hsa-miR-125b	Disputed	BACH1, CDKN2A, **E2F3**, EEF1A1, ERBB2, ETS1, KIF24, MAPK14, MYO19, PLEKHA8, PPP1R12C, RPLP0, RPS6KA1

1.4 Expression of miR-125b and Neural Stem Cell Marker Nestin Correlate with Patient Survival

Since a positive correlation between NES expression in GBM tumour tissue and the degree of malignancy has been reported, a univariate log-rank test was performed to determine any differences in NES expression between the two clusters, showing that the level of NES expression negatively correlated with patient survival ($p = 0.008$). Next, the differentially expressed miRNAs were scrutinised for known negative regulators of NES expression. From this analysis, miR-125b was found to have significant aberrant expression between the two clusters and to have Nestin as a validated mRNA target (determined with miRTarBase). Univariate analysis of the distribution of miR-125b expression showed that miR-125b expression alone positively correlated with patient survival (log-rank test, $p = 0.018$). Interestingly, the gene expression level of Nestin determined from the microarray analysis did not differ significantly between the two clusters, and no correlation between such expression and patient's survival could be found (log-rank test, $p = 0.318$).

In theory, the expression of a single miRNA and the protein product of its validated targets should be negatively correlated. Thus, the tumour samples were grouped to determine such a correlation (low miR-125b + high NES vs. high miR-125b + low NES). A Fisher's Exact test showed that the frequency of high miR-125b + low NES was significantly higher in the patients with long-term survival ($n = 9$, $p = 0.039$), and hence, this combination of molecular characteristics also correlated with patient survival (log-rank test, $p = 0.003$).

1.5 Exosome-Packaging Motifs in the Mature Sequences of Differentially Expressed microRNAs Indicate a Role in Paracrine Signalling

It was recently published that a number of miRNAs possess specific exosome-packaging motifs (EXO-motifs) in the mature sequence that guides them toward packaging and secretion in exosomes [411]. Therefore, all miRNAs with a significantly different expression between long- and short-term-surviving patients were investigated for their contents of these particular EXO-motif sequences. This analysis revealed that 61 out of 161 differentially expressed miRNAs possessed EXO-motifs in their mature sequences, indicating a role of these miRNAs in the paracrine communication between individual cells in GBM tumors (Table 5.4). Among the 61 miRNAs bearing EXO-motifs, miRNAs known to be upregulated or downregulated in GBM accounted for ∼30 % each. Approximately ten per cent had a disputed function, while the last ∼30 % has no validated dysregulation or function in GBM. Several validated tumour suppressor miRNAs contained EXO-motifs, including two miRNAs (let-7a and miR-146b) that have previously been overexpressed in donor cells to be loaded into exosomes [188, 299]. Interestingly, miR-125b, whose expression in combination with Nestin expression correlated with patient survival, contained three individual EXO-motifs along its mature sequence (Figure 5.4; Table 5.4).

Fig. 5.4: The mature sequence of miR-125b containing three individual exosome-packaging motifs

2 Discussion

In this study, we have combined clinicopathological data with molecular profiling to identify an inverse relationship between the expression of the neural stem cell marker, Nestin, and miR-125b - a relationship that correlated with survival in a cohort of primary GBM patients having not yet undergone treatment.

Several miRNA signatures that correlate to clinical progression or overall survival in GBM have been defined through the recent years. The coherency between these individual signatures is low, even though a large number of

Table 5.4: MicroRNAs upregulated in long-term-surviving patients containing exosome-packaging motifs in their mature sequences.

microRNA	miRBase accession	Mature sequence	No. of EXO-motifs	microRNAs in cluster
hsa-let-7a	MIMAT0000062	ugagguaguagguuguauaguu	1	let-7a-1 / let-7f-1 / let-7d
hsa-let-7b	MIMAT0000063	ugagguaguagguugugugguu	1	let-7a-3 / 4763 / let-7b
hsa-let-7e	MIMAT0000066	ugagguaggagguuguauaguu	2	99b / let-7e / 125a
hsa-let-7f	MIMAT0000066	ugagguaguagauuguauaguu	1	let-7a-1 / let-7f-1 / let-7d
hsa-let-7g	MIMAT0000414	ugagguaguaguuuguacaguu	1	-
hsa-miR-28-3p	MIMAT0004502	cacuagauugugagcuccugga	2	-
hsa-miR-30a	MIMAT0000087	uguaaacauccucgacuggaag	1	-
hsa-miR-30b	MIMAT0000420	uguaaacauccuacacucagcu	1	30d / 30b
hsa-miR-30c	MIMAT0000244	uguaaacauccuacacucucagc	1	30e / 30c-1
hsa-miR-93-3p	MIMAT0004509	acugcugagcuagcacuucccg	1	106b / 93 / 25
hsa-miR-98	MIMAT0000096	ugagguaguaaguuguauuguu	1	98 / let-7f-2
hsa-miR-125a	MIMAT0000443	ucccugagacccuuuaaccuguga	3	99b / let-7e / 125a
hsa-miR-125b	MIMAT0000423	ucccugagacccuaacuuguga	3	-
hsa-miR-126-3p	MIMAT0000445	ucguaccgugaguaauaaugcg	1	-
hsa-miR-127-3p	MIMAT0000446	ucggauccgucugagcuuggcu	1	337 / 665 / 431 / 433 / 127 / 432 / 136
hsa-miR-129-1-3p	MIMAT0004548	aagcccuuaccccaaaaaguau	1	-
hsa-miR-129-3p	MIMAT0004605	aagcccuuaccccaaaaagcau	1	-
hsa-miR-139-3p	MIMAT0004552	uggagacgcggcccuguuggagu	3	-
hsa-miR-140	MIMAT0000431	cagugguuuuacccuaugguag	1	-
hsa-miR-143-3p	MIMAT0000435	ugagaugaagcacuguagcuc	1	143 / 145
hsa-miR-145	MIMAT0000437	guccaguuuucccaggaaucccu	1	143 / 145
hsa-miR-146a	MIMAT0000449	ugagaacugaauuccaugguu	1	-
hsa-miR-146b	MIMAT0002809	ugagaacugaauuccauaggcu	1	-
hsa-miR-150	MIMAT0000451	ucucccaacccuuguaccagug	1	-
hsa-miR-151	MIMAT0004697	ucgaggagcucacagucuagu	1	-
hsa-miR-151-3p	MIMAT0000757	cuagacugaagcuccuugagg	2	-
hsa-miR-181a	MIMAT0000256	aacauucaacgcugucggugagu	1	181a-2 / 181b-2
hsa-miR-181c	MIMAT0000258	aacauucaaccugucggugagu	1	181c / 181d
hsa-miR-181c-3p	MIMAT0004559	aaccaucgaccguugaguggac	1	181c / 181d
hsa-miR-184	MIMAT0000454	uggacggagaacugauaagggu	1	-
hsa-miR-186	MIMAT0000456	caaagaauucuccuuuugggcu	1	-
hsa-miR-197	MIMAT0022691	cggguagagagggcagugggagg	1	-
hsa-miR-204	MIMAT0000265	uucccuuugucauccuaugccu	2	-
hsa-miR-326	MIMAT0000756	ccucugggcccuuccuccag	2	-
hsa-miR-328	MIMAT0026486	gggggggcaggaggggcucaggg	1	-

