Oligodendrogliomas with LOH 1p/19q: Identifying Genes associated with Tumourigenesis and Therapeutic Sensitivity

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Declaration

The work described in this thesis was performed by the candidate, except where due acknowledgement has been made.

I declare that no part of this work has been submitted previously for the purpose of obtaining any other degree.

Cathy A. Payne

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Abstract

Loss of heterozygosity (LOH) of the short arm of chromosome 1 (1p) and the long arm of chromosome 19 (19q) is an early event in oligodendroglioma that occurs in up to 80% of patients and is associated with therapeutic sensitivity and longer overall survival. The purpose of this study was to confirm the reported association in patients at this centre, then identify and characterise genes that were differentially expressed and may function as a tumour suppressor or contribute to therapeutic sensitivity in oligodendroglioma.

A clinical review of oligodendroglioma patients treated at Royal North Shore and North Shore Private Hospitals between 1990 and 2009 confirmed the association between LOH 1p/19q and longer overall survival in WHO grade III oligodendroglioma patients. Younger age and lower tumour grade were additionally confirmed as positive prognostic factors.

Exon microarrays were used to identify changes in gene expression between oligodendrogliomas with and without LOH 1p/19q. Seventeen oligodendroglioma specimens (5 WHO grade II with LOH 1p/19q, 2 WHO grade II without LOH, 5 WHO grade III with LOH 1p/19q, 5 WHO grade III without LOH) were examined by microarrays. Analysis in Partek identified 408 genes that were differentially expressed by LOH status. Six candidate genes (*CHI3L1, IGF2, IQGAP1, MIG-6, PDPN* and *PLAG1*), which all showed significantly lower expression in oligodendrogliomas with LOH 1p/19q, were selected for further study.

Microarray analysis of DNA methylation in seven oligodendrogliomas with LOH 1p/19q (4 WHO grade II, 3 WHO grade III) identified DNA methylation of the *PADI2* and *ALX3* genes on chromosome 1p. Bisulfite sequencing, restriction analysis and qPCR confirmed *PADI2* as a candidate tumour suppressor gene, methylated in oligodendrogliomas with LOH 1p/19q.

qPCR in forty-six oligodendroglioma specimens confirmed under-expression of each of the seven candidate genes in oligodendrogliomas with LOH 1p/19q. Survival analysis identified high Podoplanin (*PDPN*) transcript expression as a negative prognostic factor. Immunohistochemistry conducted on thirty grade III

oligodendrogliomas demonstrated that protein staining scores for CHI3L1 and IQGAP1 were significantly higher in tumours without LOH 1p/19q, but found that IGF2, MIG-6 or PDPN were not suitable as markers for LOH 1p/19q or survival. Functional analysis of MIG-6 in H423 glioblastoma cells demonstrated the ability of MIG-6 to inhibit cell invasion, validating its role as a potential tumour suppressor in glioma.

As no oligodendroglioma cell line with LOH 1p/19q was available, forty-six low grade tumour specimens were cultured to form primary cell lines. While nine cell lines retained an *IDH1* mutation typical of oligodendroglioma, no cell line maintained LOH 1p/19q.

This work has confirmed LOH 1p/19q as a positive prognostic marker for oligodendroglioma and identified seven differentially expressed genes that, through their loss following LOH 1p/19q, could contribute to the development of oligodendroglioma or confer therapeutic sensitivity to the patient.

Table of Contents

Declaration ..... **ii**

Acknowledgements ..... **iii**

Abstract ..... **iv**

Table of Contents ..... **vi**

List of Figures ..... **xiii**

List of Tables ..... **xvi**

Abbreviations used in this thesis ..... **xix**

Publications ..... **xxiii**

Presentations ..... **xxiii**

1 Review of the Literature **1**

1.1 Brain tumours **1**

1.1.1 Occurrence 1

1.1.2 Cost 1

1.1.3 Classification 2

1.2 Oligodendrogliomas **5**

1.2.1 Pathology 5

1.2.2 Histology 5

1.2.3 Prognosis 8

1.2.4 Treatment 8

1.3 Genetics of Oligodendroglioma **11**

1.3.1 LOH 1p/19q 13

1.3.1.1 Detection of LOH 1p/19q 18

1.3.1.2 Mechanism of LOH 1p/19q 22

1.3.2 *IDH1* and *IDH2* mutations 24

1.3.3 *MGMT* promoter methylation 26

1.3.4 DNA methylation (G-CIMP, CIMP) 26

1.3.5 Other genetic alterations 27

1.3.6 Impact of genetic markers on prognosis 28

1.4 LOH in other cancers **30**

1.5 Emerging technologies for cancer genetics research **31**

1.6 Oligodendroglioma candidate genes on 1p and 19q **34**

1.7 Summary and Aims **39**

2 Materials and Methods **40**

2.1 Introduction **40**

2.2 Ethics **40**

2.2.1 Brain tumour tissue collection at the Kolling Institute of Medical Research 40

2.2.2 Tumour tissue collection at Duke University Medical Center 40

2.3 Tumour specimens **41**

2.3.1 Tumour banking at the Kolling Institute 41

2.3.2 Preston Robert Tisch Brain Tumor Center Biorepository at Duke University 41

2.4 RNA **41**

2.4.1 RNA extraction from fresh frozen tumour tissue 42

2.4.2 RNA extraction from cultured tumour cell lines 42

2.4.3 RNA purification – lithium chloride 43

2.4.4 RNA quantification – Nano Drop 43

2.4.5 Quality control of RNA 44

2.4.5.1 Assessment of genomic DNA contamination: GUSβ 44

2.4.5.2 Measurement of RNA integrity on the Agilent Bioanalyzer 2100 44

2.4.6 Removal of DNA contamination 45

2.4.6.1 Acidified phenol chloroform RNA purification 45

2.4.6.2 DNase treatment 46

2.4.7 Normal brain control RNAs 47

2.4.8 cDNA Synthesis 47

2.5 DNA **48**

2.5.1 DNA extraction from fresh frozen tumour tissue 48

2.5.1.1 Phenol chloroform DNA extraction 48

2.5.1.2 QIAamp DNA mini kit DNA extraction 49

2.5.2 DNA extraction from blood 49

2.5.3 DNA extraction from paraffin embedded tumour tissue 50

2.5.4 DNA extraction from cell lines 51

2.6 PCR **52**

2.6.1 Standard PCR 52

2.6.2 Primers 53
2.6.3 cDNA sequencing for *IDH1* and *IDH2* 55
2.6.4 Sequencing 55
2.6.5 Assessment of genomic DNA contamination: *GUSβ* 55
2.6.6 Visualisation of PCR products 56
2.6.6.1 Agarose gel electrophoresis 56
2.6.6.2 Polyacrylamide gel electrophoresis 56
2.6.7 Quantitative real-time PCR 57
2.6.7.1 Standard curves 58
2.6.7.2 qPCR analysis 58
2.6.8 Detection of loss of heterozygosity by microsatellite analysis (PaLMS) 59
2.7 Immunohistochemistry 62
2.7.1 Detection of loss of heterozygosity by fluorescence *in situ* hybridisation 62
2.8 Protein and Western blot 63
2.8.1 Protein extraction from cultured cells 63
2.8.2 Protein quantification 64
2.8.3 Western blot 64
2.8.3.1 Sample preparation 64
2.8.3.2 Gel setup, loading and running 65
2.8.3.3 Protein transfer 65
2.8.3.4 Antibody hybridisation and washing 65
2.8.3.5 Signal detection 66
3 Clinical Review of Oligodendroglioma cases at Royal North Shore and North Shore Private Hospitals 67
3.1 Introduction 67
3.1.1 Overview 67
3.1.2 TP53 68
3.1.3 Aims 68
3.2 Methods 69
3.2.1 Clinical cohort 69
3.2.2 Loss of Heterozygosity of 1p and 19q 69

3.2.3 TP53 Immunohistochemistry ... 70
3.2.4 Statistical Analysis ... 70
3.3 Results ... 71
3.3.1 Loss of heterozygosity 1p and 19q (LOH 1p/19q) ... 71
3.3.2 Univariate analysis of survival for patients diagnosed with grade III oligodendroglioma ... 75
3.3.3 Multivariate analysis of survival for patients diagnosed with grade III oligodendroglioma ... 77
3.3.4 Prognostic significance of TP53 protein expression ... 77
3.4 Discussion ... 79
4 Microarray analysis of gene expression and DNA methylation in oligodendrogliomas with and without LOH 1p/19q ... 81
4.1 Introduction ... 81
4.1.1 Overview ... 81
4.1.2 Gene expression analysis of oligodendroglioma ... 81
4.1.3 DNA methylation analysis of oligodendroglioma ... 84
4.1.4 Aims ... 86
4.2 Methods ... 87
4.2.1 Exon array analysis of gene expression. ... 87
4.2.1.1 Specimens ... 87
4.2.1.2 RNA and Exon array hybridisation ... 87
4.2.1.3 Exon array data analysis ... 88
4.2.1.3.1 GeneSpring ... 88
4.2.1.3.2 Partek ... 88
4.2.1.3.3 Gene Set Enrichment Analysis ... 89
4.2.2 CpG island plus promoter array analysis of DNA methylation ... 90
4.2.2.1 Methylated DNA immunoprecipitation ... 90
4.2.2.2 CpG island plus promoter array ... 91
4.3 Results ... 92
4.3.1 Oligodendroglioma specimens analysed by exon array ... 92
4.3.2 Overview of differential gene expression ... 94
4.3.3 Differential gene expression in grade II and III Oligodendrogliomas ... 94
4.3.3.1 Principal Components Analysis ... 94

4.3.3.2 Genes differentially expressed in oligodendrogliomas separated by LOH status........ 97
4.3.4 Analysis of alternative splicing in Oligodendrogliomas 101
4.3.5 Selection of candidate genes for further study........ 103
4.3.6 Gene Set Enrichment Analysis 105
4.3.7 Analysis of DNA methylation in oligodendrogliomas with LOH 1p/19q 108
4.4 Discussion........ 112
4.4.1 Conclusion 117

5 Validation and functional analysis of candidate genes in oligodendroglioma 119
5.1 Introduction........ 119
5.1.1 Overview 119
5.1.2 Aims........ 120
5.2 Methods 121
5.2.1 Quantitative Real-Time PCR 121
5.2.2 Validation of DNA methylation........ 122
5.2.2.1 Bisulfite Sequencing 122
5.2.2.2 COBRA........ 123
5.2.3 Immunohistochemistry 124
5.2.4 Analysis and survival statistics........ 126
5.2.5 Cell lines........ 126
5.2.6 Plasmids 127
5.2.7 Transfections........ 127
5.2.8 MTT assay 127
5.2.9 Colony formation assay 128
5.2.10 Wound healing assay........ 128
5.2.11 Trans-well migration assay 129
5.3 Results 130
5.3.1 qPCR validation of differential gene expression in oligodendroglioma 130
5.3.1.1 Validation of DNA methylation 136

5.3.2 Expression of CHI3L1, IGF2, IQGAP1, MIG-6 and PDPN proteins in oligodendroglioma ... 139
5.3.2.1 Survival analysis ... 147
5.3.3 Functional analysis of MIG-6 in glioma cell lines ... 151
5.3.3.1 Endogenous MIG-6 expression ... 151
5.3.3.2 Over-expression and knockdown of MIG-6 in glioma cell lines .. 154
5.3.3.3 MIG-6 and cell proliferation ... 156
5.3.3.4 MIG-6 and cell migration ... 162
5.4 Discussion ... 165
5.4.1 Conclusion ... 168
6 Primary culture of oligodendrogliomas ... 170
6.1 Introduction ... 170
6.1.1 Overview ... 170
6.1.2 Aims ... 172
6.2 Methods ... 173
6.2.1 Ethics ... 173
6.2.2 Dissociation and culture of primary tumours ... 173
6.2.3 Cell maintenance and recording ... 173
6.2.4 DNA extraction, PCR and sequencing ... 175
6.2.5 LOH screening of cell lines ... 175
6.2.5.1 Visualisation and detection of LOH ... 177
6.3 Results ... 178
6.3.1 Primary tumour specimens ... 178
6.3.2 Primary cell culture of tumour specimens ... 178
6.3.3 *IDH1* and *TP53* mutations in primary tumours and cell cultures ... 182
6.3.4 LOH 1p/19q in primary tumours and cell cultures ... 185
6.3.5 Primary cell culture of a grade III oligodendroglioma (TB09-0655) – Detailed notes on a representative specimen and its derivative primary cell cultures ... 187
6.4 Discussion ... 190
6.4.1 Morphology and genetics of primary cell cultures ... 190
6.4.2 Known difficulties in culture of oligodendroglioma cell lines ... 191
6.4.3 Alternative techniques for primary cell culture ... 192

6.4.4 Conclusion 193

7 Discussion 194

7.1 Key findings 194

7.2 Recommendations from this thesis 197

7.3 Limitations of this study 200

7.4 Candidate genes in oligodendroglioma 201

7.5 Future directions for research 203

7.6 Conclusions 205

References 206

Appendices 223

Appendix 1: List of Reagents and Materials 224

Appendix 2: Neuroendocrine Tumour Bank Patient Consent Form and Patient Information Sheet 227

Appendix 3A: Brain tumour specimens used in this thesis for exon array, quantitative PCR, immunohistochemistry and CpG array 232

Appendix 3B: Normal Brain purchased specimens (RNA): Donors of Normal Brain RNA 234

Appendix 4: 408 genes differentially expressed in 17 oligodendrogliomas separated by LOH status 235

Appendix 5: pCMV6-Entry-MIG6 plasmid map 243

List of Figures

Figure 1.1 Oligodendroglioma imaging. 6

Figure 1.2 Haematoxylin and eosin stain of a grade III oligodendroglioma. 7

Figure 1.3 Genetic mutations and events that contribute to specific types of glioma. 12

Figure 1.4 Morphological and genetic features used to classify astrocytomas, oligoastrocytomas and oligodendrogliomas. 15

Figure 1.5 Kaplan Meier survival plot of overall survival in grade III oligodendroglioma patients stratified by LOH 1p/19q. 17

Figure 1.6 Representative images for PCR microsatellite for LOH. 19

Figure 1.7 FISH analysis of chromosomal arm 1p on isolated cell nuclei. 21

Figure 1.8 LOH 1p/19q occurs due to an unbalanced translocation. 23

Figure 1.9 *IDH1* mutations are associated with improved survival. 25

Figure 1.10 Classification of grade III oligodendrogliomas by LOH 1p/19q, *TP53* mutations and other specific genetic alterations. 29

Figure 1.11 The GeneChip™ Human Exon 1.0 ST array. 32

Figure 2.1 Interpretation of microsatellite PCR results for the detection of LOH on chromosomes 1p and 19q. 61

Figure 3.1 Survival analysis of patients diagnosed with oligodendroglioma. 74

Figure 3.2 Survival analysis of patients diagnosed with grade III oligodendroglioma. 76

Figure 3.3 Influence of TP53 intensity measured by immunohistochemistry on grade III oligodendroglioma patients without LOH 1p/19q 78

Figure 4.1 Volcano plots of differentially expressed genes between oligodendroglioma and glioblastoma. 95

Figure 4.2 Principal Components Analysis. 96

Figure 4.3 Hierarchical clustering of oligodendrogliomas. 100

Figure 4.4 Alternate splice diagram of *RUNX1*. 102

Figure 4.5 Relative transcript expression of candidate genes *CHI3L1*, *IGF2*, *IQGAP1*, *MIG-6*, *PDPN* and *PLAG1*. 104

Figure 4.6 Positional gene sets for chromosomes 1p and 19q and glioblastoma phenotypes associated with poor survival are over represented in grade III oligodendrogliomas without LOH 1p/19q. 106

Figure 4.7 *PADI2* and *ALX3* were methylated in oligodendrogliomas with LOH 1p/19q. 110

Figure 5.1 *CHI3L1*, *IGF2* and *IQGAP1* expression in oligodendrogliomas. 133

Figure 5.2 *MIG-6*, *PDPN* and *PLAG1* expression in oligodendrogliomas. 134

Figure 5.3 *EGFR*, *ALX3* and *PADI2* expression in oligodendrogliomas. 135

Figure 5.4 Bisulfite sequencing confirmed methylation of *PADI2* in three oligodendrogliomas with LOH 1p/19q 137

Figure 5.5 Methylation of *PADI2* confirmed by COBRA. 138

Figure 5.6 Expression and localisation of CHI3L1 in grade III oligodendrogliomas. 143

Figure 5.7 IQGAP1 staining in grade III oligodendrogliomas. 144

Figure 5.8 MIG-6 staining in grade III oligodendrogliomas. 145

Figure 5.9 PDPN staining in grade III oligodendrogliomas. 146

Figure 5.10 Kaplan-Meier survival curves show the prognostic value of *CHI3L1*, *IGF2*, *IQGAP1*, *MIG-6*, *PDPN* and *PLAG1* mRNA expression 149

Figure 5.11 Kaplan-Meier survival curves show prognostic value of CHI3L1, IGF2, IQGAP1, MIG-6 and PDPN protein staining in grade III oligodendroglioma patients 150

Figure 5.12 Endogenous expression of *MIG-6* in oligodendroglioma and glioblastoma cell lines. 152

Figure 5.13 Heatmap of *MIG-6* copy number data in TCGA glioblastoma samples. 153

Figure 5.14 MIG-6 knockdown in U87MG glioblastoma cells. 155

Figure 5.15 Restoration of *MIG-6* expression did not affect cell growth rate in H423 glioblastoma cells. 157

Figure 5.16 Over-expression of *MIG-6* did not affect cell growth rate in HOG or TC620 oligodendroglioma cells. 158

Figure 5.17 Knockdown of *MIG-6* did not affect cell growth rate in U87MG glioblastoma cells. 159

Figure 5.18 Overexpression of *MIG-6* did not alter colony formation in H423 glioblastoma cells. 160

Figure 5.19 Knockdown of *MIG-6* did not alter colony formation in U87MG glioblastoma cells. 161

Figure 5.20 *MIG-6* expression alters wound healing in glioma cell lines. 163

Figure 5.21 *MIG-6* alters cell migration in glioma cells. 164

Figure 6.1 Morphologies observed in SC, SD and ZO primary cell cultures............. 181

Figure 6.2 Morphology of SC cell lines derived from tumours harbouring an *IDH1* mutation. 184

Figure 6.3 Morphology of grade III oligodendroglioma (TB09-0655) cells under different culture conditions. 188

Figure 6.4 Subculture of grade III oligodendroglioma (TB09-0655) cells from SC to SD (2% FCS) media. 189

List of Tables

Table 1.1 Twelve categories of tumour affecting the Central Nervous System as defined by the World Health Organisation (Louis et al., 2007) 4

Table 1.2 Reported proportions of oligodendroglioma with LOH at 1p/19q 13

Table 1.3 Genes on 1p and 19q that have been studied in oligodendroglioma for their role in tumourigenesis or survival advantage following LOH 1p/19q 35

Table 2.1 Components of SuperScript™ Reverse Transcription master mix 47

Table 2.2 Standard PCR reaction components 52

Table 2.3 Primers used for PCR amplification and DNA sequencing 54

Table 2.4 Components of PCR reactions for *GUSβ* analysis in cDNA samples 55

Table 2.5 Standard qPCR reaction components 57

Table 2.6 TaqMan® gene expression assays used in this thesis 58

Table 2.7 Primers for PCR of microsatellite markers for the detection of loss of heterozygosity 59

Table 2.8 Components of PCR reactions for PaLMS detection of LOH 1p/19q 60

Table 2.9 Components of Protein Lysis Buffer 63

Table 2.10 Antibodies used in Western blot 66

Table 3.1 Case series of 13 grade II and III oligodendroglioma samples examined for LOH 1p/19q by PCR and FISH 71

Table 3.2 Clinical characteristics of oligodendroglioma patients 73

Table 3.3 Cox-proportional hazards model: Factors influencing survival in grade III oligodendroglioma patients 77

Table 4.1 Microarray studies of oligodendroglioma with LOH 1p/19q 83

Table 4.2 Gene set matrices used for GSEA 90

Table 4.3 Oligodendroglioma specimens selected for exon array analysis 92

Table 4.4 Additional RNA samples analysed by exon array 93

Table 4.5 Top 20 differentially expressed genes in oligodendrogliomas with LOH 1p/19q 98

Table 4.6 Top 20 genes on 1p and 19q most under-expressed (by fold change) in oligodendrogliomas with LOH 1p/19q 99

Table 4.7 Top ten alternately spliced genes 101

Table 4.8 Expression of candidate genes in analyses of grade II, grade III and all oligodendrogliomas separated by LOH 1p/19q status 103

Table 4.9 Top 20 Biological Process, Cellular Compartment and Molecular Function GO classes over represented in grade III oligodendrogliomas 107

Table 4.10 Oligodendroglioma patient specimens analysed by CpG island plus promoter array 108

Table 4.11 Candidate genes near CpG islands methylated in oligodendrogliomas with LOH 1p/19q and unmethylated in normal brain specimens 109

Table 4.12 DNA Methylation of candidate differentially expressed genes selected from gene expression microarray analysis 111

Table 4.13 Expression and methylation findings for oligodendroglioma genes identified in previous studies 114

Table 5.1 Calculated efficiency values for TaqMan gene expression assays 121

Table 5.2 Primers used for the validation of *PADI2* methylation 123

Table 5.3 Immunohistochemistry conditions 124

Table 5.4 Oligodendroglioma RNA samples used for qPCR 130

Table 5.5 Mean expression, Fold change and p-value for *CHI3L1, IGF2*, *IQGAP1*, *MIG-6, EGFR*, *PDPN, PLAG1*, *ALX3* and *PADI2* analysed by qPCR in oligodendrogliomas 132

Table 5.6 Specimens analysed by immunohistochemistry 139

Table 5.7 Protein expression scores for CHI3L1 in grade III oligodendrogliomas, classified by LOH 1p/19q 141

Table 5.8 Protein expression scores for IGF2 in grade III oligodendrogliomas, classified by LOH 1p/19q 141

Table 5.9 Protein expression scores for IQGAP1 in grade III oligodendrogliomas, classified by LOH 1p/19q status 141

Table 5.10 Protein expression scores for MIG-6 in grade III oligodendrogliomas, classified by LOH 1p/19q status 142

Table 5.11 Protein expression scores for PDPN in grade III oligodendrogliomas, classified by LOH 1p/19q status 142

Table 5.12 Survival among grade III oligodendroglioma patients 148

Table 5.13 Expression of *MIG-6* in Oligodendroglioma and glioblastoma cell lines 151

Table 6.1 Composition of Transportation media 174

Table 6.2 Composition of Stem cell (SC) media 174

Table 6.3 Composition of SD media 174

Table 6.4 Primer pairs for amplification of microsatellite markers on chromosomes 1 and 19 176

Table 6.5 Components of PCR reactions for detection of LOH 1p/19q 176

Table 6.6 Biorepository number, diagnosis and cell lines established form primary culture of tumour specimens 180

Table 6.7 Summary of *IDH1* and *TP53* mutations in primary tumours and primary cell cultures 183

Table 6.8 LOH 1p/19q in primary oligodendroglial tumours and cell lines 186

Abbreviations used in this thesis

Abbreviation	Full Term
1°	Primary Tumour
10q	Human Chromosome 10q
19q	Human Chromosome 19q
1p	Human Chromosome 1p
2°	Secondary Tumour
9p	Human Chromosome 9p
A172	A172 Human Glioblastoma Cell Line
AII	Grade II Astrocytoma
AIII	Grade III Astrocytoma
ALX3	Aristaless-Like 3
ARHGEF16	Rho Guanine Nucleotide Exchange Factor (GEF) 16
bp	Base Pair
BSA	Bovine Serum Albumin
CATNON	Phase III Trial On Concurrent And Adjuvant Temozolomide Chemotherapy In Non-1p/19q Deleted Anaplastic Glioma
CCNU	*N*-(2-Chloroethyl)-*N'*-Cyclohexyl-*N*-Nitrosourea, A.K.A. Lomustine
CDKN2A	Cyclin-Dependent Kinase Inhibitor 2A
cDNA	Complementary DNA
CEL	Cell Intensity File
CGH	Comparative Genomic Hybridisation
CHI3L1	Chitinase 3-Like-1
CIC	Capicua Homolog
CIMP	Cpg Island Methylator Phenotype
CODEL	The Phase III Randomized Study Of Radiotherapy Alone Versus Radiotherapy With Concurrent And Adjuvant Temozolomide Versus Temozolomide Alone In Patients With Newly Diagnosed 1p/19q Codeleted Anaplastic Glioma

Abbreviation	Full Term
CT	Computed Tomography
DAPI	4',6-Diamidino-2-Phenylindole (Nuclear Stain)
der(1;19)(q10;p10)	Derivative Chromosome of Unbalanced Translocation Between Chromosomes 1 and 19
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide
DPBS	Dulbecco's Phosphate-Buffered Saline
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EORTC	European Organisation for the Research and Treatment of Cancer
FDR	False Discovery Rate
FISH	Fluorescent *In Situ* Hybridisation
FUBP1	Far Upstream Element Binding Protein 1
FWER	Family-Wise Error Rate
G-CIMP	Glioma-Cpg Island Methylator Phenotype
GBM	Glioblastoma Multiforme
H423	Human Glioblastoma Cell Line H423
HOG	Human Oligodendroglioma Cell Line
IDH	Isocitrate Dehydrogenase
IDH1	Isocitrate Dehydrogenase 1
IDH2	Isocitrate Dehydrogenase 2
IGF2	Insulin-Like Growth Factor 2
IP	Immunoprecipitation
IQGAP1	IQ Motif Containing GTPase Activating Protein 1
IRB	Institutional Review Board
Kb	Kilobase
LOH	Loss Of Heterozygosity
LOH 1p/19q	Loss Of Heterozygosity Of Chromosomes 1p And 19q
M	Molar, Moles Per Litre

Abbreviation	Full Term
MGMT	O-6-Methylguanine-DNA Methyltransferase
MIG-6	ERBB Receptor Feedback Inhibitor 1
	A.K.A. Mitogen Inducible Gene 6
MO3.13	MO3.13 Human Oligodendroglioma Cell Line
MRI	Magnetic Resonance Image
mRNA	Messenger Ribonucleic Acid
NSP	North Shore Private Hospital
OAII	Grade II Oligoastrocytoma
OAIII	Grade III Oligoastrocytoma
OII	Grade II Oligodendroglioma
OIII	Grade III Oligodendroglioma
PADI2	Peptidyl Arginine Deiminase, Type II
PaLMS	Pacific Laboratory Medicine Services
PCR	Polymerase Chain Reaction
PCV	Chemotherapy Regimen Of Procarbazine, CCNU And Vincristine
PDPN	Podoplanin
PLAG1	Pleiomorphic Adenoma Gene I
PTEN	Phosphatase And Tensin Homolog
qPCR	Quantitative Real-Time Polymerase Chain Reaction
QuMA	Quantitative Microsatellite Analysis
RB1	Retinoblastoma I
RMA	Robust Multichip Average
RNA	Ribonucleic Acid
RNase	Ribonuclease
RNSH	Royal North Shore Hospital
RT	Reverse Transcription
RTOG	Radiation Therapy Oncology Group
TC620	TC620 Human Oligodendroglioma Cell Line
TCGA	The Cancer Genome Atlas
TE	Tris EDTA
TP53	Tumour Protein 53

Abbreviation	Full Term
TROG	Trans-Tasman Radiation Oncology Group
U87MG	Human Glioblastoma Cell Line U87MG
WHO	World Health Organisation
wt	Wild Type
μL	Microlitre

Publications

Duncan, C.G., Killela, P.J., Payne, C.A., Lampson, B., Chen, W.C., Liu, J., Solomon, D., Waldman, T., Towers, A.J., Gregory, S.G., McDonald, K.l., McLendon, R.E., Bigner, D.D., Yan, H. Integrated genomic analyses identify ERRFI1 and TACC3 as glioblastoma-targeted genes. Oncotarget 1(4):265-277 (2010).

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Presentations

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Payne, C.A., Parkinson, J.F., Cook, R., Wheeler, H., Robinson, B.G., and McDonald, K.L. Poster: Oligodendrogliomas with LOH 1p/19q: Identifying genes associated with therapeutic sensitivity. The 3rd Quadrennial Meeting of the World Federation of Neuro-Oncology and the 6th Meeting of the Asian Society for Neuro-Oncology, Japan, May 2009.

1 Review of the Literature

1.1 Brain tumours

1.1.1 Occurrence

Malignant brain tumours are a rare but devastating form of cancer, diagnosed in 1440 Australians each year (Chang, 2003), including 500 cases in NSW (Tracey et al., 2008). Five-year survival for brain tumours is extremely poor, remaining steady at around 20% in NSW since 1980 (Tracey et al., 2007). The five-year survival rate for patients diagnosed with glioblastoma, the most common type of primary brain tumour, is less than 5%. This high mortality rate for brain tumours results in a very great burden of disease.

Unlike some other cancers for which the risk of disease may be reduced by adopting healthier lifestyle choices, there are no known measures for the prevention of brain tumours. Early screening would be prohibitively expensive to implement and aside from exposure to ionising radiation usually received as treatment for other cancers, there are no known risk factors for brain tumours, making prevention near impossible (Wrensch et al., 2002, 2005).

1.1.2 Cost

Although brain tumours are relatively uncommon, a British study ranked central nervous system tumours first in years of life lost to cancer (Burnet et al., 2005). Brain tumours are not gender specific, affect all ages and there are no known risk factors or preventative screening measures in place. Due to the impact of central nervous system tumours on cognition, perception and movement, patients suffer high levels of disability and are often unable to return to work following treatment. For many patients, the disease affects their ability to work, drive, and care for

themselves, resulting in a greater burden of responsibility on family members and carers (Short et al., 2005; Patterson, 2007).

The economic burden faced by brain tumour patients is very high. Patients in the U.S.A. report a severe economic burden as a result of the brain tumour diagnosis. In addition to the high costs of treatment, which are often not covered by medical insurance, patients are usually unable to work and require full time care from a family member, resulting in a double loss of income (Bradley et al., 2007; Patterson, 2007).

In NSW, the financial burden on brain tumour patients has been rated as the worst of all cancers. In terms of out of pocket expenses and loss of income faced by households, brain tumours are the most expensive cancer, the average lifetime cost to patients being $149,400. It is also the second most expensive adult cancer to treat per patient, with an average cost of $60,800, which is shared between the federal and state governments, private medical insurance and the patient. The high economic burden on patients is due to a combination of medical costs and loss of present and future income due to a high mortality rate (Access Economics, 2007).

There is a great need for innovation in the treatment and management of brain tumours to improve patient survival and quality of life.

1.1.3 Classification

The World Health Organisation (WHO) Classification of Tumours of the Central Nervous System defines 12 categories of brain tumours (Table 1.1). The most common malignant tumours are astrocytoma, oligodendroglioma and oligoastrocytoma (mixed glioma), and these account for 26% of all brain tumours (Louis et al., 2007). Tumours are further categorised by grade from I to IV, with a higher grade corresponding to increased malignancy and poorer prognosis. Glioblastoma (GBM, WHO grade IV), the most common primary brain tumour in adults, is a highly malignant grade IV astrocytoma with an average survival of just 15 months.

Astrocytomas (grades II-IV), oligoastrocytomas (grades II-III) and oligodendrogliomas (grades II and III) are all diffusely infiltrating brain tumours, such that cure by surgery is futile as tumours inevitably recur. Despite the introduction of temozolomide in 1993 as a chemotherapeutic agent for GBM and recent advances in genetic analysis for treatment, prognosis for malignant brain tumours remains very grim (Hegi et al., 2005).

To further investigate an exciting area of brain tumour research, this thesis will focus on mechanisms that confer a survival advantage in a genetic subgroup of oligodendrogliomas.

Table 1.1 Twelve categories of tumour affecting the Central Nervous System as defined by the World Health Organisation (Louis et al., 2007)

	I	II	III	IV
Astrocytic tumours				
Subependymal giant cell astrocytoma	•			
Pilocytic astrocytoma	•			
Pilomyxoid astrocytoma		•		
Diffuse astrocytoma		•		
Pleomorphic xanthoastrocytoma		•		
Anaplastic astrocytoma			•	
Glioblastoma				•
Giant cell glioblastoma				•
Gliosarcoma				•
Oligodendroglial tumours				
Oligodendroglioma		•		
Anaplastic oligodendroglioma			•	
Oligoastrocytic tumours				
Oligoastrocytoma		•		
Anaplastic oligoastrocytoma			•	
Ependymal tumours				
Subependymoma	•			
Myxopapillary ependymoma	•			
Ependymoma		•		
Anaplastic ependymoma			•	
Choroid plexus tumours				
Choroid plexus papilloma	•			
Atypical choroid plexus papilloma		•		
Choroid plexus carcinoma			•	
Other neuroepithelial tumours				
Angiocentric glioma	•			
Chordoid glioma of the third ventricle		•		
Neuronal and mixed neuronal-glial tumours				
Gangliocytoma	•			
Ganglioglioma	•			
Anaplastic ganglioglioma			•	
Desmoplastic infantile astrocytoma and ganglioglioma	•			
Dysembryoplastic neuroepithelial tumour	•			
Central neurocytoma		•		
Extraventricular neurocytoma		•		
Cerebellar liponeurocytoma		•		
Paraganglioma of the spinal cord	•			
Papillary glioneuronal tumour	•			
Rosette-forming glioneuronal tumour of the fourth ventricle	•			
Pineal tumours				
Pineocytoma	•			
Pineal parenchymal tumour of intermediate differentiation		•	•	
Pineoblastoma				•
Papillary tumour of the pineal region		•	•	
Embryonal tumours				
Medulloblastoma				•
CNS primitive neuroectodermal tumour (PNET)				•
Atypical teratoid / rhabdoid tumour				•
Tumours of the cranial and paraspinal nerves				
Schwannoma	•			
Neurofibroma	•			
Perineurioma	•	•	•	
Malignant peripheral nerve sheath tumour (MPNST)		•	•	•
Meningeal tumours				
Meningioma	•			
Atypical meningioma		•		
Anaplastic / malignant meningioma			•	
Haemangiopericytoma		•		
Anaplastic haemangiopericytoma			•	
Haemangioblastoma	•			
Tumours of the sellar region				
Craniopharyngioma	•			
Granular cell tumour of the neurohypophysis	•			
Pituicytoma	•			
Spindle cell oncocytoma of the adenohypophysis	•			

1.2 Oligodendrogliomas

Oligodendrogliomas account for 5 to 15% of newly diagnosed brain tumours. The peak incidence for oligodendroglioma is between 30 and 60 years and is slightly more common in males (Louis et al., 2007).

1.2.1 Pathology

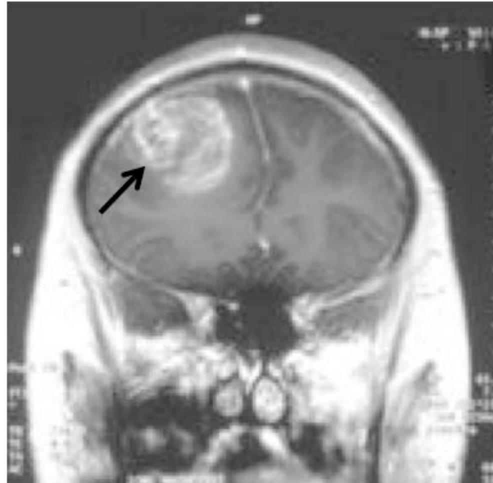
Oligodendrogliomas are traditionally diagnosed following histological examination of the tumour tissue. These tumours have a distinctive morphology (discussed below). Cytogenetic testing of tumour tissue for chromosomal rearrangements (LOH 1p/19q) is routinely conducted at most centres and now testing for *IDH1/2* mutations is also conducted at some sites to generate an accurate diagnosis and prognosis for the patient.

Suspicions of an oligodendroglial tumour first appear on a Magnetic Resonance Image (MRI) as a lesion that does not respect the grey-white junction. Using a Computed Tomography (CT) scan, an oligodendroglioma can often be identified by hypodensity and prominent calcifications (Figure 1.1).

1.2.2 Histology

The histological appearance of an oligodendroglioma shows cells containing uniform round nuclei with perinuclear haloes and vascular proliferation is evident in “chicken-wire” patterning (Figure 1.2) (van den Bent et al., 2008). In many centres, diagnosis relies solely on histopathology and remains very subjective (Coons et al., 1997). The WHO criteria define two grades of oligodendroglioma – grade II, also known as well defined or low-grade, and grade III, also known as anaplastic or high grade (Louis et al., 2007). Grade III oligodendrogliomas are those that have undergone an anaplastic change, displaying histological features such as increased mitotic activity, necrosis and microvascular proliferation. Grade III oligodendrogliomas are more aggressive tumours which are associated with shorter time to progression following treatment and poorer prognosis than grade II tumours.

A



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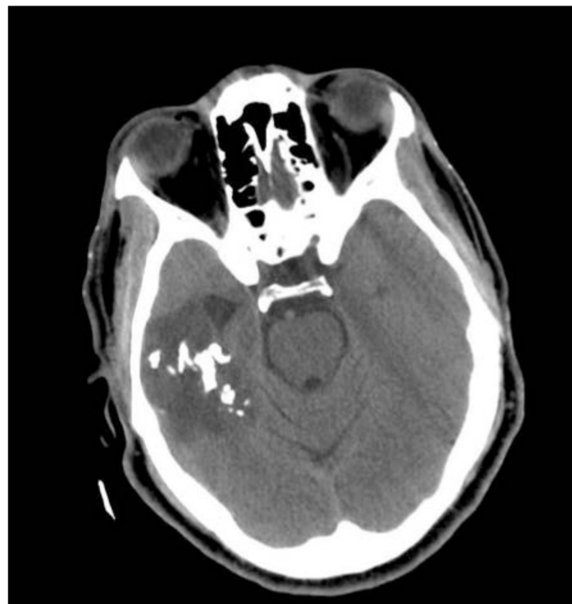


Figure 1.1 Oligodendroglioma imaging.
[A] MRI scan of a patient with an oligodendroglioma - a coronal frontal image of a lesion (indicated by arrow) that does not respect the grey-white junction of the brain matter. [B] CT scan of a patient with an oligodendroglioma - key appearance in this right temporal lobe lesion is hypodensity with prominent calcifications. Images: A. Fowler, Kolling Institute of Medical Research, RNSH.

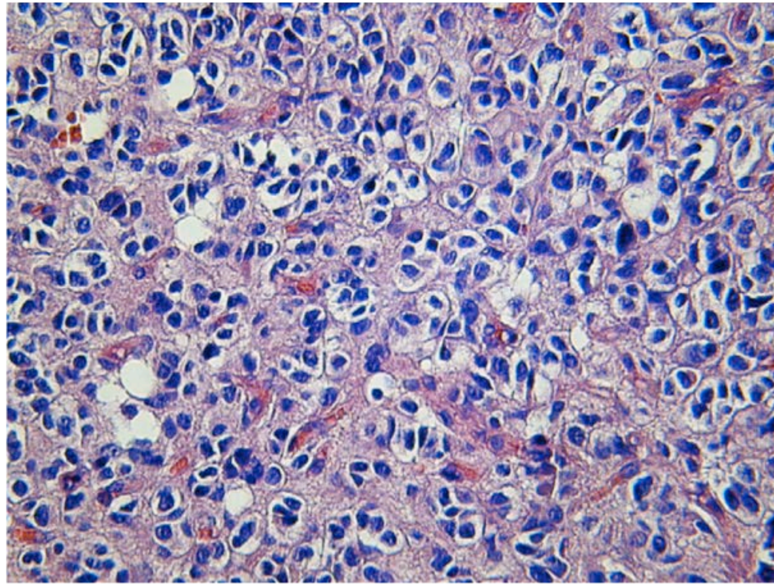


Figure 1.2 Haematoxylin and eosin stain of a grade III oligodendroglioma. High cellularity and a "fried egg" appearance (empty zones around the nuclei) are classic hallmarks of oligodendroglioma pathology (van den Bent et al., 2003).

1.2.3 Prognosis

Overall survival of oligodendroglioma patients is better than patients diagnosed with other types of glioma, however survival times are influenced by a number of genetic and demographic factors and are highly variable. Prognosis is positively influenced by younger age, low tumour grade and clinical presentation of seizures (Louis et al., 2007). Favourable outcomes have been associated with LOH 1p/19q as well as mutations of the *TP53* gene (Ino et al., 2001).

The mean survival for grade II oligodendroglioma patients is 11.6 years (Ohgaki et al., 2004) and 3.5 years for patients with grade III oligodendroglioma (Ohgaki and Kleihues, 2005). Age at diagnosis is a significant factor in survival. In one study, conducted before LOH 1p/19q was identified as a prognostic indicator, oligodendroglioma patients (grade II and III) under 20 were shown to have a median survival time of 17.5 years, compared to only 13 months (1.1 years) for patients over 60 (Westergaard et al., 1997).

1.2.4 Treatment

Patients diagnosed with an oligodendroglioma may undergo three arms of treatment: surgery, radiotherapy and chemotherapy. Treatment selection varies according to the tumour grade and type, extent of resection, results of molecular testing as well as the age, health and wishes of the patient.

Surgery is part of the recommended treatment for all brain tumour patients. A sample of the tumour is usually obtained by biopsy or surgical resection so that a pathologist can examine the histological nature of the tumour. The tumour tissue sample obtained at surgery is also examined for genetic mutations that can further classify the tumour and inform personalised treatment. Surgical debulking of the tumour also serves to relieve mass-effect symptoms suffered by the patient and usually results in an improved prognosis. Surgical resection of grade II and grade III oligodendroglioma is almost never curative, as the diffusely infiltrative nature of these lesions makes them impossible to be completely removed by surgery alone (Louis et al., 2007; van den Bent et al., 2008).

Many oligodendroglioma patients undergo post-operative radiotherapy, which may be combined with a course of chemotherapy and is administered either in the weeks following surgery or once tumour progression is evident. The benefits of radiotherapy include tumour shrinkage, better management of seizures and longer overall survival, however it can also produce negative side effects such as fatigue and hair loss. The use of radiotherapy in grade II gliomas has declined over the past 15 years (Suneja et al., 2011).

The sensitivity of oligodendroglioma to a chemotherapy regimen consisting of procarbazine, CCNU and vincristine (PCV) was described by MacDonald and Cairncross in the late 1980's and was subsequently confirmed in larger phase II studies (van den Bent et al., 1998; Cairncross et al., 1994; Cairncross and Macdonald, 1988). From this point, the standard PCV regimen was used almost exclusively as the recommended chemotherapy treatment for oligodendrogliomas until recently. The PCV regimen is only moderately well tolerated due to gastrointestinal side effects and hematologic toxicity, which limits the duration of treatment for some patients (van den Bent et al., 2006; Cairncross et al., 2006). Intensive PCV, a shorter cycle and higher dose regimen of PCV, has been used to treat oligodendroglioma with comparable success to standard PCV, however the highly toxic effects of the intensive regimen are too severe to recommend this treatment (van den Bent et al., 1998; Mason et al 1996).

Since 2000, temozolomide has also been used in the treatment of oligodendroglioma after its introduction as a well-tolerated treatment for high grade gliomas (Chinot et al., 2001; Kouwenhoven et al., 2006). Temozolomide is now the preferred chemotherapy for oligodendroglioma due its minimal side effects and ease of administration and is recommended in preference over PCV by a ratio of 5:1, however it is not yet approved or subsidised for treatment of oligodendroglioma in Australia (van den Bent et al., 2003b; Abrey et al., 2007).

A number of additional drugs and chemotherapeutic agents have been tested for treatment of recurrent oligodendroglioma. Such agents include Gliadel® wafers, a degradable wafer impregnated with the chemotherapeutic agent carmustine (Westphal et al., 2006), carboplatin and etoposide (Scopece et al., 2006).

The treatment of grade II oligodendrogliomas has not been standardised and remains controversial. Patients diagnosed with a grade II (low grade) oligodendroglioma are frequently treated first with surgery then observed to determine when to proceed with radiotherapy and/or chemotherapy. Grade II glioma patients that underwent early rather than delayed radiotherapy demonstrated longer progression free survival (5.3 years vs. 3.4 years), but not overall survival (7.4 years vs. 7.2 years) (van den Bent et al., 2005). Survival among grade II oligodendroglioma patients can reach up to 15 years and patients treated with radiotherapy may experience long term adverse effects including cognitive decline and leukoencephalopathy (Surma-aho et al., 2001). An ongoing clinical trial, European Organisation for the Research and Treatment of Cancer (EORTC) 22033-26033 and Trans-Tasman Radiation Oncology Group (TROG) 06.01, is investigating the use of either the standard radiotherapy (50.4 Gy in 28 fractions) or temozolomide (75 mg/m^2 daily x 21 days, q 28 days until progression or for max. 12 cycles) for the post-operative treatment of low grade glioma, stratified by 1p LOH status (EORTC-22033-26033, 2012).

Patients with grade III oligodendroglioma often undergo more aggressive treatment including surgery followed by adjuvant radiotherapy and chemotherapy (van den Bent et al., 2008). In 2006, two phase III clinical trials, EORTC 26951 and Intergroup Radiation Therapy Oncology Group Trial (RTOG) 9402, showed that the combination of PCV and radiotherapy improves progression free (Europe 23 vs. 13 months, North America 2.6 vs. 1.7 years), but not overall survival (Europe 40.3 vs. 30.6 months, North America 4.9 vs. 4.7 years) in newly diagnosed grade III oligodendroglioma patients when compared to treatment with radiotherapy alone (van den Bent et al., 2006; Cairncross et al., 2006). These trials identified significantly different outcomes for patients depending on their LOH 1p/19q status and advocated that future trials take LOH 1p/19q status into account.

At present, there are two ongoing phase III trials of radiotherapy and temozolomide in grade III gliomas. Patients are separated into one trial or the other based on Fluorescent *In Situ* Hybridisation (FISH) detection of derivative chromosome der(1;19)(q10;p10), which is the most accurate known indicator of LOH 1p/19q.

The Phase III trial on Concurrent and Adjuvant Temozolomide Chemotherapy in Non-1p/19q Deleted Anaplastic Glioma (CATNON) is recruiting grade III oligodendroglioma and oligoastroctyoma patients that are negative for der(1;19)(q10;p10). Patients are randomized to either temozolomide given from the start of radiotherapy or temozolomide given after the completion of radiotherapy treatment (CATNON, 2012).

The Phase III Randomized Study of Radiotherapy Alone Versus Radiotherapy With Concurrent and Adjuvant Temozolomide Versus Temozolomide Alone in Patients With Newly Diagnosed 1p/19q Codeleted Anaplastic Glioma (CODEL) is not open in Australia. This trial is recruiting patients diagnosed with grade III astrocytoma, oligoastrocytoma or oligodendroglioma with LOH 1p/19q, as evidenced by detection of der(1;19)(q10;p10) by FISH. Patients are randomized to one of three treatment arms: (1) radiotherapy alone, (2) radiotherapy plus concurrent and adjuvant temozolomide, (3) temozolomide alone (CODEL, 2012).

1.3 Genetics of Oligodendroglioma

Gliomas are characterised by specific genetic alterations - some mutations are present in the full spectrum of diffuse gliomas, whereas others define specific subgroups of glioma, including oligodendroglioma. Molecular abnormalities associated with the pathogenesis of diffuse gliomas are summarised in Figure 1.3.