hsa-miR-337	MIMAT0004695	gaacggcuucauacaggaguu	1	493 / 337 / 665 / 431 / 433 / 127 / 432
hsa-miR-338	MIMAT0004701	aacaauauccuggugcugagug	2	1250 / 338 / 3065 / 657
hsa-miR-339-3p	MIMAT0004702	ugagcgccucgacgacagagccg	1	-
hsa-miR-345	MIMAT0000772	gcugacuccuaguccagggcuc	1	-
hsa-miR-362	MIMAT0000705	aauccuuggaaccuaggugugagu	2	532 / 188 / 500a / 362 / 501 / 500b / 660 / 502
hsa-miR-376c	MIMAT0022861	gguggauauuccuucuauguu	1	1193 / 543 / 495 / 376c / 376a-2 / 654 / 376b / 376a-1 / 300 / 1185- 1 / 1185-2 / 381 / 487b / 539 / 889 / 544a / 655
hsa-miR-377	MIMAT0004689	agagguugcccuuggugaauuc	1	487a / 382 / 134 / 668 / 485 / 323b / 154 / 496 / 377 / 541 / 409 / 412 / 369 / 410 / 656
hsa-miR-381	MIMAT0022862	agcgagguugcccuuuguauau	1	376c / 376a-2 / 654 / 376b / 376a-1 / 300 / 1185-1 / 1185-2 / 381 / 487b / 539 / 889 / 544a / 655 / 487a / 382 / 134 / 668 / 485
hsa-miR-410	MIMAT0026558	agguugucugugaugaguucg	1	323b / 154 / 496 / 377 / 541 / 409 / 412 / 369 / 410 / 656
hsa-miR-433	MIMAT0026554	uacggugagccugucauuauuc	1	337 / 665 / 431 / 433 / 127 / 432 / 136
hsa-miR-484	MIMAT0002174	ucaggcucagucccucccgau	1	-
hsa-miR-485-3p	MIMAT0002176	gucauacacggcucuccucucu	1	381 / 487b / 539 / 889 / 544a / 655 / 487a / 382 / 134 / 668 / 485 / 323b / 154 / 496 / 377 / 541 / 409
hsa-miR-487b	MIMAT0026614	gugguuaucccuguccuguucg	2	376c / 376a-2 / 654 / 376b / 376a-1 / 300 / 1185-1 / 1185-2 / 381 / 487b / 539 / 889 / 544a / 655 / 487a / 382 / 134 / 668 / 485 / 323b
hsa-miR-491	MIMAT0002807	aguggggaacccuuccaugagg	2	-
hsa-miR-501	MIMAT0002872	aauccuuugucccugggugaga	3	532 / 188 / 500a / 362 / 501 / 500b / 660 / 502
hsa-miR-504	MIMAT0002875	agacccuggucugcacucuauc	1	-
hsa-miR-512-3p	MIMAT0002823	aagugcugucauagcugagguc	1	512-1 / 512-2 / 1323 / 498 / 520e

hsa-miR-517c-3p	MIMAT0002866	aucgugcauccuuuuagagugu	1	518d / 516b-1 / 518a-2 / 517c / 520h / 521-1 / 522
hsa-miR-519a	MIMAT0002869	aaagugcauccuuuuagagugu	1	20 h / 521-1 / 522 / 519a-1 / 527 / 516a-1 / 1283-2 / 516a-2 / 519a-2
hsa-miR-539	MIMAT0003163	ggagaaauuauccuuggugugu	2	376c / 376a-2 / 654 / 376b / 376a-1 / 300 / 1185-1 / 1185-2 / 381 / 487b / 539 / 889 / 544a / 655 / 487a / 382 / 134 / 668 / 485 / 323b
hsa-miR-551b	MIMAT0004794	gaaaucaagcgugggugagacc	1	-
hsa-miR-589	MIMAT0004799	ugagaaccacgucugcucugag	2	-
hsa-miR-598	MIMAT0026620	gcggugaucccgaugugugagc	1	-
hsa-miR-769	MIMAT0003886	ugagaccucuggguucugagcu	2	-
hsa-miR-885	MIMAT0004947	uccauuacacuacccugccucu	1	-
hsa-miR-1244	MIMAT0005896	aaguaguuggguuuguaugagaugguu	1	-

studies have been compiled from data extracted from similar databases and smaller scale tissue cohorts [145]. The 161-miRNA signature associated with long-term survival contained in this study included several miRNAs found in other prognostic signatures of PFS and OS in GBM. Since the overall coherency across the currently available signatures is poor, an additional step of complexity must exist with regard to miRNA dysregulation and its predictive qualities for clinical outcome in GBM. This aspect with respect to complexity on the usage of miRNA-based biomarkers was recently investigated in an elaborate study by Li et al. [241]. They identified a number of so-called hub miRNAs, which are center points in large, complex regulatory networks of miRNAs and mRNAs, and hence, their dysregulation between high-grade gliomas and normal brain tissue is associated with dysregulation of important cellular characteristics such as proliferation. Thus, these hub miRNAs had strong prognostic value with respect to patient outcome compared to dysregulated non-hub miRNAs [241].

The differences between miRNAs found to be associated with long-term survival in this study compared to similar studies, and between all available studies performed on GBM tissue in general, could possibly be due to differences in methodology (i.e., array- vs. PCR-based platforms) and varying cohort sizes. Furthermore, several subtypes of GBM together with inter- and intratumoral clonal heterogeneity have been defined [375, 407]. This study only includes data from fourteen GBM patients stringently cut back from 47 patients, but we believe that our inclusion criteria of patients being diagnosed with primary GBM resected from the first surgery before having received any treatment is very relevant, because miRNA expression is reported to change after treatment with temozolomide [401]. This might interfere with the quality of the currently published data.

Categorisation of the differentially expressed miRNAs showed that a large number of them had validated functions in GBM, while approximately a third of them had no determined functionality. Some miRNAs have been previously reported to confer either risk or protection with respect to clinical prognosis, while others have been associated with MMMI. Microarray analysis of gene expression showed that a number of validated targets of these miRNAs were differentially expressed between the long- and short-term survival clusters. Three of these genes (E2F3, CTGF and Akt2) have been identified as important miRNA targets in GBM; E2F3 is inhibited by tumour suppressor miR-128 to decrease proliferation, CTGF is regulated by the miR-17–92 cluster resulting in increased proliferation and angiogenesis and upregulation of miR-184 leads to decreased Akt2 expression, lowered invasiveness and increased apoptosis [63, 92, 268]. In addition, most of the validated gene targets of the long-term survival-associated miRNAs are found in other types of cancer, e.g., miR-125b was found to directly target E2F3 in

bladder cancer [164].

Since the scope of this study was to link miRNA signatures to clinico-pathological findings and determine their correlation with clinical outcome in GBM, we chose to investigate the neural stem cell marker, NES, because this protein was upregulated in several patients and could be correlated to OS [65]. NES is an intermediate filament, which is expressed both in proliferating and differentiating cells of the CNS during neural development. It is widely used as an early neural marker, but it can also be detected in progenitor cells in other parts of the body [175]. The expression of NES sustains through life, especially in areas, where the cellular composition contains cells with proliferative and stem-like properties, which is also the reason why NES is increasingly used as a marker for cancer stem cells [158, 435]. In GBM, NES is widely used in the clinic as a determinant of tumour grade [399]. It is recognised as an important mediator of stemness, and a lowered expression is known to decrease tumour volume in vivo [257, 442]. Brain tumour initiating cells with the ability to self-renew and to generate clonal tumour spheres were also shown to express NES [363]. The fact that we see an inverse correlation between NES expression and patient survival corresponds well with the evidence presented above.

To find a molecular link between the NES expression and patient survival, we examined our miRNA signature to identify possible regulators of NES. The examination revealed that miR-125b, which has previously been shown to target NES in neural stem and progenitor cells, was differentially expressed between the long- and short-term-surviving patients [64]. This miRNA is of particular interest, because its role in GBM pathogenesis is highly disputed. MiR-125b inhibited all-trans retinoic acid-induced cell death in U343 cells, decreased the sensitivity to temozolomide and positively correlated with tumour grade [180, 419, 443, 450]. Currently, it has been shown to directly target Bmf, MAZ, E2F2, PIAS3, connexin 43, and p38MAPK in association with GBM [180, 356, 368, 442, 443, 450]. All of these features suggest this miRNA to be oncogenic. However, in CD133- positive GBM stem cells, the true functional role of miR-125b is still debated. MiR-125b has been endowed with oncogenic potential, because it increases migration of CD133-positive GBM cells together with a concordant increase in the expression of MMP2/9 [356]. Its expression is also increased in highly invasive SU3 GBM stem cells compared with U251 cells [418]. Interestingly though, miR-125b has been characterised as the most downregulated miRNA between CD133-positive and CD133-negative GBM cells and to decrease the proliferation of CD133-positive GBM cells due to its direct targeting of transcription factor E2F2 [442]. The decrease in GBM stem cell proliferation was also associated with a decreased expression of CDK6 and CDC25A [356]. Recently, it has been reported that miR-125b was downregulated in three primary GBM stem

cell lines and targeted Lin28, hereby inhibiting GBM growth both in vitro and in vivo [419, 420]. These results did not correspond to a previous report by the same group, where only one CD133-positive GBM stem cell line was examined [356, 420]. An assessment of the staining patterns of four CD133 antibodies possibly recognising different epitopes on CD133 splice variants showed that the distribution of CD133 and concurrent cell morphology rarely corresponded between the different antibodies used [148]. This might explain some of the contradicting results reported on the role of miR-125b in GBM stem cells.

The direct targeting of NES by miR-125b is only validated in neural progenitor cells, but one study on GBM did report an inverse correlation between miR-125b and the expression of NES and CD133, a results which is also reflected in this study [64, 442]. In fact, co-expression of Nestin and CD133 was recently proposed as relevant cancer stem cell markers in GBM, which could indicate that GBM stem cells may be differentially regulated between long- and short-term-surviving patients [65]. In GBM-associated endothelial cells, the expression of miR-125b is downregulated, which may be due to the inhibitory effects of VEGF on this miRNA, hereby defining a role of miR-125b in tumour angiogenesis [368]. The role of miR-125b and how it varies across studies and between CD133-positive and CD133-negative GBM cells illustrates how complex the regulation of this miRNA must be. Therefore, miR-125b may be of great importance with regards to increase the understanding of GBM pathogenesis and how it may be valuable as a therapeutic target.

Exosomes are a family of nanoparticles with a diameter in the range of 30 to 120 nm that are secreted by all cell types of the body, and capable of carrying cargos like RNA, proteins, lipids, etc. to be shared between cells [4, 332, 413]. They can be isolated from several types of extracellular fluids including blood, urine, amniotic fluid, and saliva, but also from cell-conditioned medium [413, 439]. The growing interest of paracrine signalling between cancer cells has resulted in interesting new evidence on how cancer exosomes modulate cancer cell characteristics and the tumour niche [313, 327, 360]. For example, exosomes derived from hypoxic regions of GBM tumours or from GBM cells grown under hypoxic culture conditions potently induced angiogenesis [208]. Exosomes from GBM tumours and cell lines are known to contain a great number of miRNAs that can be shared between GBM cells, and to possess a nanofilament network, which facilitates interaction with the cell membrane and subsequently increase the cellular exosome uptake [188, 351, 366]. In a recent paper, specific exosome-packaging motifs in mature miRNA sequences was shown to bind sumoylated hnRNPA2B1 to facilitate loading into the exosome compartment [411]. The results of the present study showed that 61 miRNAs possessed these so-called EXO-motifs,

suggesting them to play a role in the paracrine signalling within tumours. This is supported by the fact that all miRNAs (miR-214, miR-146b, miR-122 and let-7a) artificially overexpressed to obtain enriched exosomes possess these EXO-motifs [51, 190, 299, 308]. Four of the 61 miRNAs containing EXO-motifs (let-7b, miR-30a, miR-30b and miR-125a) have also been shown to be abundant in glioma microvesicles [233]. Interestingly, miR-125b contained three individual EXO-motifs in its mature sequence, and hence, this miRNA is possibly communicated between GBM cells to inhibit expression of specific genes including the neural stem cell marker NES. Thus, it may be valuable to investigate the ongoing intercellular communication in GBM tumours to understand how miRNA signatures are generated and regulated, and which underlying functions are responsible for the phenotype and patient outcome. Such work could possibly identify GBM exosomes and their contents as novel therapeutic targets or biomarkers of disease [208, 269, 292].

3 Conclusion

The clustering of miRNAs to define signatures and their prognostic value is not a stand-alone entity with respect to predicting survival. It is very clear that evidence needs to be critically analysed preferentially combining clinicopathological findings with molecular profiling, which we have attempted to achieve in a smaller cohort of patients meeting stringent inclusion criteria. The inverse relationship between NES and miR-125b expression and their correlation to patient survival in GBM illustrate this eloquently. The intriguing finding that many of the differentially expressed miRNAs contained exosome-packaging motifs in their mature sequences suggest that we must expand our view to encompass the complex intercellular communication in order to identify molecular prognostic biomarkers and to increase our knowledge in the field of GBM pathogenesis.

PART II

Brain-Specific Fatty Acid Binding Protein

6

Early-on it was established that brain fatty acid binding protein (FABP7) is expressed in human malignant anaplastic astrocytoma and GBM tumour biopsies, as well as in some but not all malignant glioma cell lines [118]. Likewise, FABP7 was identified as one of the most overexpressed genes in surgical samples of GBM [242] as well as in GBM cancer stem cells [248]. Genetic manipulations of FABP7 expression in malignant glioma cell lines [243, 280] as well as glial stem cell derived neurospheres [72] showed this gene to be directly implicated in tumour proliferation and invasiveness, with similar conclusions drawn in a study on melanoma cell lines and tumours in vivo [367].

FABP7 is a member of a larger family group of fatty acid binding proteins (FABPs), which was first discovered in 1972 by Ockner et al. [295]. They are small cytosolic proteins, 14.15 kDa in size, and though they have a distinct primary structure, their tertiary structure is highly conserved.

FABPs consist of a β-barrel composed of ten antiparallel ß-strands capped by a helix-turn-helix motif. The β-barrel contains the lipid-binding cavity, rich in polar and hydrophobic amino acids, which binds various amphiphilic ligands, like fatty acids, bile aids or retinoids [136, 216, 260, 261, 279]. Initially, it was thought that the expression of the individual FABPs was confined to specific tissues, thus the FABPs were named after their first tissue of isolation. Although each FABP is primary expressed in a certain tissue, they exhibit a broader tissue distribution than originally thought, which has lead to a numerical nomenclature for the different FABPs [26, 150, 305, 378].

Even though FABPs are widely expressed in various tissues, their expression is under tight spatiotemporal regulation by regulatory elements positioned both distally and proximally to the transcriptional start site. For instance, high levels of FABP3 in muscle tissue have been shown to be ade-

quately regulated by the -400 bp promoter region, while the 1.2 kb 5' flanking region in necessary for expression in heart tissue [320]. Interestingly, knockdown of the predominantly expressed FABP can spark an increase in expression of lesser expressed FABPs as has been seen for FABP5 which in adipocytes is increase upon negative alteration in FABP4 expression [59, 91, 353], while the metabolism is relatively unaltered probably due to common functions in adipocytes [353, 377]. In contrast to this, both FABP4 and FABP5 is also expressed in macrophages where alterations in FABP4 expression does not affect FABP5 suggesting a distinct function for FABP4 and FABP5 in macrophages [91, 223].