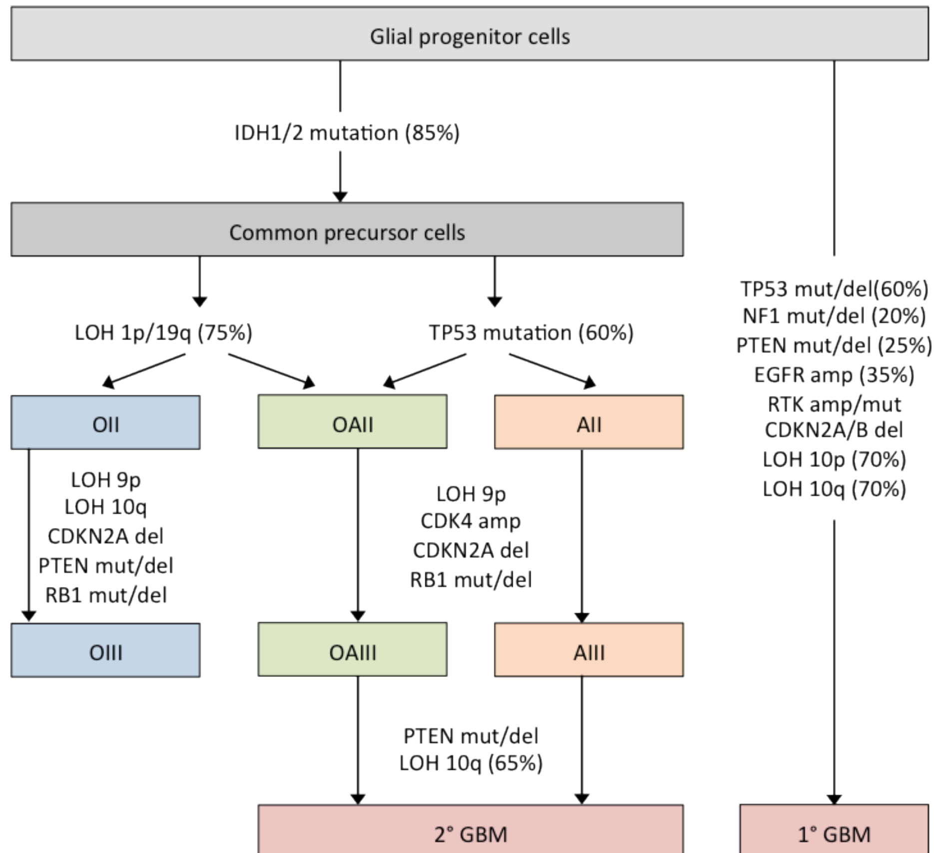


Figure 1.3 Genetic mutations and events that contribute to specific types of glioma.
Adapted from (Huse et al., 2011; Nikiforova et al., 2011; Ohgaki and Kleihues, 2011).

1.3.1 LOH 1p/19q

The loss of heterozygosity at 19q was first observed in oligodendrogliomas in the early 1990's (Ransom et al., 1992; von Deimling et al., 1992) and losses on chromosome 1p were identified in following years (Reifenberger et al., 1994; Bello et al., 1994, 1995). More than 70% grade II oligodendrogliomas harbour loss of heterozygosity at 1p and 19q (LOH 1p/19q) (van den Bent et al., 2008; Kim et al., 2010). This distinctive LOH event is an independent positive prognostic factor in gliomas and helps to distinguish oligodendrogliomas from astrocytoma (Aldape et al., 2007). The prevalence of LOH 1p/19q is lower in grade III oligodendrogliomas, occurring in approximately 50% of these tumours. LOH of either 1p or 19q occurs in up to 10% of grade II and 20% of grade III oligodendroglioma (Table 1.2). LOH 1p/19q is more common in younger oligodendroglioma patients (Myal et al., 2003).

Table 1.2 Reported proportions of oligodendroglioma with LOH at 1p/19q

Publication	Number of patients	LOH 1p/19q	LOH 1p only	LOH 19q only	No LOH
(Bello et al., 1995)	21 (11 grade II; 10 grade III)	14 (66.7%)	4 (19%)	2 (9.5%)	1 (4.8%)
(Smith et al., 1999)	33 (23 grade II; 8 grade III; 2 grade IV)	21 (63.6%)	3 (9.1%)	3 (9.1%)	6 (18.2%)
(Smith et al., 2000)	52 (34 grade II; 12 grade III; 6 grade IV)	23 (44.2%)	2 (3.8%)	10 (19.2%)	17 (32.7%)
(Ino et al., 2001)	50 (grade III only)	23 (46%)	6 (12%)	10 (20%)	11 (22%)
(Okamoto et al., 2004)	35 (grade II only)	19 (54.2%)	1 (2.9%)	5 (14.3%)	10 (28.6%)
(Cairncross et al., 2006)	201 (grade III only)	93 (46.2%)	16 (8%)	33 (16.4%)	59 (29.4%)
(van den Bent et al., 2006)	311 (grade III only)	78 (25.2%)	48 (15.4%)	38 (12.2%)	147 (47.3%)
(Kim et al., 2010)	122 (grade II only)	82 (67.2%)	8 (6.6%)	5 (4.1%)	27 (22.1%)

Around 50% of oligoastrocytomas have LOH 1p/19q. When separated by LOH status, oligoastrocytoma patients with LOH 1p/19q have a prognosis similar to oligodendroglioma patients with LOH 1p/19q (Eoli et al., 2006). Only around 10% of astrocytomas exhibit combined LOH 1p/19q, however partial losses on chromosome 1p are observed in up to 35% of astrocytomas (Idbaih et al., 2005; Kim et al., 2010).

Oligodendrogliomas with LOH 1p/19q tend to exhibit the classical histological appearance of oligodendroglioma and a clinical presentation including a long history of seizures, whereas those without LOH 1p/19q are less likely to show classical features and have a shorter clinical history (van den Bent et al., 2003a).

The diagnosis of a glioma based on histology alone can be quite subjective due to some overlap in the histologic definition of oligodendroglioma, oligoastrocytoma and astrocytoma (Coons et al., 1997; Louis et al., 2007; Gadji et al., 2009). The criteria for the classification of gliomas describe histologic features that can be placed on a spectrum from astrocytic to oligodendroglial features. The analysis of LOH 1p/19q, *TP53* mutations and other genetic alterations can help to classify tumours that fall in the middle of the morphology spectrum (Figure 1.4) (Ueki, 2005). A number of studies have advocated for LOH 1p/19q to be used in conjunction with histological examination and other clinical and genetic features to obtain an accurate diagnosis and prognosis for glioma patients (Burger, 2002; Hamlat et al., 2006; Aldape et al., 2007).

While it is widely understood that LOH 1p/19q helps to validate the diagnosis of an oligodendroglioma, the WHO Classification of Tumours of the Central Nervous System does not require LOH 1p/19q be present for the classification of oligodendrogliomas (Aldape et al., 2007; Louis et al., 2007). The addition of LOH 1p/19q should be considered as a requirement for oligodendroglioma classification in the next edition of the WHO classification guidelines.

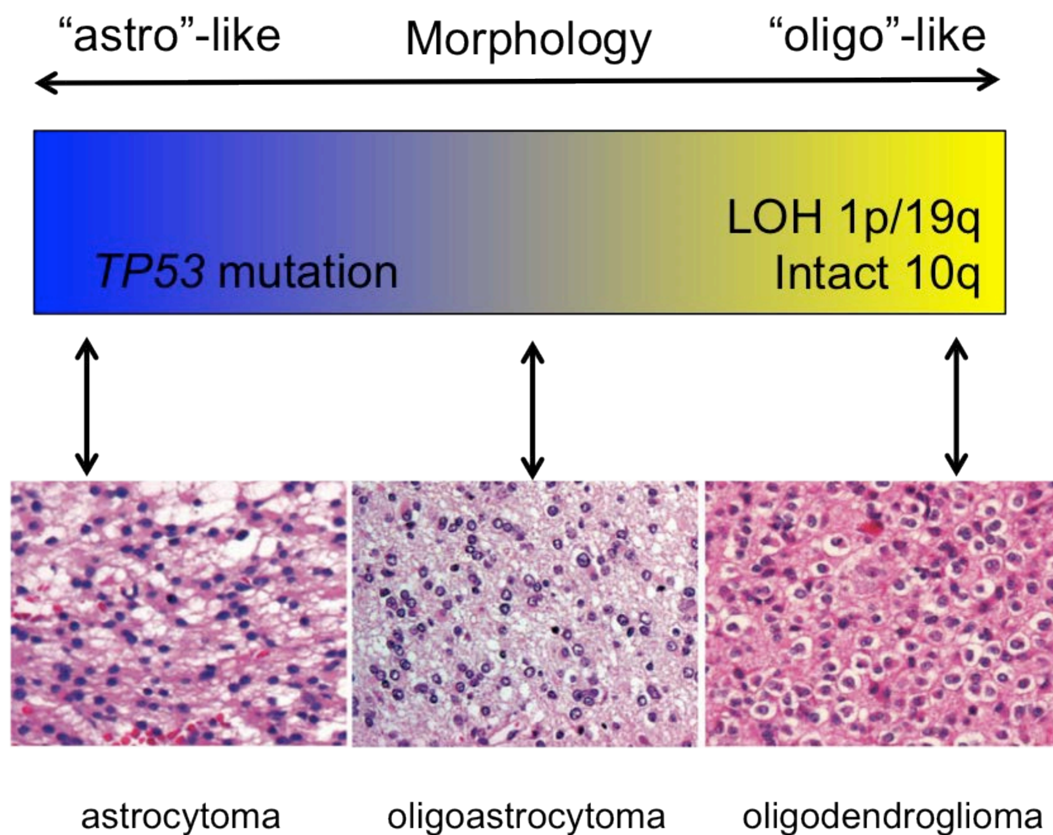


Figure 1.4 Morphological and genetic features used to classify astrocytomas, oligoastrocytomas and oligodendrogliomas.
LOH 1p/19q, intact 10q and lack of *TP53* mutations are common in oligodendroglioma. *TP53* mutations are more common in astrocytoma (Ueki et al., 2005).

Oligodendroglioma patients with LOH 1p/19q experience significantly longer survival compared to those without the combined loss of 1p and 19q (Figure 1.5). In grade III oligodendroglioma patients with LOH 1p/19q, the five-year survival rate is 70% with a median survival above 7 years, compared to 30% and 2-3 years for patients without combined LOH 1p/19q (van den Bent et al., 2006; Cairncross et al., 2006). For patients diagnosed with grade II oligodendrogliomas and LOH 1p/19q, the five-year survival rate is 96% and median survival is 13 years, compared to 70% and 10.8 years for patients without LOH (Jenkins et al., 2006).

Oligodendroglioma patients with LOH of 1p alone experience moderately good survival, however LOH of 19q alone is an indicator of poor prognosis and survival is similar to that experienced by patients without LOH of 1p or 19q (Ino et al., 2001; Eoli et al., 2006). In astrocytoma, LOH 1p/19q is relatively uncommon, however loss of 1p has been associated with long term survival (Brat et al., 2004). In a young cohort of GBM patients (age <50 years), combined 1p/19q loss was observed in 15% of long term survivors (>3 years survival) but not in any short term survivors (Burton et al., 2002).

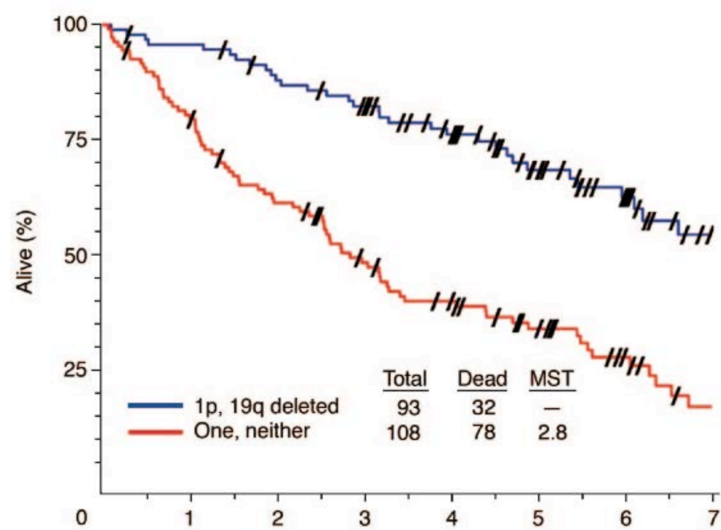


Figure 1.5 Kaplan Meier survival plot of overall survival in grade III oligodendroglioma patients stratified by LOH 1p/19q.
Patients with LOH 1p/19q are represented by the blue line. Patients without LOH or with isolated LOH of either 1p or 19q are represented by the red line (Cairncross et al., 2006).

1.3.1.1 Detection of LOH 1p/19q

Absence or presence of heterozygosity of chromosomes 1p and 19q is typically detected by FISH or PCR-based microsatellite analysis. Both FISH and PCR microsatellite analysis are widely accepted and practiced in clinical settings. Other methods for detection of LOH 1p/19q reported in the literature are comparative genomic hybridization (CGH) (Bigner et al., 1999)and quantitative microsatellite analysis (QuMA) (Nigro et al., 2001).

Microsatellite PCR employs microsatellite markers designed to amplify chromosomes 1p and 19q from DNA isolated from samples of the patients' tumour and blood. The microsatellite sequence is amplified by PCR and the products are analysed on a sequencing gel. LOH is detected when one of the two bands, resulting from the two alleles, shown in the blood sample is not present in the tumour sample. When only one allele is detected in the blood sample, that marker is designated as uninformative (Figure 1.6). Microsatellite PCR is able to detect deletion and reduplication events, which are not be distinguishable by FISH (Burger et al., 2001). To detect the extent of the 1p or 19q deletion it is necessary to examine a number of microsatellite markers on each of chromosomes 1p and 19q. The standard procedure in many centres is to examine at least three informative microsatellite loci on each chromosome as one or more markers may be uninformative (Hartmann et al., 2005). Compared to FISH, this method is more time consuming and requires greater technical skill, but can more accurately determine the extent of the chromosomal loss.

This PCR based technique has been adapted for analysis of LOH with a qPCR machine, QuMA, which compares the copy number of each allele in DNA samples from tumour and blood (Nigro et al., 2001). This method has the advantage of being faster than traditional PCR microsatellite analysis as both PCR amplification and LOH detection are combined in a single reaction.

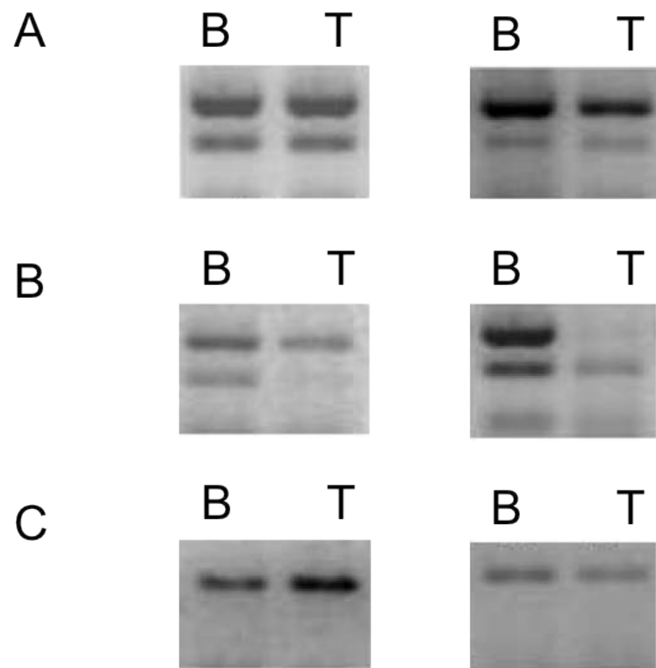


Figure 1.6 Representative images for PCR microsatellite for LOH.
[A] Both tumour and blood display two alleles for the microsatellite marker. [B] Blood shows two alleles, tumour shows one allele. [C] Both tumour and blood show one allele – non-informative at that marker. B - blood; T – tumour. Images: (Chosdol et al., 2009).

FISH employs fluorescent oligonucleotide probes that target the 1p36 and 19q13 chromosomal regions. This technique assesses absolute copy number, which is counted within each cell to calculate the ratio of 1p to 1q and 19q to 19p. In a typical clinical setting, only one or two markers on 1p and one marker on 19q are used to examine the presence or absence of these chromosomes (Figure 1.7). FISH is preferable for the examination of archived paraffin embedded tissues as peripheral blood, which is required for PCR, is not routinely collected and stored at some clinical centres. The main drawback of LOH detection by FISH is that the scale of the deletions on chromosomes 1p and 19q cannot be determined. The most commonly used kit for FISH detection LOH of 1p and 19q is the Vysis 1p36/1q25 and 19q13/19p13 FISH Probe Kit (McDonald et al., 2005b; Brandes et al., 2006; Kanner et al., 2006; Wharton et al., 2007; Gadji et al., 2009; Takeuchi et al., 2009; Buckley et al., 2011), which can only determine whether a ~435 kb region on 1p36 and/or a ~380 Kb region on 19q13 loci are deleted and thus cannot distinguish between a small focal deletion and a whole chromosome arm loss. Additionally, the number of cells that must be counted to achieve a result and the ratios of each marker have not been standardized across the discipline, so the reporting of LOH 1p/19q could be inaccurate for smaller samples (e.g. a needle biopsy) or skewed by conservative or bold cutoff ratios at individual centres.

The detection of der(1;19)(q10;p10) by FISH is a more accurate predictor of 1p/19q combined LOH than FISH using probes for 1p36 and 19q13 and can more distinctly separate patients by survival (Jenkins et al., 2006). There is evidence that the detection of this fusion chromosome may be added to the standard FISH pathology tests ordered to determine the nature of the LOH event in oligodendroglial tumours.

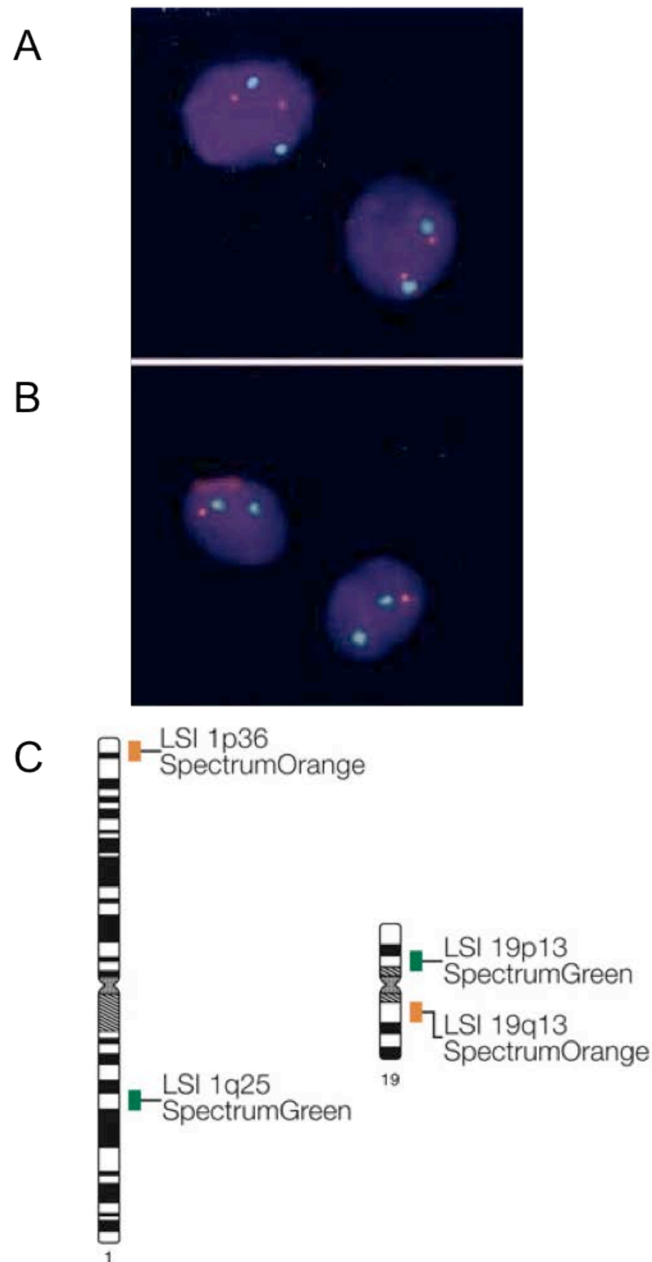
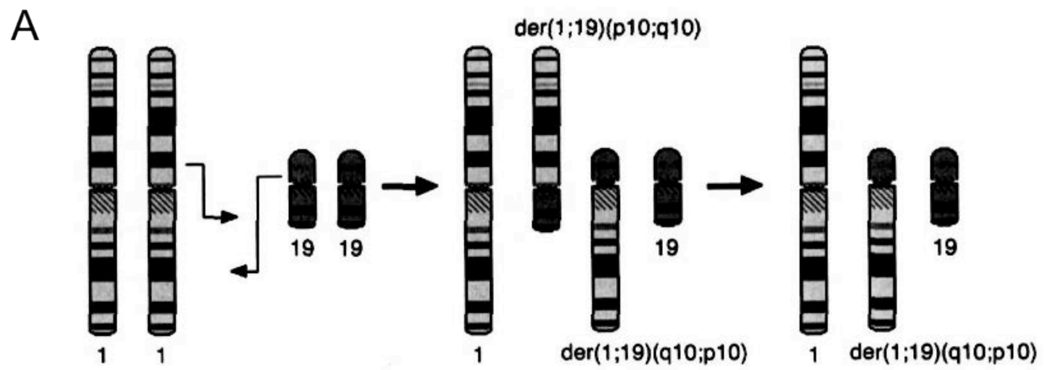


Figure 1.7 FISH analysis of chromosomal arm 1p on isolated cell nuclei. Representative hybridization results are shown in two-cell nuclei (DAPI stain), respectively. [A] normal result in a non-neoplastic control case with an equal 2:2 ratio of paracentromeric (*green*) and subtelomeric (*orange*) signals. [B] deletion status in an oligodendroglial neoplasm with a 2:1 ratio of paracentromeric (*green*) and subtelomeric (*orange*) signals. FISH (Gelpi et al., 2003). [C] Chromosomal location of probes in the Vysis 1p36/1q25 and 19q13/19p13 FISH Probe Kit (Abbot Molecular).

1.3.1.2 Mechanism of LOH 1p/19q

The mechanism of LOH 1p/19q was revealed by metaphase studies of oligodendroglioma cells. The characteristic pattern of LOH is the result of an unbalanced translocation event between chromosomes 1 and 19, in which the entire 1p and 19q chromosome arms are exchanged, resulting in unique derivative chromosomes der(1;19)(p10;q10) and der(1;19)(q10;p10). The derivative chromosome consisting of arms 1p and 19q lacks a centromere and is subsequently lost following cell division. The remaining fusion chromosome, der(1;19)(q10;p10), can be detected in patient tumour samples (Figure 1.8) (Griffin et al., 2006; Jenkins et al., 2006). The presence of this fusion chromosome is a better indicator of survival than the loss of 1p and 19q (Jenkins et al., 2006).

While the classical pattern of LOH is the loss of the entire 1p and 19q arms, some oligodendrogliomas display incomplete losses of 1p, 19q or both. Partial or incomplete losses on 1p and 19q are more frequently observed in non-classical oligodendrogliomas, oligoastrocytomas and astrocytomas (Maintz et al., 1997; van den Bent et al., 1998; Barbashina et al., 2005).



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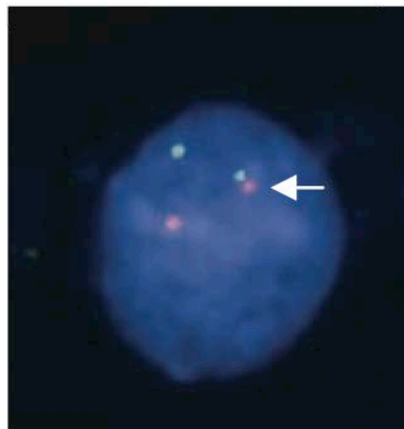


Figure 1.8 LOH 1p/19q occurs due to an unbalanced translocation.
[A] A translocation between chromosomes 1p and 19q results in two derivative chromosomes. One of the fusion chromosomes, der(1;19)(p10;q10), lacks a centromere and is lost during cell division (Griffin et al 2006). [B] The remaining fusion chromosome, der(1;19)(q10;p10), is detectable in patient tissue samples by the co-localisation of FISH probes for CEP1 and 19p12 (Jenkins et al., 2006).