In relation to the brain, three different FABPs are expressed, FABP3, FABP5 and FABP7, at varying stages of CNS development (Figure 6.1) [305]. In mouse, FABP7 is expressed during embryogenesis from E10 (Theiler stage 17) in radial glial cells found in the ventricular zone of most regions of the developing CNS, as well as in the trigeminal ganglion and the spinal dorsal root ganglia of the peripheral nervous system (PNS) [6, 8, 181, 210, 430]. FABP7 expression intensifies during neurogenesis in radial glial cells of the developing cortex to reach a maximum at E14-E17, and gradually decreases after birth, to be expressed at low levels in specific regions of the adult organism where niches of neural progenitors subside, such as the subventricular zone of the lateral ventricle and the dentate gyrus subgranular zone of the hippocampus.

In the CNS, FABP7 expression typically marks glial cells: radial glia in the neocortex and differentiated glia such as cerebellar Bergmann glia, retinal Müller cells, and cortical immature astrocytes [210]. FABP7 expression is normally not associated with neuroepithelial (NEP) cells, which are the precursors of radial glia during cortical neurogenesis, although a study in rat suggested otherwise [8]. Likewise, FABP7 expression is down-regulated in cells deriving from the radial glial lineage, such as neural progenitors and neuroblasts. Recent evidence also indicates that FABP7 is expressed prior to the formation of the neural plate, as whole-mount in-situ hybridisation showed a clear signal in the primitive streak of mouse embryo at E7.5 (Theiler stage 11) [338, 339, 386].

In embryonic neurogenesis, FABP7 fulfils at least two important functions. Firstly, it takes part in the control of radial glia growth and the projection of their cellular processes, facilitating migration of associated neurones to form the neocortex [7, 98]. Secondly, it takes part in the maintenance of and self-renewal properties of NEP cells [8]. FABP7 has been suggested to have a role in the regulation of Schwann cell-axon interaction [278] and to have a role in re-myelination in multiple sclerosis (MS) lesions [200]. During the course of MS, plaques of demyelination are enclosed by reactive astrocytes

[200], which have undergone both morphological and biochemical changes known as reactive gliosis. The mechanisms underpinning reactive gliosis are largely unknown, but includes massive proliferation, cellular hypertrophy, inflammation, leading to scar formation, and is a common hallmark of various brain pathologies [200, 350]. Work with a murine cortical stab injury model indicated a direct involvement of FABP7 in astrocyte proliferation in reactive gliosis, and it is believed that the functions of FABP7 in astrocytes during gliosis might be partly different from its role in embryonic neurogenesis [350]. Re-expression of FABP7 has been observed in ischemic monkey brains [264], again indicating that FABP7 is functional in reactive gliosis after ischemia.

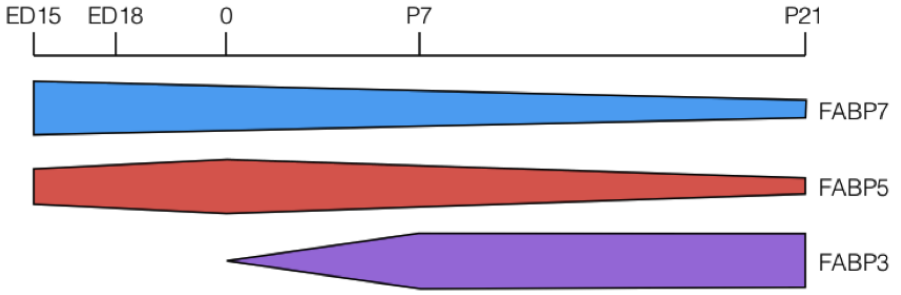

Fig. 6.1: Temporal expression patterns of FABP7, FABP5 and FABP3 in rat brain based on data from Owada et al. [306]

The involvement of FABPs in cancer is still largely elusive, however, association between tumour malignancy and FABP expression has been established. For instance, the progression of bladder, colon and liver cancer has been shown to negatively correlate with the expression of FABP2, FABP4 and FABP1, respectively [42, 68, 222], while for prostate cancer FABP5 expression has a positive correlation [2]. Elevated levels of FABP5 have also been shown for breast cancer and to promote proliferation and metastasis in vitro [229]. Another FABP member, FABP3 was initially identified as a inhibitor of growth in lactating bovine mammary gland, hence named mammary-derived growth inhibitor (MDGI), involved in mammary gland differentiation [29]. Reduced proliferation and promoted cellular differentiation upon transfection of FABP3 into breast cancer cell lines [456] as well as reduced tumour formation in nude mice [170] suggests a role of FABP3 as a tumour suppressor. LOH has been shown in the region encoding FABP3 in human breast cancer in an attempt to identify possible inactivating mutations [315]. However, no mutations were found suggesting that mutation within FABP3 is uncommon in breast tumourigenesis.

Although FABP7 is chiefly expressed in the brain, it has also been detected

in mammary tissue [155, 357] and prostate [67]. Detection of FABP7 in other tissues is mostly observed in the context of pathological conditions such as cancer, where FABP7 expression is up-regulated. Beside several neurological disorders such as bipolar disorder, schizophrenia, and Down syndrome [30, 90, 176, 278, 307, 309, 342, 405, 430], alterations of FABP7 expression have been associated with aggressive types of cancer including GBM, breast cancer, malignant cutaneous melanoma, and renal cell carcinoma [280, 367, 385, 465].

In respect to gliomagenesis, the brain specific FABP, FABP7, has been shown to have increased expression in glioma tumour tissue and speculated to be involved in migration and invasion of glioblastoma [118, 242, 280, 400]. For renal cell carcinoma, the expression of FABP7 also increased compared to normal kidney [78], while for breast cancer and melanoma it has been shown to have tumour suppressor-like functioning, being linked to better clinical outcome and decrease progression when expressed [123, 465].

7

Brain-Specific Fatty Acid Binding Protein Transcriptional Regulatory Network

Henriksen M, Duroux L, Zachar V, Duroux M

The paper has been submitted to the
BBA - Gene Regulatory Mechanisms (under review), 2016.

The layout has been revised.

Due to copyrights the full version of this chapter has been omitted. In its place, a summary has been provided highlighting the background and key findings.

1 Introduction

The expression of FABP7 is considered as a marker of the radial glial cell origin [280] and its function and regulation has been reviewed in the context of normal brain development [249] as well as in GBM [72]. FABP7 is one of the better-studied members of its family with respect to transcriptional regulation and it has been shown that the transcriptional regulation of FABP7 is under tight spatiotemporal control. The expression of FABP7 has been studied in human, mouse and rats, which all show a high degree of sequence conservation [342].

By extensively reviewing the literature and data submitted to public electronic databases, this chapter aims at providing a comprehensive overview of the regulatory elements governing FABP7 expression. The entire set of transcription factors (TFs) is assumed to be finite and the expression of FABP7 both in normal development and under pathological conditions is a result of their combination. An exhaustive list of FABP7 TFs, as well as a physical map of their interactions was established (Figures 7.1 and 7.2). Establishing such an overview will help us further understand the complexity of FABP7 transcriptional regulation both in normal development and in disease pathologies such as cancer.