1.3.2 *IDH1* and *IDH2* mutations

Isocitrate dehydrogenases 1 and 2 (IDH1 and IDH2) are enzymes that catalyse the conversion of isocitrate to α-ketoglutarate in the citric acid cycle. IDH1 is a cytoplasmic protein whereas IDH2 is present in the mitochondria.

Mutation of *IDH1* or *IDH2* is present in more than 85% of all grade II astrocytomas, oligoastrocytomas and oligodendrogliomas and is associated with longer survival (Yan et al., 2009; Kim et al., 2010). *IDH* mutations are rare in primary glioblastoma (5%), but frequent in secondary glioblastoma (85%). In oligodendrogliomas, *IDH* mutations occur almost exclusively in tumours with LOH 1p/19q. It has been suggested that the mutation of *IDH* is one of the earliest events in tumourigenesis, as other genetic aberrations such as LOH 1p/19q and *TP53* mutations are almost never present in oligodendroglioma without the mutation of either *IDH1* or *IDH2* (Watanabe et al., 2009). There are no reports of a patient that developed a *TP53* mutation or LOH 1p/19q after the acquisition of an *IDH* mutation, which provides more evidence for the case of *IDH* mutations as an initiating event in oligodendroglioma (Watanabe et al., 2009; Kim et al., 2010).

IDH1 and *IDH2* mutations are also associated with longer survival in glioma patients (Figure 1.9). As *IDH* mutations are very common in oligodendrogliomas (85-95%), all sample sets comparing survival of patients with and without *IDH* mutations consist of a mixture of oligodendroglioma, oligoastrocytoma and astrocytoma patients to obtain a statistically viable sample size for the group of patients with wild type *IDH* (van den Bent et al., 2010).

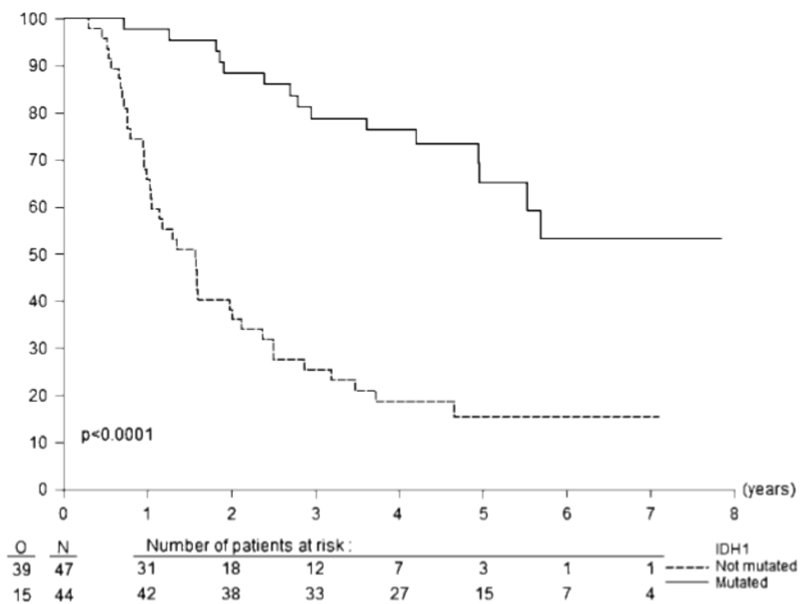


Figure 1.**9** *IDH1* mutations are associated with improved survival.
The Kaplan-Meier plot shows overall survival in grade III oligodendroglioma and oligoastrocytoma patients stratified by *IDH1* mutation status. Patients with mutant *IDH1* are represented by the solid line. Patients with wild type *IDH1* are represented by the dashed line (van den Bent et al., 2010).

1.3.3 *MGMT* promoter methylation

O(6)-methylguanine DNA methyltransferase (*MGMT*), located on 10q26, is a DNA repair protein that removes alkyl groups at the O^6 position of guanine, reversing DNA damage caused by therapeutic alkylating agents such as temozolomide (Liu et al., 1996). Epigenetic silencing due to DNA methylation of the *MGMT* promoter results in lower MGMT protein expression and a reduced ability to repair DNA damage. Methylation of the *MGMT* promoter occurs in 70 to 90% of oligodendrogliomas, most frequently in oligodendrogliomas with LOH 1p/19q (Möllemann et al., 2005; Brandes et al., 2006; McLendon et al., 2005).

MGMT promoter methylation is a positive prognostic factor in glioblastoma (Hegi et al., 2005). In a cohort of 152 grade III oligodendrogliomas and oligoastrocytomas from the EORTC 26951 study, *MGMT* promoter methylation was significantly associated with longer overall survival (HR=0.24 [0.10-0.56], $p<0.05$) but could not predict outcome to PCV chemotherapy, being equally as strong in the radiotherapy only arm as in the radiotherapy plus PCV (van den Bent et al., 2009). *MGMT* promoter methylation neared significance when analysed in a cohort of 67 grade III oligodendrogliomas and oligoastrocytomas treated with temozolomide as a positive prognostic indicator (overall survival 40.9 vs. 28.5 months) but not as an indicator of response to treatment (progression free survival 12 vs. 13 months) (Brandes et al., 2006).

1.3.4 DNA methylation (G-CIMP, CIMP)

A rapidly growing area of glioma genetics is the analysis of DNA methylation. Following methylation analysis of 272 glioblastoma DNA samples in The Cancer Genome Atlas (TCGA), an eight gene glioma-CpG island methylator phenotype (G-CIMP) methylation signature (MethylLight) was described and shown to be present in 93% of oligodendroglioma (grades II and III), 45% of astrocytoma (grades II and III) and 8% of glioblastoma samples (Noushmehr et al., 2010). Furthermore, all glioblastoma samples bearing an *IDH* mutation were shown to be G-CIMP positive. G-CIMP was also shown to be a positive prognostic factor. A similar study of grade III oligodendroglioma identified the CpG island

hypermethylation phenotype (CIMP+) as a strongly positive prognostic factor that frequently occurred with LOH 1p/19q, *IDH1* mutations and MGMT methylation (van den Bent et al., 2011).

1.3.5 Other genetic alterations

Tumour protein 53 (*TP53*) is a tumour suppressor gene that is implicated in almost every type of cancer. *TP53* mutations occur in up to 15% of oligodendrogliomas (Cairncross et al., 1998; Okamoto et al., 2004; Kim et al., 2010).

Cyclin-dependent kinase inhibitor 2A (*CDKN2A*) is a tumour suppressor gene located on chromosome 9p21, an area that is frequently lost in cancer either by loss of heterozygosity or homozygous deletion. LOH of chromosome 9p (including the *CDKN2A* gene) occurs in up to 15% of grade II and 42% of grade III oligodendrogliomas and homozygous deletion of the *CDKN2A* gene has been described in up to 37% of grade III oligodendrogliomas (Cairncross et al., 1998; Bigner et al., 1999; Hoang-Xuan et al., 2001). The loss of *CDKN2A* is associated with the progression oligodendrogliomas from grade II to grade III (Reifenberger and Louis, 2003).

Epidermal growth factor receptor (*EGFR*) is a gene that is amplified in 30 to 50% of glioblastoma patients, leading to increased *EGFR* expression, activity of the EGF pathway, cell growth and proliferation (Shinojima et al., 2003). In oligodendrogliomas, amplification of *EGFR* is a rare event, occurring only in grade III tumours at a rate of between 10 and 30% (Hoang-Xuan et al., 2001; Idbaih et al., 2008).

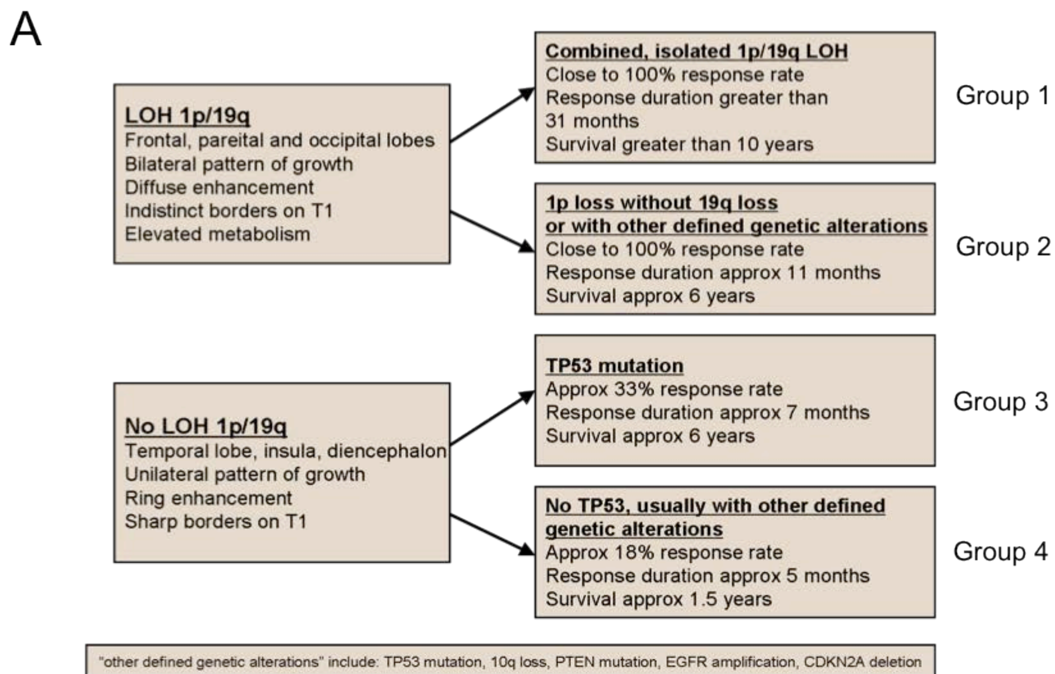
LOH of chromosome 10q is an event associated with increasing malignancy in gliomas. LOH of 10q occurs in up to 15% of oligodendrogliomas, more often in grade III than in grade II tumours (Hoang-Xuan et al., 2001; Fallon et al., 2004; Reifenberger et al., 1996). LOH 10q is an indicator of poor response to therapy, especially in oligodendrogliomas without LOH 1p/19q (Ramirez et al., 2010). Phosphatase and tensin homolog (*PTEN*), located on 10q, is mutated in fewer than 10% of grade III oligodendrogliomas (Reifenberger and Louis, 2003).

RB1 mutation/deletion may occur in up to 10% of grade III oligodendroglioma patients and disruption of the RB1 pathway occurs in up to 65% of grade III oligodendrogliomas, whereas *RB1* mutation, deletion or pathway disruption occurred in only 1/28 grade II oligodendrogliomas (Watanabe et al., 2001).

These genetic alterations are able to classify oligodendrogliomas beyond what can be known from histology alone. Oligodendrogliomas with LOH 1p/19q are also highly likely to have mutations in *IDH1*or *IDH2* and methylation of *MGMT* (Yan et al., 2009; Möllemann et al., 2005). Other genetic events in oligodendroglioma such as *TP53* mutation, LOH 10q, *CDKN2A* deletion and *EGFR* amplification, tend to occur in tumours that do not have LOH 1p/19q (Hoang-Xuan et al., 2001; Reifenberger and Louis, 2003; Ohgaki and Kleihues, 2011).

1.3.6 Impact of genetic markers on prognosis

Ino et al. separated grade III oligodendrogliomas into genetically defined subgroups that demonstrated substantially different survival and response (Figure 1.10). Group 1 consisted of patients with LOH 1p/19q and had a median survival greater than 10 years (120 months). Patients with LOH of chromosome 1p only (Group 2) and those with a *TP53* mutation (Group 3) had a median survival of 71 months. Group 4, defined by other genetic alterations including *CDNK2A* deletion, *PTEN* mutation and *EGFR* amplification, had a median survival of only 16 months (Ino et al., 2001; Nutt, 2005). It is not understood how mutations in *TP53* confer better survival in patients without LOH 1p/19q, as *TP53* mutations are typically associated with poor prognosis in many cancers, but have been associated with a less aggressive course of disease in gliomas (Kyritsis et al., 1995).



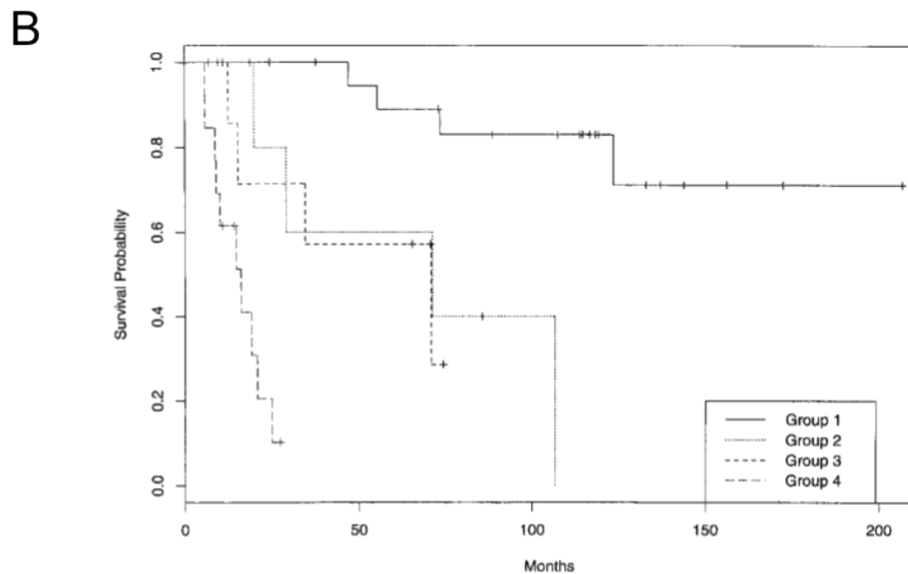


Figure 1.10 Classification of grade III oligodendrogliomas by LOH 1p/19q, *TP53* mutations and other specific genetic alterations.
[A] Key clinical and genetic features define four subgroups of grade III oligodendroglioma. [B] Kaplan Meier survival plot of patients categorised into each of the four subgroups. Adapted from (Ino et al., 2001; Nutt, 2005). T1 – T_1-weighted MR imaging.

1.4 LOH in other cancers

Knudson's "two hit hypothesis" explains the typical effect of LOH in cancer initiation and progression – the first "hit" is a mutation in a tumour suppressor gene; the second is the loss of the chromosome containing the wild type copy of that gene (Knudson, 1971). In the case of LOH 1p/19q, one "hit" is the loss of one copy of the gene. The other "hit" could be hypermethylation or a nonsense mutation of the remaining allele, resulting in loss of gene function.

In contrast with the improved prognosis for oligodendrogliomas with LOH 1p/19q, LOH in other types of cancer is generally associated with poorer prognosis. In head and neck squamous cell carcinomas, LOH at 14q (D14S995) occurs in 55% of all cases and is associated with poor prognosis (Pehlivan et al., 2008). LOH at 17p19 occurs in adrenocortical (ACC) tumours and is thought to be indicative of a malignant change in the tumour (Soon et al., 2008).

Small, focal deletions on chromosome 1p occur in astrocytoma and glioblastoma and are associated with poorer prognosis (Idbaih et al., 2005). 1p deletions have also been found in neuroblastoma, melanoma, meninigioma, phaeochromocytoma as well as cancers of the breast, thyroid, cervix and colon (Poetch et al., 2003; Piaskowski et al., 2005; Cheung et al., 2005; White et al., 1995; Bièche et al., 1993; Kleer et al., 2000; Praml et al., 1995; Moley et al., 1992). In these cancers, LOH events were believed to occur fairly late in disease progression where LOH occurs at a chromosome region that contains a tumour suppressor gene, thereby contributing to tumour initiation and growth.

1.5 Emerging technologies for cancer genetics research

Recent advances in genomic research, including completion of the Human Genome Project in 2003, have led to the development of new technologies for genomic analysis including microarrays and next generation sequencing. These technologies have permitted large scale studies of cancer and lower costs have increased the availability of microarrays for small scale studies.

Microarray technology can be utilised for numerous molecular biology applications, including copy number analysis, gene expression and DNA methylation. Gene expression microarrays have evolved very quickly, from spotted cDNA microarrays representing a small percentage of known genes, to arrays that cover the entire genome, within a decade. The gene expression microarray experiments described in this thesis were done with Affymetrix GeneChip® Human Exon 1.0 ST arrays, each of which contain 1.4 million probe sets, representing all known human exons. Four probes per exon are present on the array for 90% of exons, which permits both high quality gene expression analysis as well as alternate splicing of exons. During the preparation of this thesis, GeneChip® human exon arrays were the most advanced gene expression analysis technology available. These arrays are a significant step up from the traditional 3' gene expression microarrays, such as the GeneChip® U133 plus2.0 arrays, which contain 54000 probe sets. 3' arrays are still considered the industry standard for gene expression analysis, but lack the flexibility to analyse expression of individual exons or the entire mRNA transcript (Figure 1.11).

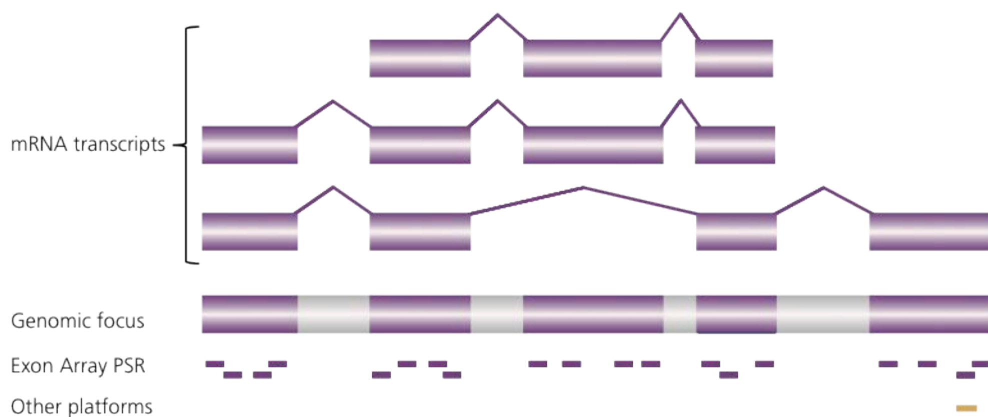


Figure 1.11 The GeneChip™ Human Exon 1.0 ST array.
GeneChip™ Human Exon 1.0 ST arrays contain probes across the entire length of the transcript. Purple regions are exons and gray regions represent introns that are removed during splicing. The short dashes underneath the genomic locus indicate individual probes representing the probe selection region (PSR).

The past five years has seen The Cancer Genome Project (TCGA) collect microarray generated gene expression, copy number, DNA methylation and gene mutation data on large numbers of patient tumour samples representing multiple cancer types. All TCGA data is publically available in annotated data sets from the TCGA website (http://cancergenome.nih.gov/), so that researchers and clinicians anywhere can access the data and analyse it for their own research questions or use it to validate their own experiments.

For glioblastoma, the pilot project of the TCGA, 582 patient tumour samples are available to download. The initial publication from the glioblastoma project identified new insights into the roles of *ERBB2*, *NF1*, *TP53* and *PIK3R1* and discovered a link between MGMT methylation and a hypermutator phenotype (McLendon et al., 2008). Another significant publication from the glioblastoma project identified four clinically relevant subtypes – Classical, Mesenchymal, Neural and Proneural – that are characterised by gene expression signatures and specific mutations in *PDGFRA*, *IDH1*, *EGFR* and *NF1* (Verhaak et al., 2010). The subtypes will enable better selection criteria for clinical trials of glioblastoma and will allow future research to focus on specific subtypes. More than 40 additional publications have utilised data from the TCGA to initiate a new study or supplement their own research.

Next-generation sequencing describes numerous recently developed technologies capable of sequencing DNA at significantly faster speeds and at a fraction of the cost of traditional Sanger sequencing (Looi, 2009; Schuster, 2008). Sequencing an entire genome or transcriptome is possible with these technologies, which are now becoming available in cancer research centres across the globe. Next-generation sequencing led to the discovery of *IDH1* mutations in secondary glioblastomas, one of the most important discoveries to affect oligodendroglioma research in recent years (Parsons et al., 2008).

This technology has the ability to bring sequencing to the clinic as patients can already apply to sequence their tumour genome to personalise their treatment, although such applications are still in their infancy. The once fictional notion of the $1000 genome may soon be a reality, as more technologies capable of cheap, rapid DNA sequencing become available.

1.6 Oligodendroglioma candidate genes on 1p and 19q

It is not yet understood how LOH 1p/19q confers a survival advantage, as oligodendrogliomas with and without LOH 1p/19q are morphologically indistinguishable. LOH 1p/19q is understood to be an early event in tumourigenesis. In line with Knudson's two hit hypothesis, it is likely that one or more tumour suppressor genes, active in early development of oligodendrogliomas is located on chromosome 1p or 19q, are lost due to LOH 1p/19q (Reifenberger and Louis, 2003). To explain the chemosensitive and slow growing nature of the tumour bearing LOH 1p/19q, it was hypothesised that chromosomes 1p and 19q are purportedly home to a gene (or group of genes) that confers resistance to therapy, which when lost during LOH 1p/19q, leaves the tumour in a chemo- and radio- sensitive state.

To identify the elusive tumour suppressor or chemoresistance gene(s), a number of studies prior to 2006 identified a common region of chromosomal loss on 1p and 19q, then analysed biologically important candidate genes from that region. As it is now known that the 1p/19q co-deletion is a distinct genetic event that involves the entire 1p and 19q arms, this approach to finding the gene of interest is unlikely to be fruitful (Jenkins et al., 2006; Griffin et al., 2006). A number of genes on chromosome 1p and 19q with potential tumour suppressor or chemoresistance functions have been identified in the past decade, but as yet there has been very little analysis of the function of these genes in the biology of oligodendrogliomas. These studies have been summarised in Table 1.3.

Microarray technology has served to discover a great number of genes that are associated with LOH 1p/19q, but there is still a lack of functional data to support these studies. Selecting genes from these vast lists for further study is difficult, as the microarray can not inform whether a gene may effect a biological change or whether it is simply a bystander whose altered expression does not affect the disease state.