2 Results

2.1 Mapping FABP7 Transcriptional Regulatory Elements and Transcription Factors

Eleven nuclear TFs were identified (figure 7.3), all of which had not been previously reported in the literature to bind upstream of the FABP7 promotor. By accessing the ENCODE project [89], over 40 TFs were found, though this was stratified to eleven TFs. This showed two initial observations (i) an absence of RNA Pol II subunits, which indicated that FABP7 transcriptional activity might globally be silenced in all cell lines investigated, and (ii) the presence of three sites occupied by a triad of factors from the chro-

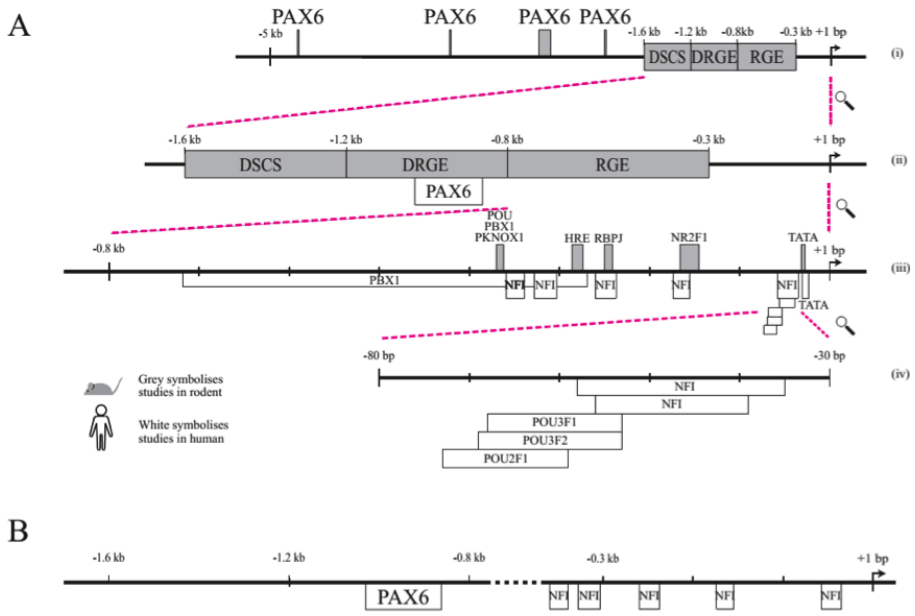

Fig. 7.1: A map of FABP7 transcription factors from peer-reviewed literature. (a) Schematic representation of FABP7 promoter. The region -1.6 kb upstream has been characterised by Feng and Heintz [98]. The -0.8 kb region (RGE) regulates FABP7 in glial cells. Regulatory elements identified in rodents (grey) and in man (white) are shown as boxes. Successive zoom levels are shown: (i) -5 kbp upstream region, (ii) -1.6 kb, (iii) -0.8 kb, and (iv) -80 bp to -30 bp. TSS is shown as an arrow at +1 bp. RGE = Radial glial element, DRGE = Dorsal root ganglion element, DSCS = Dorsal spinal cord silencer. (b) TFs involved in regulation of FABP7 in GBM.

matid cohesion complex. With regard to non-nuclear modulators of FABP7 transcription, only three have been reported to date, NOTCH, ADAM10, and EGFR.

Although several TFs are found to bind within or upstream of the FABP7 gene, only few regulatory elements have been investigated to date with respect to FABP7 expression in GBM, e.g. NFI [27, 36], EGFR [184], and PAX6 [250]. Although EGFR is not a transcription factor, it has been established that there is a correlation between FABP7 and EGFR expression [184]. However, it still remains to be understood by which signalling circuitry EGFR interacts with FABP7 regulatory elements.

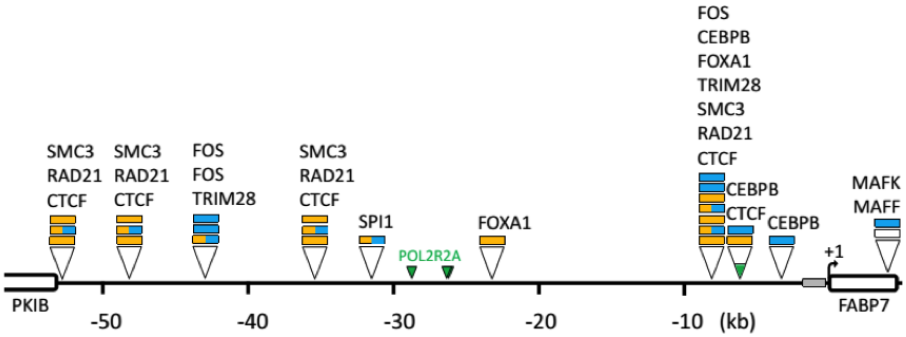

Fig. 7.2: Position of ENCODE transcription factors at FABP7 locus and upstream inter-genic region. Binding sites for TFs from the ENCODE project [391] are shown as stacks over an arrowhead. In orange are TFs controlling chromatin dynamics and modelling. In blue are TFs driving inflammatory or immune responses. Sites for PolII large subunit POL2R2A are shown as small green arrowheads. The grey box adjacent to FABP7 represents the regulatory region from -1.6 kb to -0.3 kb shown on Figure 7.1. Note the presence of 4 sites with the cohesin complex CTCF/RAD21/SMC3.

2.2 FABP7 Regulation in Astrocytic Tumour and Associated Transcription Factor Regulatory Network

A physical map of protein-protein interactions between the regulatory elements of FABP7 and three core regulatory pathways controlling onset of GBM [274] was generated. This revealed that none of the factors controlling FABP7 expression reported here appeared within any of the three core regulatory modules, except for EGFR. However, the majority of the TFs were associated with the regulatory modules, supporting previous findings about FABP7 being a target gene in GBM.

Additionally, the FABP7 promoter shows several CTCF sites occupied by members of the cohesin complex, which might control FABP7 silencing. Finally, it also appears that several factors are driving inflammatory and immune responses, confirming a recently discovered role for FABP7 in these processes.

3 Conclusion

The extensive literature review and database mining resulted in a more comprehensive picture of FABP7 transcriptional regulatory network, based on already known (from published experimental evidence) or newly discovered (from ENCODE) regulatory factors. Although most FABP7 TFs physically

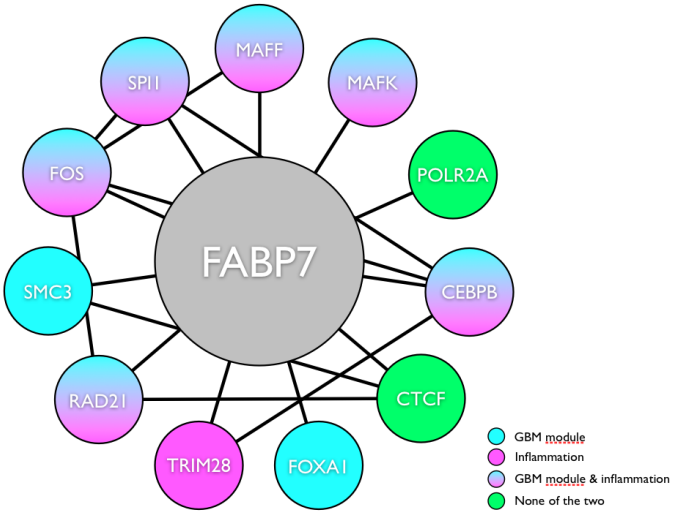

Fig. 7.3: Small interaction network of the eleven newly identified FABP7 TFs. Light blue is those connected to one of the GBM modules, pink is those involved in inflammation and those with a gradient are associated with both, the two in green are not associated with any of the two.

interact with one another and are closely connected to transcriptional regulators driving GBM through physical interaction, further experimental evidence will be required to confirm those newly discovered TFs and their effect on FABP7 transcriptional regulation. Furthermore, the presence of TFs classically associated to inflammation and immune response confirms the link between FABP7 and these processes, and will deserve further investigations.

8

The Elusive Expression of FABP7 and its Association to the Molecular Subtype of GBM

Henriksen M, Duroux L, Pilgaard L, Agger R, Moos T, Zachar V, Duroux M

(in preparation).

The layout has been revised.

Due to copyrights this chapter has been summarised in the form of an extended abstract and a full version has been placed in Appendix **??**.