Table 1.3 Genes on 1p and 19q that have been studied in oligodendroglioma for their role in tumourigenesis or survival advantage following LOH 1p/19q

Reference	Year	1p	19q	Genomic mutation	Putative function
(Husemann et al., 1999)	1999	1p32 - Cyclin dependant kinase inhibitor (*CDKN2C* aka *p18INK4C*)	-	No mutation	Not studied (hypothetical tumour suppressor)
(Dong et al., 2002)	2002	1p36.3 - Tumour Protein 73 (*TP73*)	-	Hypermethylation of remaining allele	Not studied (hypothetical tumour suppressor)
(Mukasa et al., 2002)	2002	1p21 - Collagen type IX α 1 (*COL11A1*) 1p – Retinoblastoma-binding protein 4 (*RBBP4*)	-	-	None studied
(Wolf et al., 2003)	2003	-	19q13.3 - Glucocorticoid receptor DNA binding factor 1 (*p190RhoGAP*)	-	Putative tumour suppressor (inhibits cell proliferation)
(Hong et al., 2003)	2003	-	19q13 - Zinc finger protein 342 (*ZNF342*)	Hypermethylation of remaining allele	Not studied (hypothetical tumour suppressor)
(Trouillard et al., 2004)	2004	-	19q.13.3 - Paternally expressed gene3 (*PEG3*)	Methylation of remaining maternal allele	Not studied
(Barbashina et al., 2005)	2005	1p36 - Calmodulin binding transcription activator 1 (*CAMTA1*)	-	No mutation	Not studied (hypothetical tumour suppressor)
(McDonald et al., 2005a)	2005	1p36.3 - DNA fragmentation factor, 40kDa, beta polypeptide (*DFFB*)	-	-	Not studied
(Tews et al., 2006)	2006	1p36 – Isoprenylcysteine carboxyl methyltransferase (*ICMT*) 1p36 – Solute carrier family 25, member 33 (*MGC4399, SLC25A33*)	19q13 – Ribosomal protein L18 (*RPL18*) 19q13 – Phospholipase A2, group IVC (*PLA2G4C*)	-	None studied
(McDonald et al., 2006; Cogdell et al., 2011)	2006, 2011	1p36.32 - Adherens junctions associated protein 1 (*AJAP1* aka *SHREW1*)	-	-	Inhibits cell migration and adhesion
(Tews et al., 2007)	2007	1p34.2 - Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 4 (*CITED4*)	-	Hypermethylated in oligodendrogliomas	Not studied
(Ngo et al., 2007)	2007	1p36 - Stathmin (*STMN* aka oncoprotein 18)	-	-	Chemoresistance to nitrosoureas
(Kunitz et al., 2007)	2007	-	19q13.3 – Epithelial membrane protein 3 (*EMP3*)	Hypermethylated in oligodendrogliomas	Not studied
(Riemenschneider et al., 2008)	2008	1p31 - DIRAS family, GTP-binding RAS-like 3 (*DIRAS3*)	-	Hypermethylated in oligodendrogliomas	Not studied

1p – Chromosome 1p; 19q – Chromosome 19q

Studies published by four different groups used microarrays to identify numerous genes on 1p, 19q and throughout the genome that are significantly differentially expressed between oligodendrogliomas separated by LOH 1p/19q and that may contribute to the tumourigenesis or survival advantage in oligodendrogliomas (Mukasa et al., 2002; French et al., 2005; Tews et al., 2006; Ferrer-Luna et al., 2009). There is significant overlap in the lists of genes identified, however each group selected a different panel of genes to validate by quantitative real-time PCR.

Generally, changes in gene expression profiles due to LOH 1p/19q were global, rather than small differences restricted to 1p and 19q. Oligodendroglial tumours with LOH 1p/19q were shown to have a "pro-neural" phenotype with higher expression of genes related to cell adhesion and cell cycle inhibition, whereas tumours that retained both copies of 1p and 19q were characterised by higher expression of genes involved in inflammatory and immune responses, cell migration, proliferation and cell cycle progression (Ferrer-Luna et al., 2009).

Two studies examined the gene expression profiles in oligodendrogliomas with LOH 1p/19q in comparison to high-grade glioma with *EGFR* amplification. The first study used Affymetrix Human Exon 1.0 ST arrays to identify 715 novel exons that were differentially expressed between the two types of glioma (French et al., 2007). The second used Affymetrix HG U133-plus 2.0 expression arrays and demonstrated that tumours with LOH 1p/19q exhibited a proneural gene expression profile (Ducray et al., 2008). The proneural molecular signature in oligodendrogliomas was confirmed in an analysis of microarray data from astrocytoma, oligodendroglioma and glioblastoma specimens accessed from the Rembrandt database (Cooper et al., 2010).

The main translatable result from microarray experiments of the past decade was the discovery of numerous biomarkers that have potential to act as surrogate markers for LOH 1p/19q or as indicators of prognosis or response to treatment. Internexin (*INA*), a gene on 10q24 that encodes a neurofilament interacting protein, has high expression in oligodendrogliomas with LOH 1p/19q and has been proposed as a biomarker for LOH 1p/19q. Initially identified by two independent microarray studies, positive immunohistochemistry for INA can identify tumours

with LOH 1p/19q with 86% specificity and 96% sensitivity (Mukasa et al., 2002, 2004; Ducray et al., 2008, 2009).

Numerous biomarkers for LOH 1p/19q or response to treatment that can be analysed by qPCR in tumour samples have been identified following microarray studies. Mukasa et al. confirmed *MYTIL*, *PTPRN*, *SCG2*, *SNCB*, *ALDH1A1* and *L1CAM* as markers of intact 1p and 19q, and *COL11A1*, *RBBP4* and *KCND2* as markers of LOH 1p/19q by semi-quantitative RT-PCR (Mukasa et al., 2002). French et al. identified *F3*, *IQGAP*, *PPAP2B* and *GNG12* as markers of 1p LOH; *CASP3*, *ZNF222* and *DCDT* as markers of 19q LOH; and *MAN1C1*, *IQGAP1*, *TRIM56* and *AQP1* as markers of response to treatment, each validated by qPCR (French et al., 2005). Tews et al. confirmed *RPL18*, *MGC4399* and *ICMT* by qPCR as markers of tumours with intact 1p and 19q (Tews et al., 2006). Even though each of these qPCR-validated biomarkers was significantly associated with either LOH 1p/19q or response to treatment, qPCR is not a method that can be adapted easily to clinical settings. qPCR is technically challenging, requires significant operator skill and has stringent requirements for the collection, storage and processing of tumour samples. Additional research and further development must be done for each gene panel before they can be integrated into a clinical setting.

The advent of next-generation sequencing has also aided the discovery of new candidate genes in oligodendroglioma. The genes Capicua homolog (*CIC*, 19q13.2) and Far upstream element binding protein 1 (*FUBP1*, 1p31.1) were recently identified as potential tumour suppressor genes in oligodendroglioma as they were frequently mutated in oligodendrogliomas with LOH 1p/19q (Bettegowda et al., 2011). Both *CIC* and *FUBP1* fit the pattern of a tumour suppressor gene as they were mutated only in tumours with LOH of the other allele and the majority of mutations were inactivating (nonsense mutations). As yet, little is known about the function of either gene, but the mutations in each gene may function as a "second hit" in the path to tumourigenesis following LOH 1p/19q. A similar study resequenced all exons, splice sites, microRNA's and promoter regions on 1p and 19q in seven oligodendroglioma samples and found just one mutation in the gene *ARHGEF16*, a gene in which no mutation was identified in by Bettegowda et al. (Bralten et al., 2011).

While many candidate genes have been validated by sequencing, qPCR or immunohistochemistry, functional analysis of potential biologically important genes discovered on 1p and 19q is lacking. So far, Adherens Junctions Associated Protein 1 (*AJAP1* aka *SHREW1,* 1p36) is the only gene candidate to account for the role of LOH 1p/19q in tumourigenesis of oligodendrogliomas. The restoration of *AJAP1* expression was shown to inhibit cell migration and adhesion, indicating that the loss of this gene may contribute to the development of an oligodendroglioma following LOH 1p/19q (McDonald et al., 2006). Down-regulated *AJAP1* was associated with poorer survival in a cohort of 343 gliomas from the Rembrandt database (Cogdell et al., 2011), further implicating it as a tumour suppressor gene that may be involved in the development of oligodendroglioma.

Stathmin (*STMN1*, 1p36) was the only gene demonstrated to be important in the chemosensitive genetic makeup of tumours with LOH 1p/19q. Also known as Oncoprotein 18, *STMN1* acts to stabilize microtubule. A single study showed that *STMN1* conferred chemoresistance to nitrosoureas in malignant brain tumours (Ngo et al., 2007).

It is still critical to identify and characterise genes on 1p and 19q that confer a survival advantage in oligodendrogliomas with LOH 1p/19q as it cannot fully be explained by the loss of 1p genes *STMN1* and *AJAP1*. In addition, genes located on other chromosomes that are trans-activated or repressed following the loss of transcription factors or repressors coded on chromosomes 1p and 19q due to LOH 1p/19q warrant further study to identify whether they contribute to the survival advantage. Very few such genes have been identified by microarray studies and none have been subject to functional analysis to determine their role in oligodendroglioma biology. Such genes, which are expressed at much higher levels in tumours without LOH 1p/19q, could present as valuable new targets for therapy.

1.7 Summary and Aims

LOH 1p/19q in oligodendroglioma has been extensively studied, but there is little understanding of how this genetic alteration functions in either tumourigenesis or chemosensitivity of oligodendrogliomas. The overall aim of this thesis is to identify and investigate individual genes that contribute to tumourigenesis or sensitivity to therapy in oligodendroglioma.

The specific aims for this thesis are:

2 To conduct a clinical review of oligodendroglioma patients treated at Royal North Shore and North Shore Private Hospitals between 1988 and 2009, determine the frequency of LOH 1p/19q in this patient cohort and identify whether LOH 1p/19q or any other genetic and clinical factors influence prognosis.

3 To identify new gene candidates for use as biomarkers or targets for therapy by analysing gene expression and DNA methylation in a selected cohort of oligodendrogliomas with and without LOH 1p/19q.

4 To validate the differential expression of selected gene targets by quantitative PCR and immunohistochemistry in the original and extended patient cohorts and determine the value of the gene targets as prognostic indicators.

5 To further investigate the function of one gene target, MIG-6, in glioma cell lines to better determine its role in tumourigenesis and/or chemosensitivity of oligodendroglioma.

6 To culture primary oligodendroglioma samples with the aim of generating a cell line that retains the LOH 1p/19q genetic marker of oligodendroglioma.

This investigation has the potential to highlight genes that can be targeted or mimicked by therapeutic agents and permit the development of safe and highly effective treatments for brain tumour patients. This research may also identify genetic events in the development of oligodendrogliomas, which will contribute to a greater understanding of the nature of this complex disease.

2 Materials and Methods

2.1 Introduction

This chapter describes the materials and general methods pertaining to the results chapters of this thesis. Specific methods are described in the respective chapters. Appendix 1 contains a complete list of reagents and materials used.

2.2 Ethics

2.2.1 Brain tumour tissue collection at the Kolling Institute of Medical Research

Ethics approval for the collection and use of brain tumour tissue was granted by the Northern Sydney Health Human Ethics Committee on June 19, 2007 (Protocol 0707-116M). Patients eligible for this study gave informed consent for the storage and use of tumour tissue obtained during surgery for research purposes (a copy of the patient information sheet and consent form can be found in Appendix 2).

2.2.2 Tumour tissue collection at Duke University Medical Center

Brain tumour tissue samples were obtained from the Preston Robert Tisch Brain Tumor Center Biorepository at Duke University Medical Center by an IRB-approved protocol. All samples were obtained in accordance with the Health Insurance Portability and Accountability Act. Each patient in this study gave informed consent for the use of his or her tumour tissue.

2.3 Tumour specimens

2.3.1 Tumour banking at the Kolling Institute

Tumour tissue was snap-frozen in liquid nitrogen at the time of surgery, within five minutes of its being removed from the patient. Tumour specimens were stored at –80 °C in the Neuroendocrine Tumour Bank of the Kolling Institute of Medical Research. The diagnosis for each patient specimen was provided following a pathological review of the tissue by Dr Janice Brewer (RNSH, Neuropathology). Tumour specimens were de-identified by tumour bank numbers to protect patient details. Fresh frozen tumour tissue was used for RNA and DNA extractions. Formalin fixed tumour tissue was embedded in paraffin for histological review. These blocks were used to cut slides for immunohistochemistry. Paraffin shavings from these blocks were used for DNA extraction for LOH analysis by PCR.

2.3.2 Preston Robert Tisch Brain Tumor Center Biorepository at Duke University

Tumour specimens were obtained for the purposes of culturing primary tumour cells and developing a cell line. Tumour tissue was obtained fresh from surgery and dissociated to put into culture. Tumours were snap frozen and stored at -80°C for RNA and DNA extraction.

Clinical information for all patients was kept in the central Brain Tumor Biorepository database, deidentified by sample numbers.

2.4 RNA

RNA was extracted from fresh frozen tumour tissue and cultured human tumour cells. All RNA was resuspended in RNase free water and stored at -80°C. RNA was used in this study for microarray and qPCR gene expression analysis.

2.4.1 RNA extraction from fresh frozen tumour tissue

RNA was extracted from tumour tissue with QIAzol lysis reagent (Qiagen). All pipettes, ball bearings, racks and other equipment used in RNA extractions were cleaned with RNaseZAP (Ambion) before each use.

Tumour tissue was cut with a sterile, disposable scalpel in a sterile plastic petri dish on a bed of dry ice. A small section was removed from the bulk of the tumour (~5-10 mg) and was placed in 10% formalin for histological review. Approximately 10-50 mg of the tumour was placed in a pre-cooled 2 mL tube. QIAzol reagent (1 mL) and two 3 mm tungsten ball bearings were added to the sample tube.

The sample was homogenised at 30/sec for 2 x 2 minutes using a tissue pulveriser (Model 200 Mixer Mill, Retsch Technology, GmbH, Haan, Germany). The homogenate was transferred to a fresh 1.7 mL tube and incubated for 5 minutes at room temperature to permit dissociation of nucleoprotein complexes, then centrifuged at 12000 g for 10 minutes at 4°C to remove fat, debris and other insoluble matter. The supernatant was transferred to a fresh 1.7 mL tube.

Chloroform (200 µL) was added to the sample, which was then shaken vigorously for 20 seconds and incubated at room temperature for 10 minutes, then centrifuged at 12000 g for 15 minutes at 4°C. The aqueous layer containing the RNA was carefully removed and transferred to a new tube. The RNA was precipitated with isopropanol (500 µL) and mixed by inversion, incubated at room temperature for 10 minutes, then centrifuged at 12000 g for 10 minutes at 4°C.

The RNA sample was washed twice with 75% ethanol (1 mL per wash), air dried for 5 minutes then resuspended in RNase free water (20 µL) and stored at -80°C.

2.4.2 RNA extraction from cultured tumour cell lines

RNA was extracted from cells derived from cultured human brain tumours with the RNeasy mini kit (Qiagen).

Media was removed from cells, which were then washed in Dulbecco's Phosphate-Buffered Saline (DPBS). Cells were detached from the flask or well with trypsin (0.25% in DPBS). Cells were collected in ice cold DPBS and centrifuged to form a pellet (300 g for 5 minutes). Supernatant was removed and cells were resuspended in Buffer RLT (350 µL), then homogenised by repeatedly drawing the cell suspension through a 27-gauge needle and 1 mL syringe. Ethanol (350 µL) was added and mixed with the sample by pipetting. The entire sample was loaded onto the filter of an RNeasy spin column, resting in a 2 mL collection tube. The sample was centrifuged at 8000 g for 15 seconds and the flow-through discarded. Buffer RW1 (700 µL) was added to the column, then centrifuged at 8000 g for 15 seconds and the flow-through discarded. To wash the column, Buffer RPE (500 µL) was added to the column, centrifuged at 8000 g for 15 seconds and the flow-through discarded. The wash stop was repeated with a second aliquot of Buffer RPE (500 µL). The column was placed in a fresh 2 mL collection tube and centrifuged at 13200 g for 1 minute to remove any residual ethanol. The column was placed in a fresh 1.5 mL tube. The sample was eluted from the column in RNase free water (30 µL), which was added to the column and incubated for 1 minute at room temperature, then centrifuged at 8000 g for 1 minute. RNA was stored at -80°C.

2.4.3 RNA purification – lithium chloride

RNA extracted by QIAzol was put through an additional purification step to remove any residual phenol and potential DNA contamination. Lithium chloride (7.5 M, Ambion) was added to RNA samples at a final concentration of 3.75 M. Samples were incubated at -20°C for 2 hours. RNA was pelleted by centrifugation (>12000 g for 20 minutes at 4°C) and washed twice with 75% ethanol. RNA was air dried for 5-10 minutes, dissolved in RNase free water and stored at -80°C.

2.4.4 RNA quantification – Nano Drop

Quantity and quality of RNA was measured using the NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). RNA was diluted 1:20 in RNase free water prior to measurement. A 2 µL volume of RNase

free water was used as a blank measurement. A 2 µL volume of diluted RNA was pipetted onto the pedestal, the lever arm was lowered and the sample measured. RNA quality was measured by the A260/280 ratio. A ratio of 2.00 is considered optimal purity for RNA. A lower ratio indicated phenol, ethanol, salt or other contamination, and such samples were subjected to a second lithium chloride purification step.

2.4.5 Quality control of RNA

RNA was assessed for purity from genomic DNA contamination by PCR for GUSβ and for degradation on the Agilent Bioanalyzer 2100.

2.4.5.1 Assessment of genomic DNA contamination: GUSβ

RNA was assessed for contaminating genomic DNA by PCR for *β-Glucuronidase* (*GUSβ*). The primers target a section of the *GUSβ* gene spanning an exon boundary with a 200 base pair intron. The cDNA for *GUSβ* produces a 200bp PCR product and the genomic DNA for *GUSβ* produces a 400bp PCR product. The presence of a 400bp PCR product was indicative of genomic DNA contamination of the RNA sample.

RNA was converted to cDNA (see cDNA synthesis 2.4.8) and 2µL cDNA was used as the template for the *GUSβ* PCR reaction (for PCR see section 2.6.5).

2.4.5.2 Measurement of RNA integrity on the Agilent Bioanalyzer 2100

The Agilent Bioanalyzer 2100 (Agilent Technologies, CA) was used with the Agilent RNA 6000 Nano Assay (Agilent Technologies, CA) to calculate the quality of RNA samples. The RIN cut off (minimum) for RNA samples to be used for microarray analysis was 7.0.

Before use, the gel was purified by adding gel (550 µL) to a spin filter, which was then centrifuged at 1500 g for 10 minutes. The gel was then aliquoted into 8 tubes

(65 µL per tube). The RNA ladder was incubated at 70°C for 2 minutes, then cooled on ice. Other reagents were brought to room temperature before starting.

RNA (1 µL) was mixed with RNase free water (1 µL) in a 0.5 mL tube and incubated at 70°C for 2 minutes to denature tertiary structure of RNA, then cooled on ice. The Gel-Dye mix was prepared by combining dye (1 µL) and gel (65µL), which were mixed by vortex and centrifuged at 13000 g for ten minutes.

The RNA 6000 Nano chip was loaded with gel-dye (9 µL) and primed with a syringe plunger for 30 seconds. Gel-dye (9 µL) was then added to each of the two other gel-dye wells. RNA 6000 Nano marker (5 µL) was pipetted into each of the 12 samples wells and the ladder well. RNA ladder (1 µL) and samples (1 µL each) were pipetted into individual wells.

The chip was vortexed for 1 minute, then run in the Agilent 2100 Bioanalyzer. Results were analysed by the Agilent 2100 Expert software (Version B.02.05.SI360).

2.4.6 Removal of DNA contamination

2.4.6.1 Acidified phenol chloroform RNA purification

This procedure was used for RNA samples over 100 µg.

The contaminated RNA sample was brought up to a total volume of 200 µL with RNase free water. An equal volume (200 µL) of Acidified Phenol Chloroform was added to the sample and vortexed briefly, then centrifuged at 12000 g for 5 minutes at 4°C. The aqueous layer was transferred to a new tube and mixed with Chloroform: Isoamyl Alcohol (24:1) (250 µL). The sample was mixed vigorously by hand then centrifuged at 12000 g for 1 minute at 4°C. The aqueous phase was transferred to a fresh tube, containing 3M Sodium Acetate (20 µL). Ethanol (400 µL) was added to the sample, mixed by pipetting and incubated for 5 minutes at room temperature, then centrifuged at 12000 g for 5 minutes at 4°C. The supernatant was discarded and the RNA pellet was washed twice in 75% ethanol. For each wash, 75% ethanol (1 mL) was added to the RNA pellet, vortexed then

centrifuged at 12000 g for 5 minutes at 4°C. Ethanol was removed with a pipette. RNA was air dried for 5-10 minutes then resuspended in RNase free water.

2.4.6.2 DNase treatment

This procedure was used for RNA samples under 100 μg. The RNeasy mini kit (Qiagen) was used to purify RNA, with the RNase free DNase Set (Qiagen) to remove contaminating DNA. Before starting, DNase I stock solution (10 μL) was diluted in Buffer RDD (70 μL) and mixed by inversion.

RNA was brought up to a total volume of 100 μL with RNase free water. Buffer RLT (350 μL) was added to the sample and mixed by pipetting. Ethanol (250 μL) was added to the sample and mixed by pipetting. The sample was applied to an RNeasy Mini column, resting in a 2 mL collection tube. The column was centrifuged at 10000 g for 15 seconds and the flow through discarded. Buffer RW1 (350 μL) was added to the column and centrifuged at 10000 g for 15 seconds and the flow through discarded. Diluted DNase I (80 μL) was applied to the column and incubated at room temperature for 15 minutes, to digest contaminating DNA.

Buffer RW1 (350 μL) was added to the column and centrifuged at 10000 g for 15 seconds. The flow through was discarded and the column placed in a fresh collection tube. To wash, Buffer RPE (500 μL) was added and the sample centrifuged at 10000 g for 15 seconds. The flow through was discarded and a second wash was performed with a fresh aliquot of Buffer RPE (500 μL). The column was placed in a fresh collection tube and centrifuged at 8000 g for 2 minutes to dry the column.

To elute the purified RNA, the column was placed in a fresh 1.5 mL tube. RNase free water (30 μL) was applied to the column, incubated for 1 minute then centrifuged at 10000 g for 1 minute.

2.4.7 Normal brain control RNAs

RNA from normal brain was purchased from Ambion. Normal brain RNA was used for microarray and qPCR analysis. The brain regions and donors for the normal whole brain RNA specimens are listed in Appendix 3B.

2.4.8 cDNA Synthesis

Reverse transcription was performed with the SuperScript® III First-Strand Synthesis System (Invitrogen), according to the manufacturer's instructions. The starting material for each sample was 1 µg total RNA.

RNA was mixed with RNase Free water to a volume of 8 µL in a 0.5 mL tube. Random hexamers (1 µL, 50 ng/µL) and dNTPs (1 µL, 10 mM) were added to the sample, mixed by pipetting, and incubated at 65°C for 10 minutes. The reaction was set on ice for one minute, then the SuperScript™ Reverse Transcription master mix (Table 2.1, 10 µL per reaction) was added. Samples were mixed by gentle pipetting, incubated for 10 minutes at 25°C to allow binding of the random hexamers, 50 minutes at 50°C to permit reverse transcription of RNA to cDNA and finally 5 minutes at 85°C to denature the enzyme.

cDNA was stored at -80°C, or short term at -20°C, for use within 4 weeks.

Table 2.1 Components of SuperScript™ Reverse Transcription master mix

Component	Volume (per reaction)	Final Concentration (20 µL reaction)
RT Buffer (10X)	2 µL	1X
$MgCl_2$ (25 mM)	4 µL	12.5 mM
DTT (0.1 M)	2 µL	0.01 M
RNaseOUT (40 U/µL)	1 µL	40 U/Rxn
SuperScript™ III RT (200 U/µL)	1 µL	200 U/Rxn
Total	**10 µL**	

RT – Reverse transcription; Rxn – Reaction; U – Units of enzyme.

2.5 DNA

DNA was stored at 4°C in Tris EDTA pH 7.0 (TE) buffer. DNA extracted by phenol chloroform from fresh frozen tumour tissue was used for CpG island plus promoter array analysis (Chapter 4.2.2). DNA extracted from blood and paraffin embedded tissue was used for analysis of LOH by PCR conducted on specimens from the Neuroendocrine Tumour Bank of the Kolling Institute of Medical Research, to identify the LOH 1p/19q status for specimens examined by exon array (Chapter 4.3.1), qPCR and immunohistochemistry (Chapter 5.3.1 and 5.3.2). The QIAamp DNA Mini Kit was used to extract DNA frozen tumour tissue and cell lines to use for PCR and sequencing of *IDH1*, *IDH2* and *TP53* (Chapter 6.3.3).

2.5.1 DNA extraction from fresh frozen tumour tissue

2.5.1.1 Phenol chloroform DNA extraction

A 500 mm^3 piece of tumour tissue was minced with a sterile scalpel, placed on a sterile wire mesh over a sterile glass petri dish and teased through the mesh with a sterile glass rod. The tumour fragments were washed and collected in DPBS (2.8 mL) and placed in a 10 mL tube. Triton-X 100 (5% in water, 1.2 mL) was added and gently mixed with the sample, before centrifugation at 1500 g for 5 minutes. The supernatant was discarded and Proteinase K buffer (1 mL), Sodium Dodecyl Sulfate (10% in water, 100 µL) and Proteinase K enzyme (10 mg/mL, 100 µL) were added. The sample was incubated for 16 hours at 220 rpm at 37°C.