1 Introduction

FABP7 expression in radial glial cells has been proposed to be the malignant glioma cell of origin [280] and has been identified as one of the most distinct genes over expressed in glioblastoma multiform (GBM) cancer stem cells [248]. It has been associated with regions of GBM tumour infiltration, tumour relapse and reduced survival [72, 184, 242, 280, 281]. The invasive nature could be a function of FABP7 overexpression, which emphasises the importance of understanding the complex regulatory mechanisms underpinning FABP7 expression in tumour growth dynamics.

We observed that the high level of FABP7 expression in tumour tissue was not mirrored in vitro in primary cell cultures at the transcriptional or the protein level. This finding, supported by the re-expression of FABP7 when grown as neurosphere culture and intracranial xenograft tumours, highlighted the fact that the tumour microenvironment was supporting FABP7 expression.

2 Results

2.1 Differential expression of FABP7 and transcriptional regulatory factors

Combining, qPCR, microarray, and bioinformatic analysis, FABP7 expression essentially grouped the tumours into low and high FABP7 expression. This differential expression of FABP7 was further investigated with respect to transcriptional regulatory factors. Several regulatory elements and their respective binding factors have been mapped in the promoter region of FABP7 (see Chapter 7). These are partially responsible for the temporal spatial regulation of FABP7. A number of TFs showed a significant correlation with the expression of FABP7 in our tumour tissue cohort or databases, though only few of these have been investigated to date with respect to FABP7 expression in glioma and GBM (figure 8.1).

2.2 Molecular subtypes and FABP7 expression

Since only few of the TFs showed a correlation with the expression of FABP7 it was further investigated whether the molecular subtype could influence the inherent variation in FABP7 expression. FABP7 was found to be associated with the classical subtype, though several of the samples exhibiting high FABP7 expression were of mesenchymal subtype. This was consolidated through public electronic databases (TCGA and Rembrandt). Several of the TFs linked to FABP7 regulation were also found to be associated with the classical subtype (figure 8.1).

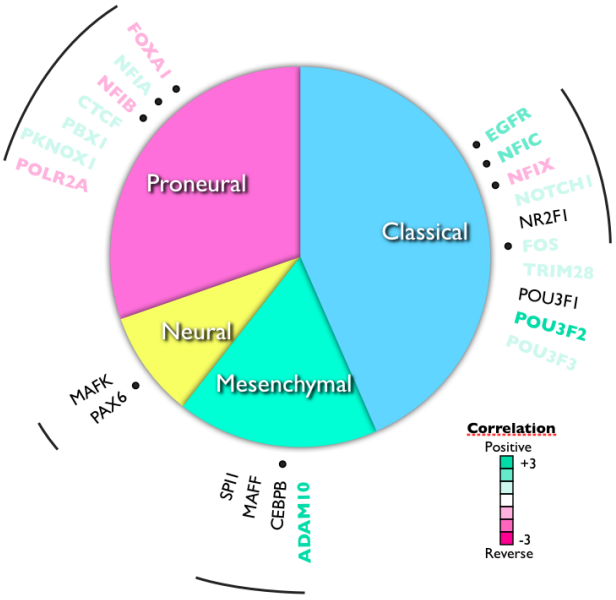

Fig. 8.1: The distribution of molecular subtypes among the TFs regulating FABP7. The individual TFs are highlighted whether their expression correlates with the expression of FABP7 (possitive correlation: green; reverse correlation: red) with a shaded effect, dependent on whether they correlated in one, two or tree set. The TFs are grouped and those associated with a GBM module (see Chapter 7) are indicated with a bar above them. A dot below the TF signifies that the TF is associated with Glioma/GBM

3 Conclusion

The elusive behaviour of FABP7 was investigated and the results suggested that FABP7 expression could be dependent on the culturing environment and the context of the tumour niche. Although the tumour samples exhibit a large variation in FABP7 expression, only few TFs showed a correlation with

respect to FABP7. Thus it was hypothesised that their molecular subtype could influence this inherent variation. Here, FABP7 was associated with the classical subtype along with ten of the TFs linked to FABP7 regulation. While seven out of nine TFs linked to glioma correlated with FABP7, only three TFs correlated with FABP7 in all three datasets. One of the TFs, ADAM10, is furthermore linked to inflammation.

General Discussion

9

This thesis has spanned the molecular landscape of GBM, looking at the gene regulation, pre- and post-transcriptional (microRNA), and how this could be linked in some way to the expression of stem cell like proteins that are dysregulated. By looking at these in combination with clinicopathological features, we have learnt more about the underlaying pathology of this disease and its progression.

From the start, the objective was to look at the regulation of FABP7, a protein that is highly dysregulated in GBM and to link this to the invasive nature, survival and molecular subtypes. However, often the simplest task can be more complex, the deeper you dig. The challenge was to investigate the somewhat elusive behaviour of this protein in vitro and how this could subsequently be reversed when changing the culturing milieu and microenvironment in vivo. The transcriptional regulatory network of this highly interesting protein was gradually unfolded, and the complexity visualised with the help of in vitro studies and extensive literature and bioinformatics analysis. The role of miRNAs as modulators of key regulatory mechanisms in GBM was reviewed, their potential as markers of risk and protection with respect to disease progression was investigated and finally, this was linked to clinicopathological features that aid us forward in our understanding of the biology of GBM.

1 The Role of MicroRNA in Glioblastoma

Variation in miRNA profiles is a hallmark for almost all cancer types compared to non-malignant tissue [254]. Because these aberrantly expressed miRNAs provide valuable information of the tumour state, e.g., with respect to

proliferative or invasive capacity, they are able to serve as prognostic indicators, and provide a basis for deciding on the treatment strategy [38, 384, 454]. Since our review in 2012 [282], there has been a steady increase in the number of studies analysing different miRNAs and their functionality in GBM. In fact, dysregylated miRNA expression profiles in GBM can differentiate between different tumour grades and stages, and the specific function of individual miRNAs has expanded our knowledge on disrupted signalling pathways, hereby providing an additional regulatory level to take into account when characterising the tumour. This has lead to a more in depth understanding of GBM pathology [197].

Many miRNAs have been functionally characterised in numerous studies, based on their specific targeting of mRNA transcripts involved in cancer-associated signalling pathways. However, we know that the vast majority of miRNAs known to be differentially expressed in GBM tumour tissue and normal tissue have no functional characterisation, and thus may be of great importance for disease progression and relevant for usage in prediction of patient outcome, if their functionalities were to be characterised [282]. The most extensively studied miRNA in GBM is miR-21. miR-21 is over expressed in GBM in a grade-specific manner, and known to target many important tumour suppressor mRNA transcripts [44, 48, 57, 60, 62, 79, 109, 114, 126, 168, 211, 212, 215, 221, 234, 239, 240, 268, 310, 328, 333, 334, 343, 346, 355, 361, 447, 462, 473, 475, 476]. In addition to regulating key apoptosis and proliferation pathways, high expression of miR-21 is also linked to shorter progression-free survival, and is therefore regarded as an oncogenic miRNA [46, 215, 325]. As for miR-21, other microRNAs such as miR-17, miR-93, miR-221 and miR-222, have the same oncogenic potential and all are known to be over expressed in GBM and to inhibit the translation of important tumour suppressors [282]. With the use of microRNA target prediction software which has proven to be a powerful tool, a number of predicted target sites in mammals have been reliably validated in numerous experiments [205, 232].