An equal volume of phenol-chloroform (1:1 mixture, 1.2 mL) was added and mixed vigorously by shaking for 10 seconds. The sample was allowed to stand for 1 minute, then shaken again and centrifuged at 1500 g for 5 minutes. The aqueous phase was transferred to a new tube and the extraction was repeated with a second aliquot of phenol-chloroform (1.2 mL). The aqueous phase was transferred to a new tube. The extraction was repeated twice with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1 mixture, 1.2 mL per extraction). The aqueous phase was removed to a fresh tube and mixed with a one-tenth volume of

Ammonium acetate (7.5 M, pH 5.5, 120 µL), followed by an equal volume of isopropanol. The sample was mixed by inversion and incubated at -20°C for 16 hours.

The sample was then centrifuged at 1500 g for 30 minutes at 4°C. The supernatant was removed and the DNA pellet washed in 70% ice-cold ethanol, then centrifuged for 20 minutes at 4°C. The supernatant was discarded and the DNA pellet was dried at room temperature. DNA was resuspended in TE buffer and stored at 4°C.

2.5.1.2 QIAamp DNA mini kit DNA extraction

A 25 mg piece of fresh frozen tumour tissue was cut into small pieces with a sterile scalpel and placed in a 1.5 mL tube. Buffer ATL (180 µL) and proteinase K (20 µL) was added. The sample was mixed by vortexing and incubated overnight at 56°C in a thermomixer until the sample was completely lysed. RNase A (4 µL) was added to the sample, which was then mixed by vortexing and incubated at room temperature for 2 minutes. Buffer AL (200 µL) was added to the sample followed by vortexing.

Ethanol (100%, 200 µL) was added to the sample and mixed by vortexing. The entire sample was loaded onto a DNeasy mini spin column resting in a 2 mL collection tube, then centrifuged for 1 minute at 6000 g and flow-through discarded. The column was washed with the addition of Buffer AW1 (500 µL), centrifugation at 6000 g for 1 minute, then the flowthrough discarded. The wash was repeated with Buffer AW2 (500 µL). Following the second wash, the column was centrifuged for 3 minutes at 20000 g to dry the DNeasy membrane. DNA was eluted with Buffer AE (200 µL), which was added to the column and incubated for 1 minute at room temperature. The column was centrifuged for 1 minute at 6000 g.

2.5.2 DNA extraction from blood

DNA extraction from peripheral blood leucocytes was performed using the PureGene DNA extraction kit (Gentra Systems, Minneapolis, MN, USA).

Blood samples (stored at -20°C) were thawed and an aliquot of blood (300 µL) placed in a 1.5 mL tube. To remove red blood cells, RBC Lysis solution (900 µL) was added. The sample was mixed by inversion, then incubated for 10 minutes at room temperature and centrifuged at 15000 g for 20 seconds. The supernatant was discarded and the white cell pellet was vortexed to resuspend cells in residual liquid.

Cell Lysis Solution (300 µL) was added to the sample and mixed by vortex. Protein Precipitation solution (100 µL) was added and the sample mixed by vortex and centrifuged at 15000 g for 3 minutes to remove the precipitated proteins.

The supernatant was removed to a fresh 1.5 mL tube and mixed with isopropanol (300 µL). Precipitated DNA was pelleted by centrifugation at 15000 g for 1 minute. The supernatant was discarded and the DNA pellet washed with 70% ethanol. The ethanol was removed and the DNA pellet allowed to air dry for 10 minutes, then dissolved in TE buffer.

2.5.3 DNA extraction from paraffin embedded tumour tissue

DNA was extracted from paraffin embedded tumour tissue with the QIAamp DNA mini kit (Qiagen, CA).

Paraffin embedded tissue was scraped from the block with a scalpel and placed in a 1.5 mL tube. To dissolve the paraffin, xylene (600 µL) was added to the tube and vortexed. The sample was placed on a rotating wheel (60 rpm) for 30 minutes at room temperature followed by centrifugation (12000 g, 5 minutes). The supernatant was removed by pipette and the procedure repeated with a second aliquot of xylene (600 µL).

1200 µL ethanol (100%) was added to the sample and the tube vortexed and centrifuged at 12000 g for 5 minutes. The supernatant was removed with a pipette, and the remaining ethanol evaporated by placing the open tube in a 37°C heat block for 15 minutes. Buffer ATL (180 µL) and Proteinase K (600 mAU/mL, 20 µL) were added to the tube, which was then vortexed and incubated at 55°C for 48

hours. At 24 hours into this incubation, an additional aliquot of Proteinase K (20 µL) was added to the tube.

Buffer AL (200 µL) was added to the tube which was then vortexed and incubated for 18 hours at 70°C.

Following incubation, ethanol (210 µL) was added to the tube. The sample was vortexed then loaded into a Qiagen spin column, which was placed in a 2 mL collection tube. The column was centrifuged at 6000 g for 1 minute and the flow through discarded. To wash the column, Buffer AW1 (500 µL) was added to the spin column then centrifuged at 8000 g for 1 minute and the flow through discarded. A second wash was done with Buffer AW2 (500 µL). The flow through was discarded and the spin column was replaced in the collection tube and centrifuged at 20000 g for 3 minutes to dry the membrane. To elute the DNA, the spin column was placed in a fresh 1.5 mL tube and Buffer AE (50 µL warmed to 37°C) was added to the top of the column. The column was incubated at 70°C for 5 minutes and centrifuged at 6000 g for 1 minute. The eluate was loaded again to the top of the column, incubated at 70°C for 5 minutes and centrifuged at 6000 g for 1 minute. The DNA sample was stored at 4°C.

2.5.4 DNA extraction from cell lines

DNA was extracted from cultured cell lines with the QIAamp® DNA Mini kit. Before starting, ethanol (100%) was added to Buffers AW1 and AW2.

Cells were washed with DPBS and trypsinized. Cells were collected by centrifugation (300 g for 5 minutes). Supernatant was removed. Cells were resuspended in PBS (200 µL) and transferred to a 1.5 mL tube. Proteinase K (20 µL) and RNase A (4 µL, 100 mg/mL) were added to the tube, mixed by vortexing, and incubated for 2 minutes at room temperature. Buffer AL (200 µL) was added to the tube, mixed thoroughly by vortex and then incubated at 56°C for ten minutes. Ethanol (200 µL) was added to the sample, which was mixed by vortexing, then loaded onto a DNeasy spin column, resting in a 2 mL collection tube. The tube was centrifuged at 6000 g for 1 minute, then the column placed in a fresh collection tube.

Buffer AW1 (500 µL) was added to wash the column, which was then centrifuged (6000 g for 1 minute) and placed in a fresh collection tube. A second wash was performed with Buffer AW2 (500 µL). The column was centrifuged for at 20000 g for 3 minutes to dry the membrane.

To elute the DNA, the column was placed in a clean 1.5 mL tube. Buffer AE (200 µL) was added directly to the column membrane and incubated for 1 minute at room temperature. The column was centrifuged at 6000 g for 1 minute. The eluted DNA was stored at -20°C.

2.6 PCR

2.6.1 Standard PCR

PCR reactions were assembled in a sterile biosafety hood. The working area was pre-irradiated with UV light for 15 minutes to sterilize the pipettes and work surface. Filtered pipette tips were used to prevent aerosol contamination of pipettes. PCR reactions were assembled as per Table 2.2.

Table 2.2 Standard PCR reaction components

Component (Stock concentration)	Volume (µL)	Final Concentration
$H_2 0$	8.94	
5x KAPA buffer A	3	1x
dNTPs (10 mM, 2.5 mM each)	0.3	0.2 mM (0.05 mM each)
DMSO	0.9	6%
Forward Primer (100 µM)	0.15	1 µM
Reverse Primer (100 µM)	0.15	1 µM
KAPA taq (5 U/µL)	0.06	0.3 U/rxn
DNA (5 ng/µL)	1.5	7.5 ng/rxn
Total	**15**	

rxn – Reaction; U – Units of enzyme.

A touchdown PCR program was used for all standard PCRs. Reactions were denatured at 94°C for 2 minutes, followed by 3 cycles of denaturation at 94°C for 20 seconds, annealing at 64°C for 15 seconds and extension at 70°C for 10 seconds; 3 cycles of denaturation at 94°C for 20 seconds, annealing at 61°C for 15 seconds and extension at 70°C for 10 seconds; 3 cycles of denaturation at 94°C for 20 seconds, annealing at 58°C for 15 seconds and extension at 70°C for 10 seconds; 35 cycles of denaturation at 94°C for 20 seconds, annealing at 57°C for 15 seconds and extension at 70°C for 10 seconds. A final extension step at 70°C was performed for 5 minutes.

2.6.2 Primers

Oligonucleotide primers were ordered from Integrated DNA Technologies Inc (Iowa, USA). Primers were resuspended in RNase/DNase free water at a concentration of 100 μM. Several primer pairs were designed to incorporate the M13F(-21) sequence into the 5' end of the forward primer, for ease of processing for sequencing. A summary of all primers used in this thesis is provided in Table 2.3.

Table 2.3 Primers used for PCR amplification and DNA sequencing

Gene	Primer Name	Sequence	Exon, direction
GUSβ	GUS F	ACTATCGCCATCAACAACACACTCACC	F
	GUS R	GACGGTGATGTCATCGATGT	R
IDH1	IDH1-Seq-F(M13)	GTAAAACGACGGCCAGTTGAGCTCTATATGCCATCACTGC	4F
	IDH1-Seq-R	CAATTTCATACCTTGCTTAATGGG	4R
	IDH1 cDNA Seq F (551)	GTAAAACGACGGCCAGCGGTCTTCAGAGAAGCCATT	F
	IDH1 cDNA Seq R (784)	TATACATCCCCATGGCAACA	R
IDH2	IDH2-Seq-F(M13)	GTAAAACGACGGCAGTGTCTGGTGTTGTTGCTTG	4F
	IDH2-Seq-R	CAGAGACAAGAGGATGGCTAGG	4R
	IDH2 cDNA seq F	TGTAAAACGACGGCCAGTAGCCCATCATCTGCAAAAAC	F
	IDH2 cDNA Seq R	GAGATGGACTCGTCGGTGTT	R
TP53	TP53x1u (M13)	GTAAAACGACGGCCAGTCCATCTTGATTTGAATTCCCG	11f
	TP53x1r	ATTGCAAGCAAGGGTTCACCG	11r
	TP53x2u (M13)	GTAAAACGACGGCCAGTATCTGCCTTTGACCATGAAG	10r
	TP53x2r	ATTGCACCATTGCACTCCC	10f
	TP53x3u (M13)	GTAAAACGACGGCCAGTGGAGCACTAAGCGAGGTAAGC	9f
	TP53x3r	TTGTCTTTGAGGCATCACTGC	9r
	TP53x4u (M13)	GTAAAACGACGGCCAGTTTGGGCAGTGCTAGGAAAGAG	8r
	TP53x4r	GTTGGGAGTAGATGGAGCCTG	8f
	TP53x5u (M13)	GTAAAACGACGGCCAGTAGAAATCGGTAAGAGGTGGGC	7r
	TP53x5r	CATCCTGGCTAACGGTGAAAC	7f
	TP53x6u (M13)	GTAAAACGACGGCCAGTCTGCTCAGATAGCGATGGTG	6f
	TP53x6r	AGGCCCTTAGCCTCTGTAAGC	6r
	TP53x7u (M13)	GTAAAACGACGGCCAGTGGGCCAGACCTAAGAGCAATC	5r
	TP53x7r	AAGCTCCTGAGGTGTAGACGC	5f
	TP53x8u (M13)	GTAAAACGACGGCCAGTGAGGAATCCCAAAGTTCCAAAC	4r
	TP53x8r	ACGTTCTGGTAAGGACAAGGG	4f
	TP53x9u (M13)	GTAAAACGACGGCCAGTCAGTCAGATCCTAGCGTCGAG	3f
	TP53x9r	AAATCATCCATTGCTTGGGAC	3r
	TP53x10u (M13)	GTAAAACGACGGCCAGTAGGGTTGGAAGTGTCTCATGC	2f
	TP53x10r	AGCCCAACCCTTGTCCTTAC	2r
	p53x7f62	CTGCTTGCCACAGGTCTCC	7f (seq)
n/a	M13F(-21)	TGTAAAACGACGGCCAGT	sequencing

F – Forward; R – Reverse.

2.6.3 cDNA sequencing for *IDH1* and *IDH2*

Sequence analysis of the Isocitrate Dehydrogenase 1 (*IDH1*) and Isocitrate Dehydrogenase 2 (*IDH2*) genes was performed on 45 cDNA samples. cDNA was diluted 1:20 with molecular grade water and PCR reactions were assembled as per the Standard PCR mixture (Table 2.2).

PCR products were visualised on a 1% agarose gel, diluted in ultrapure water and submitted for sequencing.

2.6.4 Sequencing

Sequencing was performed by Eton BioScience Inc. 20ng/µl DNA (unpurified PCR product) was submitted. The sequencing primer was supplied at a 5 µM concentration, diluted in ultrapure water. PCR products were purified and sequenced at the Eton BioScience facility in Morrisville, NC, USA. Sequences were analysed with 4Peaks software (version 1.7. Mekentosj, The Netherlands).

2.6.5 Assessment of genomic DNA contamination: *GUSβ*

The PCR reaction was assembled as per Table 2.4.

Table 2.4 Components of PCR reactions for *GUSβ* analysis in cDNA samples

Component	**Volume (µL)**	**Final Concentration**
H_2O	18	
10X PCR Buffer	2.5	1X
$MgCl_2$ (25 mM)	0.8	0.8 mM
dNTP (10 mM)	0.5	0.2 mM
Forward Primer – GUS F (20 µM)	0.5	0.4 µM
Reverse Primer – GUS R (20 µM)	0.5	0.4 µM
Taq polymerase (AmpliTaq Gold) 5 U/µL (Applied Biosystems)	0.2	1 U/rxn
cDNA	2 µL	
Total	**25**	

rxn – Reaction; U – Units of enzyme.

Reactions were denatured at 94°C for 5 minutes, followed by 25 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds and elongation at 72°C for 30 seconds. Following the reaction, samples were maintained at 4°C. PCR products were visualised on a 4% polyacrylamide gel.

The presence of a 200 bp band indicated a successful PCR. The presence of a 400 bp band indicated contamination of the RNA sample by genomic DNA. RNA samples that displayed a 400 bp band were purified by acidified phenol chloroform (Section 2.4.6.1) or DNase treatment (Section 2.4.6.2).

2.6.6 Visualisation of PCR products

2.6.6.1 Agarose gel electrophoresis

Agarose (1% w/v) was dissolved in 1X Tris-Acetate-EDTA buffer (TAE buffer; Bio-Rad) at high temperature. When the agarose was completely dissolved, the flask was allowed to cool for five minutes. The gel tray was prepared by clamping it in a mould. The agarose was poured into the gel tray, a comb was inserted and then the gel was allowed to cool for 30 minutes.

The gel tray, containing the gel, was removed from the mould and placed into a gel tank. The tank was filled with 1X TAE buffer until it just covered the entire gel and the comb was removed. DNA (2 µL) was mixed with 1X loading dye (10 µL, 1:10000 SYBR Green) and pipetted into the wells. A 1 Kb ladder (5 µL) was loaded into the first lane as a standard. The gel was run for 60 minutes at 5 Volts/cm (75 Volts for a small [15 cm] tank, 150 Volts for a large [30 cm] tank). The gel was visualised at 460 nM using the BioDoc-It system (UVP, CA) and photographed with a digital camera.

2.6.6.2 Polyacrylamide gel electrophoresis

A gel was prepared by adding 13.5 mL 0.5X Tris-Borate-EDTA (TBE) buffer (0.045 m Tris-borate, 0.045 M boric acid and 0.001 M EDTA), 1.5 mL polyacrylamide (acrylamise/bis 19:1 solution), 22µL TEMED (Bio-Rad) and 120 µL Ammonium

persulfate (10% solution in water) to a 50 mL tube. Reagents were mixed by inversion and poured into gel plates set in the Mini PROTEAN 3 gel casting apparatus (Bio-Rad), and 15-well combs inserted. After 20 minutes, the gels were transferred to the Mini PROTEAN 3 Ready Gel Cell (Bio-Rad) electrophoresis unit, which was then filled with 0.5X TBE buffer. 2µL DNA was mixed with 2µL 2X neutral loading buffer (20% sucrose [w/v], 10 mM Tris-HCl, 1 mM EDTA, 0.1% bromophenol blue [w/v]) and loaded into a well. pUC19/*HpaII* (Bresatec) ladder was used as a standard. Gels were run at 120 volts for 30 minutes. Gels were post stained in ethidium bromide solution (100 µL/L) for ten minutes. Gels were scanned on the FUJIFILM Image Reader FLA-3000 and the images processed with FUJIFILM Science Lab 2001, Image Gauge 4.0 software.

2.6.7 Quantitative real-time PCR

RNA was extracted as described above in (RNA extraction). cDNA was diluted 1:25 with RNase free water before addition to the PCR reaction. Quantitative real-time PCR (qPCR) was conducted on the Corbett Rotorgene (Corbett Life Sciences, now Qiagen). The qPCR reactions were assembled in triplicate for each sample as per Table 2.5. The gene expression assays used in this thesis are summarised in Table 2.6.

Table 2.5 Standard qPCR reaction components

Component	Volume (µL)	Final Concentration
2X TaqMan® Universal PCR Master Mix, no UNG	10	1X
20X 18S TaqMan® assay (see Table 2.6)	1	1X
20X gene of interest TaqMan® assay (see Table 2.6)	1	1X
Water	6	
cDNA (diluted 1:25)	2	
Total	**20**	

Reactions were pipetted by robot (Eppendorf EpMotion) into 0.1 mL tubes (Corbett Research) compatible with the Corbett Rotorgene. The tubes were loaded

into the rotor of the qPCR machine and run through the following thermocycling sequence: Denaturation for 10 minutes at 95°C, then 40 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 60 seconds. Signal data was gathered on the FAM (green) and VIC (yellow) channels.

Table 2.6 TaqMan® gene expression assays used in this thesis

Gene	Assay ID	Amplicon length (bp)
ALX3	Hs00197798_m1 (Inventoried)	65
CHI3L1	Hs00609691_m1 (Inventoried)	77
EGFR	Hs01076092_m1 (Inventoried)	103
IGF2	HS01005964_g1 (Inventoried)	123
IQGAP1	Hs00182622_m1 (Inventoried)	113
MIG-6 (ERRFI1)	Hs00219060_m1 (Inventoried)	57
PADI2	Hs00247108_m1 (Inventoried)	79
PDPN	Hs01089982_g1 (Inventoried)	72
PLAG1	Hs00231236_m1 (Inventoried)	78
18S	18S r RNAmgB - 4319413E	187

bp – base pairs.

2.6.7.1 Standard curves

The efficiency of each gene expression assay was calculated by performing a standard curve. A serial dilution (1:5; 1:50, 1:500, 1:5000, 1:50000) was generated for one normal brain cDNA sample and one oligodendroglioma cDNA sample. The five dilutions of each cDNA was used as the template for qPCR, with each standard conducted in triplicate.

The Corbett RotorGene software was used to calculate the efficiency value for each probe pairing (gene of interest and 18S) from the quantification cycle (C_q) values and the dilution factors for each standard curve.

2.6.7.2 qPCR analysis

Relative Expression Software Tool (REST v.2008) software was used for the analysis of gene expression by qPCR. The efficiency values for each probe and C_q

values for each sample were entered, and the relative expression value for each sample was obtained. Each sample was run in triplicate in a minimum of two qPCR reactions. The relative expression value gained from each of these repeats was averaged to obtain the relative expression value reported in the results here.

2.6.8 Detection of loss of heterozygosity by microsatellite analysis (PaLMS)

Testing for LOH 1p/19q on chromosomes 1p and 19q was conducted in the PaLMS Department of Laboratory and Community Genetics.

Five microsatellite markers located on or near 1p36.3 and four microsatellite markers on or near 19q13.3 were amplified by PCR in parallel patient germline (blood, buccal swab) and brain tumour DNA specimens. Microsatellite markers and the primers used are detailed in Table 2.7. DNA was prepared as described in sections 2.5.2 and 2.5.3. PCR reactions were assembled as per Table 2.8.

Table 2.7 Primers for PCR of microsatellite markers for the detection of loss of heterozygosity

Location	Marker	Temp (°C)	Primer	Sequence 5'-3'	Expected range (bp)
1p	D1s508	60	Fwd	AGCTGGGGAATATATGTNTCATAT	73-85
			Rev	TGTGGAAGGCCAACTC	
	D1S2743	60	Fwd	GGTTCAAGGGATTCTCCTG	108-134
			Rev	TGGCACTCAGACCTCAA	
	D1s2783	60	Fwd	CCCTACCCTAATTCCACTG	119-151
			Rev	GTTTATGTTTCACCTCCTATCC	
	D1s226	50	Fwd	GCTAGTCAGGCATGAGCG	69-115
			Rev	GGTCACTTGACATTCGTGG	
	D1s162	60	Fwd	GGGGGAAGAAGTCCGAGTAG	115-175
			Rev	ATAAGGGGAACAGGTCTGGG	
19q	D19s219	64	Fwd	GTGAGCCAAGATTGTGCC	160-190
			Rev	GACTATTTCTGAGACAGATTCCCA	
	D19s412	64	Fwd	GAATGAGACTCTGTCTCAAAACA	89-113
			Rev	ACCTCATGTGTCTCCTCCTT	
	D19s596	64	Fwd	CCACAGAGCAAGACTCGAT	172-190
			Rev	GCCAGAGCCACTGTGT	
	D19s918	55	Fwd	AAAGGCTTGATTACCCCCGA	130-185
			Rev	GATTACAGGCGTGAGCACCG	

bp – Base pairs.

Table 2.8 Components of PCR reactions for PaLMS detection of LOH 1p/19q

Component	Volume (µL)	Final Concentration
H_20	14.4	
10x PCR AMP buffer	2	1x
dNTPs (2 mM each)	2	0.2 mM each
Forward Primer (20 µM)	0.2	0.2 µM
Reverse Primer (20 µM)	0.2	0.2 µM
Platinum *Taq* (5 U/µL)	0.2	1 U/rxn
DNA (5 ng/µL)	1	5 ng/ rxn
Total	**20**	

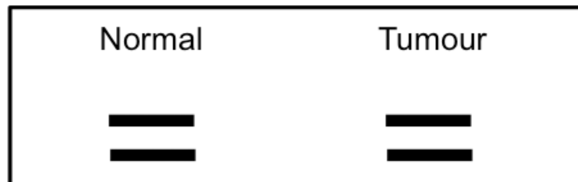
rxn – Reaction; U – Units of enzyme.

Reactions were denatured at 94°C for 3 minutes, followed by 35 cycles of denaturation at 94°C for 15 seconds, annealing at the optimal temperature for each primer pair (Table 2.7) for 15 seconds and extension at 72°C for 15 seconds. A final extension step was carried out at 72°C for 5 minutes. Completed reactions were stored at 4°C.

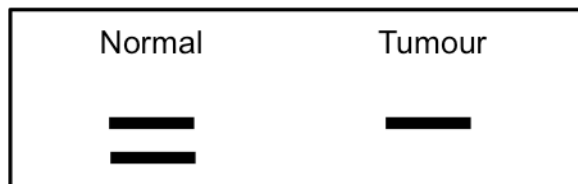
Following amplification, reactions were denatured (95°C/5 min), loaded on a denaturing gel (7 M Urea 5% Polyacrylamide [0.3 x TBE]) in the GelScan 2000 apparatus, electrophoresed for 45 minutes and analysed in 1DSCAN. Results were interpreted as per Figure 2.1.

A. Uninformative at this locus – both homozygous for the same allele

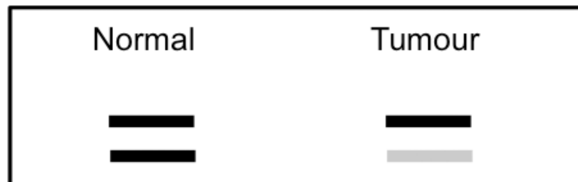
B. Informative at this locus and retention of both alleles in tumour tissue – Heterozygous for different alleles



C. Informative at this locus and loss of heterozygosity in tumour tissue – Heterozygous for different alleles



or



D. Informative at this locus and loss of heterozygosity in tumour tissue at both alleles OR not amplified in tumour tissue – Heterozygous for different alleles

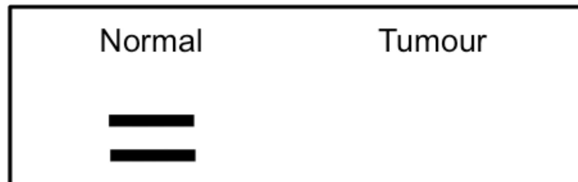


Figure 2.1 Interpretation of microsatellite PCR results for the detection of LOH on chromosomes 1p and 19q.

2.7 Immunohistochemistry

2.7.1 Detection of loss of heterozygosity by fluorescence *in situ* hybridisation

Detection of LOH 1p/19q by Fluorescence In Situ Hybridisation (FISH) was conducted at St Vincents Hospital (50 samples tested) and Duke University Medical Center (standard test for LOH 1p/19q in oligodendroglioma patients). The Vysis 1p36/1q25 and 19q13/19p13 FISH Probe Kits (Abbott Molecular) were used at St Vincents Hospital. The same kits were used at Duke University Medical Center. The methods used here were previously published (Ellis et al., 2005). The 1p36 and 19p13 probes are located in the most common regions of loss in oligodendroglioma (Figure 1.7).

4μm thick paraffin sections were mounted on Superfrost Ultra slides™. Sections were deparaffinised in xylene (2 x 10 minutes), dehydrated in ethanol (5 minutes), washed in 0.2 N HCl (20 minutes), washed in distilled water, then incubated in Zymed heat pre-treatment solution (50 mL) at 95°C for 95 minutes. Following pre-treatment, sections were washed twice in distilled water.

Zymed digestion enzyme solution (90 μL) was applied to the sections, which were then overlayed with a coverslip and incubated at 38°C in a humidified chamber for 60 minutes. Sections were then washed three times in distilled water then dehydrated through graded ethanols (70%, 95%, 100%) and air dried.

PathVysion probes (sample DNA and Probe DNA) were denatured at 72°C for 5 minutes, then applied to the sections and hybridised at 37°C overnight. Sections were washed in wash solution (0.4x saline sodium citrate, Tween 20) at 73°C for 2 minutes, then transferred to 2x wash solution at room temperature for 30 seconds. Sections were dehydrated in graded ethanols and air dried in darkness.

Fluorescence mounting medium was mixed with DAPI counterstain (50:1) and applied to the sections, which were then coverslipped and viewed under a fluorescent microscope.

Scoring was done by counting 60 cells in the area of greatest tumour content, and deriving a ratio of 1p/1q and 19q/19p expression. Cut-off scores were assessed using the method of Smith et al (Smith et al., 2000).

2.8 Protein and Western blot

2.8.1 Protein extraction from cultured cells

Cell media was removed and cells were washed with DPBS. Cells were detached from the culture flask with trypsin (0.2%) or by mechanically scraping the cells with a cell scraper. Cells were resuspended in DPBS then centrifuged at 300 g for 3 minutes. The supernatant was removed and cells were resuspended in Protein Lysis Buffer (Table 2.9), incubated on ice for 15 minutes, before centrifugation (12000 g for 10 minutes at 4°C) to remove DNA and other cell debris. An aliquot of the cell lysate (2 µL) was removed to calculate the protein concentration of the sample. The rest of the cell lysate was transferred to a fresh tube and stored at -80°C.

Table 2.9 Components of Protein Lysis Buffer

Component	Volume/amount	Final Concentration
Tris pH 7.5 (1 M)	20 mL	50 mM
Sodium Chloride	8.766 g	150 mM
Sodium Fluoride (40 g/L)	5.25 mL	5 mM
Nonidet P-40 (IGEPAL® CA-630)	5 mL	0.5 %
Water	969.75 mL	
Total	**1000 mL**	

2.8.2 Protein quantification

Protein concentration was determined by the Bradford assay, with a BSA standard curve.

Bio-Rad Protein Assay Dye Reagent Concentrate was diluted to 1x concentration in water. Pre-diluted protein assay BSA set Standards (Bio-Rad) were diluted in protein assay dye to create a BSA standard set, with a concentration range of 5 to 90 µg/mL. 200 µL of each standard was added to two wells of a 96-well plate.

Protein samples (cell lysates) were diluted in protein assay dye (1:210) and 200 µL of each sample was added to two wells of a 96-well plate. Absorbance was measured on the OPTIMA plate reader at 595 nm. (POLAR star OPTIMA BMG Labtech)

A standard curve for the BSA standards was calculated, and used to determine the concentration of each protein sample.

2.8.3 Western blot

2.8.3.1 Sample preparation

To make 2x protein loading dye, β-mercaptoethanol was diluted in BioRad Laemmli Sample Buffer (1:20). Cell lysate (10 to 40 µg protein) was mixed with 2x protein loading dye, to a maximum volume of 20 µL. Protein samples were incubated at 95°C for 10 minutes, then placed on ice to await loading on the gel.

Protein standards: MagicMark™ XP Western Standard (2.5 µL, Invitrogen) was mixed with SeeBlue® pre-stained standard (1.5 µL, Invitrogen) in a 1.5 mL tube and incubated at 37°C for 5 minutes. This mixture results in a protein ladder that is visible on the membrane under normal light and also on film following ECL detection.

2.8.3.2 Gel setup, loading and running

Protein gels were run using the NuPAGE gel system (Invitrogen). A NuPAGE 4-12% BisTris gel was set in the gel box. The gel tank was filled with NuPAGE MES SDS Running Buffer, diluted to 1x with RO water.

Samples (10 to 40 µg protein, up to 20 µL) and standards (4µL) were loaded into lanes of the gel, which was then run at 150 volts for 60 minutes.

2.8.3.3 Protein transfer

The gel was removed from its cassette and soaked briefly in 1x NuPAGE transfer buffer. A nitrocellulose membrane and two sheets of thick blotting paper were also soaked in the transfer buffer for 5 minutes. The transfer buffer was used to wet the entire surface of the BioRad semi-dry transfer apparatus.

One sheet of thick blotting paper was placed on the transfer apparatus. The membrane was placed on top of the paper and the gel placed on top of the membrane. Bubbles were removed by rolling a 10 mL plastic pipette across the top of the gel. The remaining piece of thick blotting paper was placed on top of the gel. The lid was placed on the transfer apparatus and run at 5 volts for 30 minutes.

2.8.3.4 Antibody hybridisation and washing

The membrane was taken out of the transfer apparatus and placed in wash solution (10 mM Tris pH 7.5; 100 mM NaCl; 0.1% TWEEN20). The membrane was blocked in blocking solution (wash solution with 5% skim milk powder) for 2 hours at room temperature, or overnight at 4°C.

The primary antibody was diluted in blocking solution as per Table 2.10. The membrane was incubated with the primary antibody for 90 minutes at room temperature on a shaking platform. The membrane was washed in wash solution (5 x 5 minutes, then 3 x 10 minutes, minimum volume 50 mL per wash).

The secondary antibody was diluted in blocking solution as per Table 2.10. The membrane was incubated with the secondary antibody for 30 minutes at room temperature on a shaking platform. The membrane was washed in wash solution (5 x 5 minutes, then 3 x 15 minutes).

Table 2.10 Antibodies used in Western blot

Name	Indication	Supplier	Animal of origin	Dilution
Anti GAPDH	Primary	Santa Cruz: GAPDH antibody, Rabbit polyclonal #sc-25778	Rabbit	1:10000
Anti MIG-6	Primary	Proteintech Group, Inc: ERRFI1 antibody, Rabbit polyclonal #11630-1-AP	Rabbit	1:2500
Anti-Mouse	Secondary	GE Healthcare: ECL™ Anti-mouse IgG, HRP linked whole antibody #NA931	Sheep	1:5000
Anti Rabbit	Secondary	GE Healthcare: ECL™ Anti-rabbit IgG, HRP linkes whole antibody #NA934V	Donkey	1:5000

2.8.3.5 Signal detection

Excess wash solution was removed and the membrane was then placed on a clean plastic sheet. The SuperSignal® West Femto Maximum Sensitivity Substrate kit (Thermo Fisher Scientific) was used for detection of the signal. The kit components were brought to room temperature. Component A (1 mL) was mixed with component B (1 mL) in a 15 mL tube. The detection reagent was applied to the membrane for 5 minutes. Excess detection reagent was poured off the membrane. The membrane was sandwiched between two sheets of plastic and fixed in place in a film cassette with lab tape.

In the dark room, HyBlot CL™ Autoradiography film (Denville Scientific, NJ) was exposed to the blot for a set period of time (15 seconds to 5 minutes). Film was developed on the Kodak X-OMAT 2000A processor (Carestream, Rochester, NY).

3 Clinical Review of Oligodendroglioma cases at Royal North Shore and North Shore Private Hospitals

3.1 Introduction

3.1.1 Overview

The presence of LOH 1p/19q in grade II and III oligodendroglioma and oligoastrocytoma, first identified in 1989, is associated with longer progression free and overall survival (Reifenberger et al., 1994; Jenkins et al., 1989; Smith et al., 2000). The routine testing of LOH 1p/19q for patients diagnosed with oligodendrogliomas has been ongoing at Royal North Shore (RNS) and North Shore Private (NSP) hospitals since 2005. This clinical audit was conducted to investigate the reproducibility of the survival advantage in patients with the co-deletion treated at this centre.

Other factors have been shown to improve prognosis and include younger age at presentation, good Karnofsky Performance Score (KPS), tumour grade, lack of contrast on a MRI scan, low mitotic index and gross macroscopic resection of the tumour (Daumas-Duport et al., 1997; Schiffer et al., 1997). It has also been reported that patients who initially present with seizures survive longer than those who present with other symptoms such as neurological deficit, intracranial hypertension and mental deterioration (Hamlat et al., 2005). The effect of these additional factors on survival in RNS and NSP patients with intact 1p/19q is also investigated in this chapter.

3.1.2 TP53

Analysis of grade III oligodendrogliomas by Ino et al found that among patients that lacked LOH of chromosome 1p, the presence of a *TP53* mutation conferred a distinct survival advantage (Ino et al., 2001). *TP53* sequencing is not available at all clinical centres, which has led to the use of immunohistochemistry to detect TP53 abnormalities in oligodendroglioma specimens.

High TP53 immunoreactivity (> 75% of cells) in oligodendrogliomas was indicative of extremely poor prognosis (Kros et al., 1993). Wild type TP53 has a short half-life (20 minutes) compared to the most commonly occurring TP53 mutants (1 to 7 hours) (Hinds et al., 1990). Unless present in uncommonly high levels, wild type TP53 is unable to be detected by immunohistochemistry. Based on this information, it has been long assumed that positive immunohistochemistry is indicative of mutant TP53, and a negative result is indicative of wild type TP53 (Nayak et al., 2004).

3.1.3 Aims

1. Conduct an audit of patients diagnosed with oligodendroglioma at Royal North Shore and North Shore Private Hospital to determine if LOH 1p/19q is associated with better survival.

2. Determine which additional factors may be associated with improved prognosis in those oligodendroglioma patients with intact 1p/19q.

3.2 Methods

3.2.1 Clinical cohort

A total of 105 patients diagnosed with an oligodendroglioma were identified for this cohort. All patients were operated on and subsequently treated at Royal North Shore (RNS) and North Shore Private (NSP) Hospitals between April 1987 and November 2007. Central histopathological diagnosis was performed by an experienced neuropathologist (Dr Janice Brewer) at RNS Hospital. The retrospective clinical audit was conducted with approval from the local human ethics committee, as described in chapter 2.2.1. Twenty-nine patients were diagnosed as grade II oligodendroglioma (OII), 62 patients were diagnosed as grade III oligodendroglioma (OIII), 5 patients were diagnosed with a grade II oligoastrocytoma (OAII) and 9 patients were diagnosed with a grade III oligoastrocytoma (OAIII). Due to low numbers of oligoastrocytomas, these patients were excluded from further analyses.

The clinical information collected for each patient included date of diagnosis, presenting symptoms (specifically documentation of seizures), tumour location, extent of surgical resection (debulk or biopsy), treatment received after surgery (no treatment, radiotherapy only or radiotherapy and chemotherapy) and LOH 1p/19q. Overall survival was determined from the primary surgery date to the date of death or the census date.

3.2.2 Loss of Heterozygosity of 1p and 19q

Loss of heterozygosity for 1p/19q was determined at one of two sites: RNS and St Vincent's Hospital. The method for LOH 1p/19q analysis at RNS is a PCR based microsatellite length assay (chapter 2.6.8). St Vincent's Hospital uses a fluorescent in-situ hybridization (FISH) method (chapter 2.7.1) (Smith et al., 2000). Where LOH 1p/19q testing had not been conducted prospectively, this was performed retrospectively where formalin-fixed paraffin-embedded tissue was available.

3.2.3 TP53 Immunohistochemistry

TP53 protein expression was evaluated on formalin-fixed paraffin-embedded sections. The NCL-L-TP53-DO7 clone (Novacastra) was used which detects both the wild type and mutant forms of TP53. The complete absence of nuclear staining, or very occasional staining (≤ 5%), was regarded as negative. This value was based upon the spread of the observed staining percentages, where positive staining typically encompassed percentage nuclear staining in greater than 60% of counted cells. TP53 staining was conducted by Adele Clarkson (Department of Anatomical Pathology, RNSH).

3.2.4 Statistical Analysis

The primary end point was overall survival. Cox proportional hazards regression analysis was performed to assess LOH 1p/19q as a prognostic marker for survival, adjusted for age, tumour grade, extent of tumour resection, treatment and TP53 immunoreactivity. Kaplan-Meier survival analysis was used to generate survival curves and estimates of median survival times, with the use of two-sided log rank statistics (SPSS, version 18.0, Chicago IL, U.S.A).

3.3 Results

3.3.1 Loss of heterozygosity 1p and 19q (LOH 1p/19q)

A case-series of 13 samples were assessed using both microsatellite PCR and FISH methods for detection of LOH 1p/19q. The result was identical for both methods in all 13 cases (Table 3.1).

Table 3.1 Case series of 13 grade II and III oligodendroglioma samples examined for LOH 1p/19q by PCR and FISH

IHC Study number	Tumour bank specimen number	Diagnosis	LOH result (FISH)	LOH result (PCR)
3912	B3843	OIII	LOH 1p/19q	LOH 1p/19q
3915	B3108	OII	No LOH	No LOH
3923	B3679	OIII	LOH 1p/19q	LOH 1p/19q
3932	B3757	OII	LOH 1p/19q	LOH 1p/19q
3943	B3042	OIII	No LOH	No LOH
3947	B3002	OIII	LOH 1p/19q	LOH 1p/19q
3950	B3633	OIII	No LOH	No LOH
3966	B3561	OII	No LOH	No LOH
1	B120501B1	OIII	LOH 19q only	LOH 19q only
3985	B3578	OIII	No LOH	No LOH
3939	B3540	OIII	No LOH	No LOH
4283	B3590	OIII	No LOH	No LOH
3934	B117801B1	OIII	LOH 1p/19q	LOH 1p/19q

OII – grade II oligodendroglioma; OIII – grade III oligodendroglioma.

LOH 1p/19q data was available for 23 out of 29 grade II and 60 out of 62 grade III oligodendroglioma patients. The clinical data, categorized by LOH 1p/19q is summarised in Table 3.2. Forty-eight per cent of patients with grade II (12 out of 25) and 43% of patients with grade III oligodendroglioma (26 out of 60) showed LOH 1p/19q co-deletion.

Intriguingly, one grade III oligodendroglioma patient demonstrated gain of 1p and gain of 19q. As expected, patients diagnosed with grade II oligodendroglioma survived significantly longer than patients diagnosed with grade III oligodendroglioma ($p<0.001$; Figure 3.1A). The median survival for grade II patients was 16.3 years whilst the median survival for patients diagnosed with a grade III oligodendroglioma was 2.0 years (not stratifying for LOH 1p/19q). The survival time of patients with and without LOH was also examined, irrespective of tumour grade. The median survival of patients with LOH 1p/19q was 10.9 years compared to 2.0 years for patients without LOH 1p/19q ($p<0.001$; Figure 3.1 B).

Table 3.2 Clinical characteristics of oligodendroglioma patients

	LOH 1p/19q (%)	No LOH (%)	LOH 1p only (%)	LOH 19q only (%)
Grade II Oligodendroglioma (n=23)				
Patients (total number)	12	11		
Average age (years)	42	39		
[range]	[21-69]	[21-59]		
Tumour resection (Debulk)	9/12 (75)	10/11 (91)		
Tumour location				
Frontal	7/12 (58)	8/11 (73)		
Temporal	2/12 (17)	2/11 (18)		
Parietal	1/12 (8)	-		
Other	2/12 (17)	1/11 (9)		
Presenting with seizures	7/12 (58)	6/11 (55)		
TP53 protein expression [≥5%]	1/8 (12)	2/6 (33)		
Treatment				
No Treatment	4/11 (36)	6/9 (66)		
Radiotherapy only	3/11 (27)	2/9 (22)		
Radiotherapy plus chemotherapy*	4/11 (36)	1/9 (11)		
Grade III Oligodendroglioma (n=59)				
Patients (total number)	26	25	4	4
Average age (years)	47	58	43	56
[range]	[25-73]	[27-80]	[31-58]	[31-69]
Tumour resection (Debulk)	21/26 (81)	22/25 (88)	4/4 (100)	4/4 (100)
Tumour location				
Frontal	12/26 (46)	16/25 (64)	3/4 (75)	1/4 (25)
Temporal	4/26 (15)	7/25 (28)	1/4 (25)	2/4 (50)
Parietal	6/26 (23)	3/25 (12)	-	1/4 (25)
Other	4/26 (15)	-	-	-
Presenting with seizures	14/24 (58)	7/25 (28)	1/3 (33)	2/3 (75)
TP53 protein expression [≥5%]	2/17 (12)	12/20 (60)	1/2 (50)	1/3 (33)
Treatment				
No Treatment	4/26 (15)	1/23 (4)	-	1/4 (25)
Radiotherapy only	5/26 (19)	9/23 (39)	1/3 (33)	2/4 (50)
Radiotherapy plus chemotherapy**	17/26 (65)	13/23 (57)	2/3 (66)	1/4 (25)

*Radiotherapy plus chemotherapy – chemotherapy added after recurrence of tumour. ** Radiotherapy plus chemotherapy – chemotherapy added following radiotherapy or recurrence of tumour.

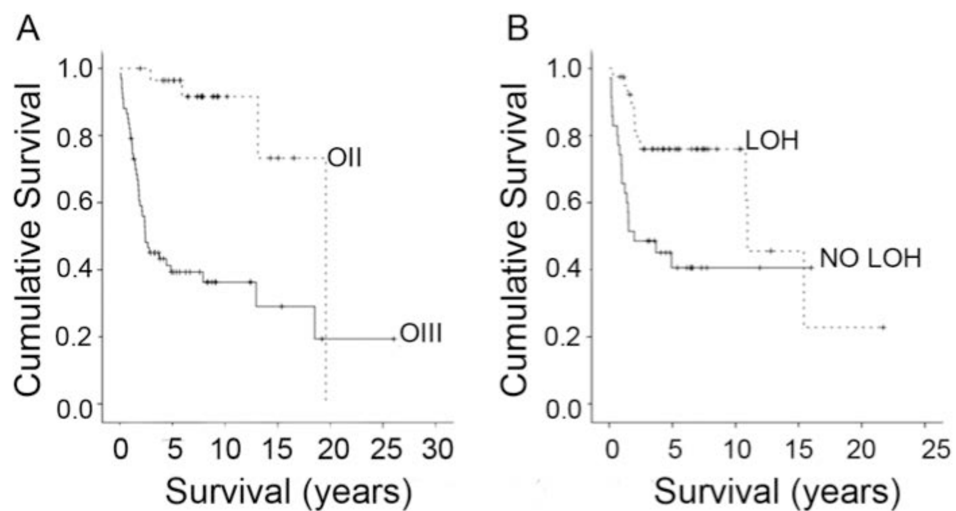


Figure 3.1 Survival analysis of patients diagnosed with oligodendroglioma. Kaplan-Meier estimates of overall survival in the entire series (n= 91). (A) Disease-specific survival of oligodendroglioma patients stratified by grade: OII (n=29), OIII (n=62); LogRank p<0.001; (B) Disease-specific survival of oligodendroglioma patients stratified by LOH 1p/19q: LOH (n=38), No LOH (n=48); LogRank p<0.001.

3.3.2 Univariate analysis of survival for patients diagnosed with grade III oligodendroglioma

Due to the lack of survival census data in the grade II oligodendroglioma group (21 out of 27 patients were still alive at the time of this study) all further analyses were specific to the grade III patient group only. The effect of LOH 1p/19q, increased age, treatment (surgery only, surgery plus radiotherapy or surgery plus radiotherapy and chemotherapy) and the presence of seizures at clinical presentation on survival was assessed and Kaplan Meier survival curves were generated for each variable (Table 3.2). The median survival was 15.4 years for grade III oligodendroglioma patients with LOH 1p/19q tumours and 1.2 years for patients without LOH 1p/19q (Figure 3.2 A). The unadjusted hazard ratio (HR) for death in the patient group without LOH was 3.5 (95%CI: 1.6-7.5; $p<0.001$). Increase in patient age (measured in decades) was also associated with significantly poorer survival (HR, 1.7 [95%CI: 1.3-2.3]; $p<0.001$, Figure 3.2 B). When the patients were stratified according to treatment, the group size for patients who received no treatment was very small (n=5). No median survival could be deduced from this group. A significant survival benefit was observed in grade III oligodendroglioma patients who were treated with both radiotherapy and adjuvant chemotherapy treatment (n=36) compared to patients who received radiotherapy only (n=16) (10.8 years compared to 1.45 years; $p=0.026$; Figure 3.2C). The unadjusted HR for death in the grade III oligodendroglioma patients who were treated with radiotherapy only was 2.4 (95% CI: 1.1-5.3; $p=0.030$). Patients were also categorized into groups according to clinical presentation of a seizure (simple partial, complex partial or generalised). The median survival of patients who presented with a seizure was 10.8 years compared to 1.5 years for patients who did not (Figure 3.2 D). The unadjusted HR for death in patients who did not present with a seizure was 2.4 (95% CI: 1.5-5.0, $p=0.016$).