2 MicroRNA Defined as Risk-associated vs Protective as Predictors of Clinical Outcome

Due to the appertaining dysfunction in key cancer-associated signalling pathways, the patterns of dysregulated miRNA expression in GBM are now being recognised as a tool to stratify GBM patients with respect to predictive clinical outcome [16, 146, 197, 322, 374, 469]. Thus, many studies are providing miRNA signatures (with single or multiple miRNAs) that are associated with longer or shorter survival times. In the review concerning this subject

(Chapter 4), all literature was revised pertaining to the use of miRNA expression profiles as prognostic and/or diagnostic biomarkers [145]. The analysis showed that numerous signatures had been proposed to aid in clinical decision-making, but the same overall coherency of the signatures was poor, even though a number of studies utilised data derived from the same publicly available databases, like TCGA. Studies investigating individual tissue cohorts to define miRNA signatures also lacked strong coherency, probably because of differences in the number of miRNAs investigated, type of array platform utilised, cohort size and especially the choice of control tissue. Several studies chose to use control tissue from other diseased brains, such as epilepsy brains, which must be expected to possess miRNA dysregulation as well, therefore making true comparison between the individual studies difficult.

Interestingly, several studies were found to identify specific miRNAs as being associated with either risk or protection with respect to the clinical outcome of GBM. For example, high expression of known oncogenic miRNAs, such as miR-221 and miR-222, were found to correlate with short-term survival, whereas high expression of tumour suppressor miRNAs, such as miR-181d and miR-328, were associated with long-term survival. However, even though the association to survival correlated well with the known functionality of these particular miRNAs, many miRNAs had validated functions in vitro and in vivo (xenograft models) opposite to what would be expected from their association with clinical outcome. This pointed to the fact that assessment of individual miRNAs to define their function is not necessarily a good indicator for the multifactorial pathogenesis and progression of GBM. This may be due to the way miRNAs bind to and inhibit numerous target mRNAs, encompassing both oncogenes and tumour suppressors [145]. Nevertheless, some miRNAs associated with either risk or protection were identified in several miRNA signatures, suggesting that these specific miRNAs could be relevant as biomarkers of disease, in prognosis, and as therapeutic targets.

3 MicroRNA Expression, Prognosis and Linking this to Clinical Findings

The association between microRNA expression profiles and survival was highlighted by the analysis of tumours from a cohort of fourteen patients diagnosed with primary GBM (Chapter 5) [146]. A microRNA signature was determined with the potential of predicting either long-term or short-term survival. When comparing the expressional profile of the miRNAs included

in the signature in the GBM tumours to the available clinicopathological data, a negative correlation between the expression of miR-125b and the stem cell marker, NES, was found. This correlation could predict survival in the GBM patient cohort, pointing towards the need of combining molecular profiling with clinicopathological features in order to confidently determine patient prognosis.

4 The Potential of MicroRNA Modulating the Tumour Niche

Interestingly, the study in chapter 5 (table 5.4) showed that many of the differentially expressed miRNAs possessed specific exosome-packaging motifs (EXO-motifs) in their mature sequences, including some already known to be abundant in glioma microvesicles [233]. This suggested that these microRNAs potentially participate in the intercellular communication within the GBM tumour to modulate the behaviour of recipient cells [146, 188, 366]. Since exosomes have recently been shown to possess the potential of mediating delivery of exogenous drugs (e.g., interfering RNAs), this could eventually be an interesting concept to pursue by combining the knowledge on microRNA expression profiles associated with survival in GBM, and the possibility of interfering with these aberrant expression profiles by delivering tumour suppressor microRNA via exosomes to GBM tumours.

5 FABP7: A Complex Regulatory Landscape

The loss of FABP7 expression in primary cultures pointed toward a selection pressure with respect to cell type or niche adaptation where the culture environment no longer supported the expression of FABP7. Why this loss of expression? This was a major question given the gene expression and corresponding protein levels is tissue. Since the FABP7 promoter region has been shown to be deprived of CpG islands, the role of hypermethylation was not of compelling interest. But whether it was a change in the availability of transcription factors or the involvement of miRNAs was more difficult to deduce. The poor correlation between transcription factors and FABP7 expression, however, suggested that the answer was broader than first expected. It was no longer a question whether a certain transcription factor was present, but apparently also a question whether the culture conditions were favourable since the elusive behaviour of FABP7 was reversed when grown in vivo (Figure **??**, Chapter **??**).

6 Piecing Together the Biological Dots: FABP7 Linked to Molecular Subtype

To this end the molecular subtype of GBM was a compelling starting point, since it is known that the subtypes behave differently when grown in culture, especially when grown as a monolayer and the majority of the primary cultures were initially grown with FCS, in monolayer format [253, 329, 349]. Clearly, the tissue levels of FABP7 were high (see Figure **??** in appendix **??**) and FABP7 was observed in all tumour tissue but to a varying degree. Through gene profiling, it was found that FABP7 belonged to the classical subtype, based on the mRNA signature reported by Verhaak et al. [407]. However, molecular subtypes based on miRNA signatures have also been reported [197]. This has revealed five classes of GBM; neural, oligoneural, multipotent, astrocytic, and neuromesenchymal. These five groups were found to have gene markers corresponding to oligoneural, radial glial, neural, neuromesenchmal, and astrocytic. FABP7, a marker of the radial glial group, was found to belong to the classical subtype [197]. This supported the finding that FABP7 belonged to the classical subtype of GBM. Other genes such as PAX6, NES, SOX2 and GFAP, all of which are related in one way or another to FABP7 expression, was represented in this classical subtype [8, 117, 118, 181, 243, 250, 285, 350]. Interestingly, EGFR was also associated with the classical subtype, being amplified or expressed in the vIII mutated form. Overexpression of EGFR has been associated with nuclear FABP7 localisation, migration and poor survival [184, 243] and FABP7 expression is downstream of Ras-independent EGFR signalling in Schwann cells [278]. The correlation between EGFR overexpression and FABP7 in GBM could therefore simply be a question of molecular subtype. FABP7 could still be regulated by EGFR in a yet undetermined fashion, and EGFR seems to have an effect on the localisation and properties of FABP7, all of which further strengthens the association of FABP7 to the classical subtype. The subtyping of FABP7 as classical fits with its role in supporting tumour growth, however, the mesenchymal subtype has been associated with invasion [304] and stem cell markers [316], both properties attributed to FABP7. In addition, the mesenchymal class is furthermore associated with inflammation [9], and tumour immunomodulation. Through database mining (chapter 6), FABP7 was found to be regulated in some way (yet to be identified) by transcription factors linked to inflammation, all of which fall in the mesenchymal subtype (Chapter 7 and 8, respectively. Tables **??** and **??**). The inflammatory response mediates malignant gliomagenesis shaping the tumour microenvironment [61]. Reactive gliosis and scar formation, a result of inflammation, are also observed in infiltrative forms of glioma [340]. It appears that FABP7 participate in the homeostasis of CD4+ T-cells [397] and it recently emerged

that FABP7 can function as an intracellular endocannabinoid transporter, an important modulator of the immune system [21, 182, 311]. Based on this information, it could have been expected that FABP7 would belong to the more mesenchymal grouping, however, tumours with high FABP7 expression were of either classical or mesenchymal subtype in the tissue cohort, which lead to the speculation of FABP7 having a dual role in GBM pathogenesis. Could FABP7 belong to both classes, but have different functional roles or properties? This question can in part be answered by the fact that FABP7, though predominantly expressed in the brain, is found in mammary tissue where it has been shown to have properties of inhibiting tumour growth [155, 357].

7 Refining the Transcriptional Regulatory Landscape to Include the Role of MicroRNA and FABP7 Expression

The elusiveness of FABP7 and the poor correlation between FABP7 expression and TFs analysed suggested that other "factors" are at play. Here, we have considered the role of miRNA and there are no known miRNA that are directly involved in FABP7 regulation. However, one miRNA has been functionally characterised to target FABP7 in mouse, a microRNA that is not predicted to target the human FABP7 counterpart. There are several microRNA candidates that could potentially target FABP7, which would need to be confirmed experimentally. In a preliminary study, two microRNAs (miR-181 and miR-382) identified through miRBase and known to be expressed in GBM tissue were investigated for their impact on FABP7 expression in vitro. Preliminary studies has shown that down regulation of FABP7 expression was observed in the model cells upon miRNA transfection . However, further validation was needed to substantiate this finding with the aid of a reporter gene system.

8 Tying Part One and Two Together

The functional role of many differentially regulated microRNAs are still to be elucidated, however, it is apparent that microRNAs have a huge impact on the biology of GBM. Of the microRNAs that were found differentially regulated across the tumour samples, 36 were predicted to target FABP7. However, three or more algorithms predicted only four microRNAs, namely miR-181a, miR-181c, miR-382 and miR-383. That is not to say that these four functionally target FABP7 or that any of the remaining 32 does not. How-

ever, these four candidates could be interesting to further study with respect to the FABP7 regulatory landscape. However, the involvement of microRNAs has a role in cell-to-cell communication and facilitating the progression of tumour growth. The paracrine effect through miRNAs transported by exosomes could very well target either FABP7 or any of its TFs. The interconnected web of regulatory elements is vast, and to this end, this thesis only scratches the surface.

9 Future Perspectives

The complex web of regulation has only begun to be revealed. The findings of this thesis has (i) gathered a basic understanding of the role of miRNAs in GBM and given a comprehensive overview of all the available literature, (ii) evaluated miRNA signatures as a function of prognosis and survival, (iii) investigated the possibility of combining miRNA expression with clinicopathological features, (iv) provided an up-to-date account of regulatory elements of the FABP7 gene and (v) investigated the expression of FABP7 in GBM to correlated it with the expression of known regulatory elements. However, there are still several lines which are of great interest to pursue.

Based on the discovery that a large part of the differentially regulated miRNAs in GBM contained EXO-motifs, an indication of cell-to-cell communication and paracrine signalling, it would be

[1] valuable to investigate the intercellular communication in GBM tumours. This could provide further understanding on how miRNA signatures are generated and regulated, and which underlying functions are responsible for the phenotype and patient outcome. Such work could possibly identify GBM exosomes and their content as novel therapeutic targets or biomarkers of disease.

Since miRNAs has a huge impact on the biology of GBMs and due to their connection with the clinicopathological features, it would be highly relevant to

[2] validate miRNAs found to target FABP7, since only one miRNA to date (miR-21) has been found to regulate FABP7 in mouse.

This would expand the regulatory landscape of FABP7 which could further explain the elusive behaviour of FABP7 in primary cell cultures and could be combined with molecular subtypes og GBM which could shed light on the diversity of GBM. With regard to the expression of FABP7 and the regulatory map generated, the following would also be relevant to investigate further

[3] Further experimental evidence will be required to confirm those newly discovered TFs (from ENCODE) and their effect on FABP7 transcriptional regulation.

[4] The intriguing presence at FABP7 locus of a cohesin complex and TFs involved in chromatin dynamics opens new perspectives to study their impact on FABP7 develpment expression and overexpression in aggressive cancers.

[5] If it is assumed that FABP7 upregulation in non-neoplastic reactive glioma is under control of TFs coordinating inflammatory or immune responses, it would be interesting to determine whether these TFs also control FABP7 re-expression in GBM.

Appendices

A

Table A.1: MicroRNAs upregulated in cluster 1 (long survival) and reported upregulated in GBM compared to normal tissue.

hsa-let-7a	hsa-miR-93*	hsa-miR-194	hsa-miR-381	hsa-miR-519d
hsa-let-7b	hsa-miR-99a	hsa-miR-200b	hsa-miR-383	hsa-miR-520d-3p
hsa-let-7f	hsa-miR-125b-2*	hsa-miR-200c	hsa-miR-411	hsa-miR-532-3p
hsa-miR-16	hsa-miR-125b	hsa-miR-204	hsa-miR-455-3p	hsa-miR-532-5p
hsa-miR-20a	hsa-miR-140-3p	hsa-miR-222	hsa-miR-455-5p	hsa-miR-589
hsa-miR-20b	hsa-miR-140-5p	hsa-miR-324-3p	hsa-miR-484	hsa-miR-589
hsa-miR-23b	hsa-miR-146a	hsa-miR-345	hsa-miR-500	hsa-miR-625
hsa-miR-24	hsa-miR-150	hsa-miR-361-3p	hsa-miR-501-5p	hsa-miR-885-5p
hsa-miR-26a	hsa-miR-152	hsa-miR-361-5p	hsa-miR-517c	
hsa-miR-30a-5p	hsa-miR-191	hsa-miR-362-5p	hsa-miR-518f	
hsa-miR-30d	hsa-miR-192*	hsa-miR-374b	hsa-miR-519a	

Table A.2: MicroRNAs upregulated in cluster 1 (long survival) and reported downregulated in GBM compared to normal tissue.

hsa-miR-29b	hsa-miR-127-3p	hsa-miR-139-5p	hsa-miR-326	hsa-miR-422a
hsa-miR-29c	hsa-miR-128	hsa-miR-146b-5p	hsa-miR-328	hsa-miR-454
hsa-miR-34a	hsa-miR-129-3p	hsa-miR-149	hsa-miR-330-3p	hsa-miR-487b
hsa-miR-95	hsa-miR-129-5p	hsa-miR-181a	hsa-miR-342-3p	hsa-miR-491-5p
hsa-miR-98	hsa-miR-129*	hsa-miR-181c	hsa-miR-342-5p	hsa-miR-495
hsa-miR-100	hsa-miR-132	hsa-miR-184	hsa-miR-369-5p	hsa-miR-543
hsa-miR-103	hsa-miR-134	hsa-miR-197	hsa-miR-370	hsa-miR-574-3p
hsa-miR-124	hsa-miR-137	hsa-miR-203	hsa-miR-376a	hsa-miR-598
hsa-miR-125a-5p	hsa-miR-138	hsa-miR-218	hsa-miR-379	hsa-miR-628-3p
hsa-miR-126	hsa-miR-139-3p	hsa-miR-323-3p	hsa-miR-410	

Table A.3: MicroRNAs upregulated in cluster 1 (long survival) and with disputed reports about their regulation in GBM compared to normal tissue.

hsa-let-7d	hsa-miR-27b	hsa-miR-143	hsa-miR-195	hsa-miR-338-5p	hsa-miR-26b
hsa-miR-30c	hsa-miR-145				

Table A.4: MicroRNAs upregulated in cluster 1 (long-term survival) with no reported dysregulation in GBM.

hsa-let-7e	hsa-miR-99a*	hsa-miR-365	hsa-miR-512-3p	hsa-miR-874
hsa-let-7g	hsa-miR-99b*	hsa-miR-376c	hsa-miR-539	hsa-miR-935
hsa-miR-31	hsa-miR-126*	hsa-miR-377*	hsa-miR-551b*	hsa-miR-1179
hsa-miR-628-5p	hsa-miR-151-3p	hsa-miR-382	hsa-miR-592	hsa-miR-1180
hsa-miR-26b*	hsa-miR-151-5p	hsa-miR-409-3p	hsa-miR-642	hsa-miR-1244
hsa-miR-27b*	hsa-miR-181c*	hsa-miR-433	hsa-miR-655	hsa-miR-1253
hsa-miR-29a	hsa-miR-186	hsa-miR-485-3p	hsa-miR-664	hsa-miR-1260
hsa-miR-29b-1*	hsa-miR-213	hsa-miR-488	hsa-miR-758	hsa-miR-1271
hsa-miR-29b-2*	hsa-miR-331-5p	hsa-miR-488	hsa-miR-769-5p	
hsa-miR-30b	hsa-miR-339-3p	hsa-miR-502-3p	hsa-miR-770-5p	

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