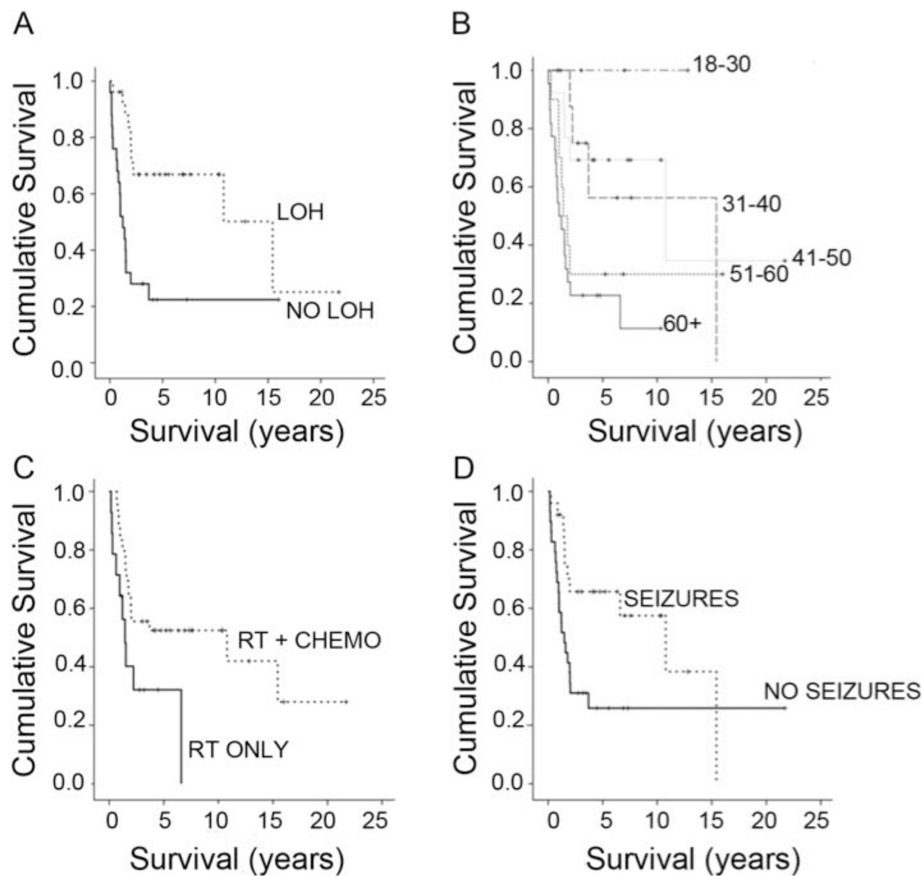


Figure 3.2 Survival analysis of patients diagnosed with grade III oligodendroglioma.
Kaplan-Meier estimates of overall survival in OIII patients according to LOH, age, treatment received and presence of seizures at clinical presentation. (A) Disease specific survival of OIII patients with LOH (n=26) compared to patients with no detectable LOH (n=25); LogRank p<0.001. (B) Disease specific survival of patients with increasing age: 18-30 years (n=3), 31-40 years (n=10), 41-50 years (n=13); 51-60 years (n=10) and 61years+ (n=22); LogRank p<0.001. (C) Disease specific survival of OIII patients treated with radiotherapy only (n=14) compared to patients treated with radiotherapy and chemotherapy (n=36); LogRank p=0.030. (D) Disease specific survival of OIII patients with documented seizures at clinical presentation: with seizures (n=25), no seizures (n=29); LogRank p=0.016.

3.3.3 Multivariate analysis of survival for patients diagnosed with grade III oligodendroglioma

Cox survival analysis, including variables that were significant in univariate analyses (LOH, treatment and the presence of seizures), revealed that LOH 1p/19q (HR, 3.4; p=0.015) was an independent marker of prognosis when adjusted for treatment and seizures (Table 3.3).

Table 3.3 Cox-proportional hazards model: Factors influencing survival in grade III oligodendroglioma patients

Variable	HR (95% C.I.)	p-value
LOH status	3.4 (1.3 – 9.0)	0.015
Treatment (radiotherapy only)	1.9 (0.8 – 4.8)	0.176
Seizures	1.3 (0.5 – 3.1)	0.588

HR - Hazard Ratio; C.I. - Confidence Interval

3.3.4 Prognostic significance of TP53 protein expression

Immunohistochemical detection of the TP53 protein and its impact on survival in grade III oligodendroglioma patients was also examined. TP53 expression was categorized as negative (expression in ≤5% of tissue) or positive (>5%). No significant difference in survival was observed between patients based on TP53 status (p=0.776). The median survival of patients with positive TP53 protein expression was 1.8 years compared to 2.0 years for patients negative for TP53.

However when considering only patients without LOH 1p/19q, those positive for TP53 immunoreactivity demonstrated a survival advantage compared to patients that were negative for TP53 expression. The median survival of patients without LOH 1p/19q and positive for TP53 (n=11) was 2.0 years compared to 11.8 months (0.99 years) for patients without LOH and negative for TP53 (n=8; Figure 3.3). This survival advantage did not reach significance (p=0.221).

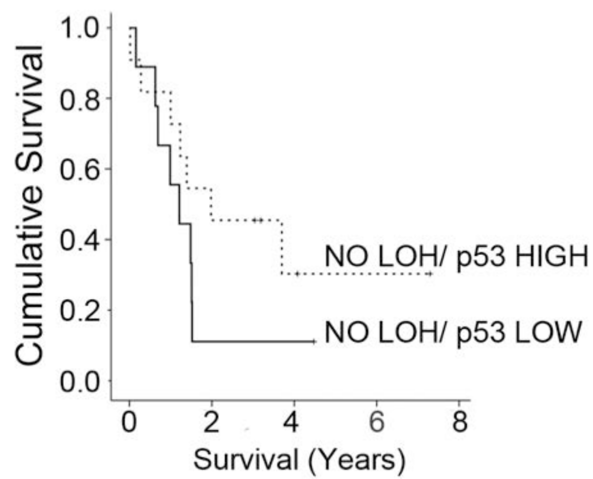


Figure 3.3 Influence of TP53 intensity measured by immunohistochemistry on grade III oligodendroglioma patients without LOH 1p/19q
Disease specific survival of patients with negligible TP53 (n=9) compared to patients with high TP53 staining (n=11); LogRank p=0.171.

3.4 Discussion

In this cohort, patients diagnosed with grade III oligodendroglioma and harbouring LOH 1p/19q survived significantly longer than those with intact 1p and 19q alleles. Treating patients with chemotherapy and the clinical presentation of seizure were also associated with better survival. This study confirms the findings of similar audits elsewhere and supports the routine testing of all newly diagnosed oligodendrogliomas for LOH 1p/19q (Louis et al., 2007).

The proportion of grade III oligodendroglioma patients that have LOH 1p/19q (43%) is in keeping with numbers reported in the literature (van den Bent et al., 2006; Cairncross et al., 2006). However in the cohort of OII patients, the number with LOH 1p/19q (48%) is lower than reported elsewhere (see Table 1.2).

Survival of grade III oligodendroglioma patients without LOH 1p/19q in our cohort was 1.2 years, which is similar to the established survival for glioblastoma patients, which is 12-15 months (Wen and Kesari, 2008). This is striking, as patients diagnosed with oligodendroglioma will likely be given a more hopeful prognosis and less aggressive treatment than those diagnosed with glioblastoma. The survival time for patients with grade III oligodendroglioma without LOH 1p/19q is generally given as 2 to 3 years (van den Bent et al., 2008), which is about twice as long as found here.

TP53 expression been shown to be a useful marker in previous studies. Patients with high TP53 immunoreactivity were shown to have poor survival (Kros et al., 1993). Testing for LOH 1p/19q was not done at the time of the Kros study.

Ino et al. stratified grade III oligodendroglioma patients by 1p/19q and other genetic alterations, showing that patients with intact 1p had significantly longer survival if they also harboured a *TP53* mutation, as revealed by DNA sequencing (Ino et al., 2001). Here it was shown that positive TP53 immunoreactivity correlated with longer survival. Assuming that positive TP53 immunoreactivity is due to a *TP53* mutation, our data is in agreement with the stratification of oligodendroglioma patients proposed by Ino et al.

This study is the first of its kind in Australia, and confirms the prognostic and predictive effect of LOH 1p/19q on survival in Australian patients with grade III oligodendroglioma. Other significant factors influencing survival were presentation with seizures and treatment. Whilst the role of LOH 1p/19q status as a requirement in diagnosis or in dictation of patient treatment is still developing, it clearly impacts upon patient counselling regarding prognosis, and should be mandatory in the assessment of all newly diagnosed gliomas.

4 Microarray analysis of gene expression and DNA methylation in oligodendrogliomas with and without LOH 1p/19q

4.1 Introduction

4.1.1 Overview

Chapter three of this thesis confirmed LOH 1p/19q as a robust predictor of patient survival in the cohort of oligodendroglioma patients treated at Royal North Shore and North Shore Private hospitals. The loss of chromosomes 1p and 19q may result in reduced gene expression of genes located in the regions of loss as well as changes in the expression of genes located throughout the genome.

This chapter describes the analysis of gene expression in oligodendrogliomas with and without LOH at 1p/19q using gene expression profiling and subsequent data analysis to identify genes that are highly differentially expressed between the two genotypes. DNA methylation in grade III oligodendrogliomas with LOH at 1p/19q was also examined using methylation arrays to identify genes on 1p and 19q that are lost due to LOH 1p/19q and methylated on the remaining allele.

4.1.2 Gene expression analysis of oligodendroglioma

Gene expression levels in cancer are highly variable and can provide valuable clues as to which oncogenic pathways are overactive, altered or susceptible to drug targeting. Microarray technology is used to examine global gene expression, as it is a high-throughput method for assessing expression throughout the genome. Previous studies have employed a variety of microarray platforms to profile gene expression in oligodendrogliomas with and without LOH 1p/19q (Mukasa et al.,

2002, 2004; French et al., 2005; Tews et al., 2006; French et al., 2007; Ducray et al., 2008; Ferrer-Luna et al., 2009) (Table 4.1). Each of these studies published a list of differentially expressed genes and validated a set of handpicked genes as candidates for either a tumour suppressor or chemo-resistant gene in oligodendrogliomas with LOH 1p/19q. While there was some overlap in the published gene lists, no gene was further investigated by more than one study. Global changes in gene expression due to LOH 1p/19q were also noted, as neural genes and genes involved in brain development were more highly expressed in oligodendrogliomas with LOH 1p/19q (Mukasa et al., 2002; Tews et al., 2006; Ferrer-Luna et al., 2009).

Table 4.1 Microarray studies of oligodendroglioma with LOH 1p/19q

Reference	Platform	Objective	Results	Oligodendroglioma		Oligoastrocytoma		Other
				Grade II	Grade III	Grade II	Grade III	
(Mukasa et al., 2002)	GeneChip® HG U133 plus 2.0	Identify candidate genes on 1p and 19q.	209 differentially expressed genes, 9 validated.	3 LOH 1p/19q 4 No LOH	2 LOH 1p/19q 1 No LOH	1 LOH 1p only		
(French et al., 2005)	GeneChip® HG U133 plus 2.0	Identify genes associated with LOH 1p, 19q, patient response and survival.	60 genes identified by LOH 1p/19q status, 16 genes identified by patient response.	1 LOH 1p/19q 1 No LOH	12 LOH 1p/19q 10 No LOH 1 LOH 1p only 3 LOH 19q only			
(Tews et al., 2006)	Custom made arrays, enriched for 1p and 19q regions	Identify candidate genes on 1p36 and 19q13.	37 genes differentially expressed by LOH status, 4 validated.	6 LOH 1p/19q 1 No LOH	8 LOH 1p/19q 6 No LOH		1 LOH 1p/19q 5 No LOH	
(French et al., 2007)	GeneChip® Human exon 1.0 array	Identify differentially expressed exons due to LOH 1p/19q.	715 splice variants and differentially expressed exons, 17 validated.	2 LOH 1p/19q	20 LOH 1p/19q			18 GBM (EGFR+) 8 GBM (2°)
(Ducray et al., 2008)	GeneChip® HG U133 plus 2.0	Compare gene expression profiles between tumours with LOH 1p/19q and tumours with EGFR amp.	Proneural gene signature enriched in OIII with LOH 1p/19q. 22 genes validated.		4 LOH 1p/19q		1 No LOH (EGFR+)	5 GBM (EGFR+) 3 AIII (EGFR+)
(Ferrer-Luna et al., 2009)	GeneChip® HG U133 plus 2.0	Identify gene expression changes due to LOH 1p/19q.	In tumours with LOH 1p/19q, increased expression of neurogenesis genes, decreased expression of cell proliferation and motility genes.	3 LOH 1p/19q 12 No LOH 1 LOH 1p only	2 LOH 1p/19q 1 LOH 1p only	5 LOH 1p/19q 1 No LOH 1 LOH 1p only	1 LOH 1p/19q 1 No LOH 1 LOH 19q only	

While previous microarray studies in oligodendroglioma have either defined LOH 1p/19q specific gene signatures or identified numerous biomarkers relating to LOH status, no follow-up studies have been published on the function of these genes in oligodendroglioma biology. In this study, gene expression in oligodendrogliomas with LOH 1p/19q will be compared to oligodendrogliomas with intact 1p and 19q chromosomes, using exon array technology. The study will include analysis of gene expression patterns and Gene Set Enrichment Analysis (GSEA) to identify previously published gene sets that are enriched in one or the other group of oligodendrogliomas. This study differs from the standard practice in the literature of defining gene signatures or to identify yet more biomarkers. Instead this study is designed to identify individual genes that contribute significantly to the biological differences due to LOH 1p/19q, using exon microarray technology.

The GeneChip® Human Exon 1.0 ST array is a specialised technology to profile gene expression at both the whole transcript and the exon level. The exon microarray is a single colour chip, containing 1.4 million probes, representing all known and putative genes at the time of array design. 90% of known exons are each represented by four probes on the array and the remaining 10% of exons are each represented by 2 or 3 probes, due to short or repetitive sequence. The exon array was selected for this study because it produces more robust gene expression data than the standard 3 prime arrays, such as the GeneChip® HG U133 plus 2.0 array, which only contain probes located at the 3' end of the gene. Expression of individual exons can be used to determine patterns of alternate gene splicing, which is known to play a key role in biology contributing to significant protein diversity. This is the first study to use exon array technology to investigate changes in gene expression due to LOH 1p/19q in oligodendroglioma.

4.1.3 DNA methylation analysis of oligodendroglioma

Epigenetic activity is typically associated with loss of heterozygosity events and can result in the silencing of the single remaining copy of a gene within the region of loss. In oligodendroglioma with LOH at chromosomes 1p and 19q, the methylation status of the genes on the remaining allele has been analysed in many

studies and is key in determining whether these genes are transcriptionally silenced. Genes *TP73* (Dong et al., 2002), *PEG3* (Kohda et al., 2001), *ZNF342* (Hong et al., 2003), *CITED4* (Tews et al., 2007), *EMP3* (Alaminos et al., 2005; Kunitz et al., 2007) and *DIRAS3* (Riemenschneider et al., 2008), all located on 1p of 19q, have each been identified as potential tumour suppressor genes following detection of DNA methylation of the remaining allele. Candidate genes *PEG3* (19q13.4) and *EMP3* (19q13.3) are both methylated on the remaining 19q allele and demonstrate tumour suppressor characteristics, illustrating the significance of DNA methylation in detection and validation of candidate genes and their function (Trouillard et al., 2004; Maegawa et al., 2001; Kohda et al., 2001; Jiang et al., 2010; Kunitz et al., 2007; Alaminos et al., 2005)

Another methylation event that has a significant impact on glioma is methylation of the *MGMT* promoter, identified as a predictor of chemosensitivity in both glioblastoma and oligodendroglioma (Hegi et al., 2005; Ney and Lassman, 2009). In oligodendrogliomas, *MGMT* promoter methylation occurs most frequently in oligodendrogliomas with LOH 1p/19q (van den Bent et al., 2009). A possibly related phenomenon is the CpG island methylation phenotype (CIMP^{+}), which also occurs frequently in oligodendroglioma with LOH 1p/19q (Noushmehr et al., 2010; van den Bent et al., 2011). Both CIMP^{+} and *MGMT* promoter methylation are associated with longer patient survival.

Here we aim to identify novel methylation events in oligodendroglioma that may result in the loss of function of genes on 1p and 19q and contribute significantly to oligodendroglioma biology. The CpG island plus promoter array (Roche NimbleGen), selected as the microarray platform for this analysis, contains oligonucleotide probes (50- to 75-mers) targeted to all UCSC-annotated CpG islands and promoter regions for genes in the RefSeq database (7–24 probes per CpG island). DNA methylation at CpG islands will be compared between oligodendrogliomas with LOH 1p/19q and normal brain samples, focusing on CpG islands and associated genes located on chromosomes 1p and 19q.

4.1.4 Aims

Aim 1: To use exon arrays to profile gene expression in oligodendrogliomas with and without loss of heterozygosity at 1p and 19q, normal brain samples and glioblastomas. Microarray analysis software programs will be used to analyse and identify differential patterns of gene expression

Aim 2: To identify genes that are differentially expressed and alternately spliced between oligodendrogliomas with and without LOH 1p/19q, that may be used as biomarkers, functional targets for therapy or may contribute significantly to the biology of oligodendroglioma.

Aim 3: To assess epigenetic changes in oligodendrogliomas with LOH 1p/19q and normal brain specimens. The CpG island plus promoter array will be used to identify genes that are transcriptionally silenced in oligodendroglioma with LOH 1p/19q.

4.2 Methods

4.2.1 Exon array analysis of gene expression.

4.2.1.1 Specimens

Grade II oligodendroglioma (2 with LOH 1p/19q, 5 without LOH 1p/19q), grade III oligodendroglioma (5 with LOH 1p/19q, 5 without LOH 1p/19q), glioblastoma (n=8) and normal brain (n=4) specimens were analysed by exon arrays. ANOVA analysis of the patient group for age bias was conducted in SPSS Statistics (version 18.0, Chicago IL, U.S.A.).

4.2.1.2 RNA and Exon array hybridisation

RNA was extracted from selected oligodendroglioma tissue samples and with the purity and concentration of RNA samples assessed with the NanoDrop system. RNA samples were assayed for DNA contamination by PCR and purified if necessary. The integrity of RNA samples was analysed on the Agilent Bioanalyser. RNA samples with an RNA Integrity Number (RIN) over 7.0 were acceptable for microarray analysis. For full details of RNA processing, see chapter 2.4.

Total RNA from 7 grade II oligodendroglioma, 10 grade III oligodendroglioma, 8 glioblastoma and 4 normal brain samples was supplied to the Clive and Vera Ramaciotti Centre for Gene Function Analysis, University of NSW, Australia for additional quality control checks, whole transcript sense target labelling and hybridization to the Affymetrix GeneChip® Exon arrays.

Total RNA (1μg) was used as starting material. Briefly, poly-A RNA controls, rRNA reduction and RNA concentration steps were preformed in the GeneChip ® Fluidics Station 450. The WT cDNA synthesis and Amplification Kit was used to synthesise and purify cDNA. cDNA was labelled with the WT terminal labelling kit. Hybridisation of cDNA, controls and stain cocktails with the Affymetrix GeneChip® Exon ST arrays was performed in the GeneChip® Hybridisation Oven 640. The

GeneChip® Scanner 3000 7G (Affymetrix) was used to scan the arrays and convert to CEL data files.

4.2.1.3 Exon array data analysis

4.2.1.3.1 GeneSpring

GeneSpring GX 7.3.1 (Agilent Technologies Inc, CA) was used to obtain a global perspective of gene expression in the samples arrayed. CEL files were imported into GeneSpring and Robust Multichip Average (RMA) normalisation was used to normalise signal level for probe GC content. Probe sets were condensed to gene assignments for analysis of gene expression. Data were analysed with respect to tumour grade, LOH status and sample type using one-way ANOVA parametric tests. Volcano plot graphing tools were used to visualise ANOVA results.

4.2.1.3.2 Partek

Partek Genomics Suite (version 6.10.1020) was used to analyse exon array data for both gene expression and alternative splicing. CEL files were imported and normalised by RMA to adjust for GC content of array probes. Probeset, transcript, gene ontology and gene annotation libraries for the exon arrays were automatically downloaded by Partek for data interpretation.

Using the gene expression workflow, CEL files were imported and annotated for tumour grade, LOH status and sample number. A gene summary was conducted to summarise probe data to whole genes. Principal Components Analysis (PCA) was performed to present the multidimensional array data in two or three principal components. A one-way ANOVA was used to calculate the fold change and p-values for differential expression of each gene. A list of differentially expressed genes was generated from these values, including genes with a p-value below 0.05 and fold change above 2. Hierarchical clustering was invoked based on the gene lists generated. Dotplots were generated for individual genes of interest, displaying expression data for each sample. Genes were ranked by the T-statistic, which

combines the p-value and the fold change into a single statistical value, ranking the impact of differential expression.

Under the exon workflow, CEL files were imported as core exons and annotated. PCA analysis was performed and 1-way ANOVA was used to calculate genes that displayed differential exon expression. Gene view summaries were generated for genes of interest.

4.2.1.3.3 Gene Set Enrichment Analysis

Gene set enrichment analysis (GSEA) was performed using GSEA software version 2.07 (Mootha et al., 2003; Subramanian et al., 2005). GSEA is a computational method that determines whether a defined gene set has enriched expression or over representation in a phenotype class within a data set. GSEA can be used to compare new data sets with previously published gene lists.

Expression data for all genes for all oligodendroglioma samples, normalised to normal brain baseline expression in Partek, was used for GSEA. Microarray data were converted to .txt format. Phenotype classes were created, categorising samples by LOH status. Gene sets were downloaded from the Broad Institute website or created from published gene lists. The three file types (expression data, phenotype, gene set) were loaded into the GSEA software, then run (Parameters: Number of Permutations: 1000; Permutation type: Gene Set; Chip platform: SEQ_EXPRESSION.chip; Gene Set size - min 15, max 500). Gene sets used in the analysis are listed in Table 4.2. A gene set was considered significantly enriched in a phenotype class if it had a Nominal p-value below 0.05 and a False Discovery Rate (FDR) q-value below 25% (0.25).

Table 4.2 Gene set matrices used for GSEA

Gene Set matrix	Type	Gene sets
Phillips, Cancer Cell 2006	Published gene set	3
C1.all.v3.0.symbols.gmt	Positional Gene sets	326
C2.all.v3.0.symbols.gmt	Curated gene sets	3272
C3.all.v3.0.symbols.gmt	Motif gene sets	836
C4.all.v3.0.symbols.gmt	Computational gene sets	881
C5.all.v3.0.symbols.gmt	Gene Ontology Gene sets	1454

4.2.2 CpG island plus promoter array analysis of DNA methylation

This experiment was conducted in collaboration with Dr Michael Buckland (The University of Sydney). DNA was extracted from fresh frozen oligodendroglioma tissue (4 grade II, 3 grade III) or normal brain tissue (n=4) with phenol chloroform (See chapter 2.5.1.1). Tumour blocks were analysed by a neuropathologist (Dr Michael Buckland) to ensure each oligodendroglioma sample contained at least 90% tumour tissue, to minimise sample contamination by non-tumour tissue.

The DNA samples were sent to Roche Nimblegen (USA) for Methylated-DNA immunoprecipitation (MeDIP) and CpG island plus promoter array hybridization. Details of these methods are described briefly below.

4.2.2.1 Methylated DNA immunoprecipitation

DNA was enriched for methylated regions by immunoprecipitation (IP) as previously described (Weber et al., 2005).

Genomic DNA was sonicated to produce random fragments ranging in size from 300 to 1,000 bp. Fragmented DNA (4 µg) was denatured for 10 minutes at 95°C and immunoprecipitated for 2 hours at 4 °C with monoclonal antibody against 5-methylcytidine (Eurogentec cat# BI-MECY-0100; 10 µL) in IP buffer (10 mM sodium phosphate (pH 7.0), 140 mM NaCl, 0.05% Triton X-100; final volume 500 µL). The mixture was incubated with Dynabeads M-280 sheep antibody to mouse IgG (Dynal Biotech; 30 µL) for 2 hours at 4 °C and washed three times with IP

buffer (700 μL per wash). The beads were then treated with proteinase K for 3 hours at 50 °C. Methylated DNA was recovered by phenol-chloroform extraction followed by ethanol precipitation.

4.2.2.2 CpG island plus promoter array

MeDIP-enriched and input DNAs were differentially labelled with Cy5 and Cy3 dyes, respectively, and co-hybridised to a CpG island plus promoter array (Roche NimbleGen; Madison, WI). The CpG island plus promoter array contains oligonucleotide probes (50- to 75-mers) targeted to all UCSC-annotated CpG islands and the promoter regions for RefSeq genes (7–24 probes per CpG island). Peak methylation calls were made by the manufacturer and provided to us. Methylation calls were viewed using the SignalMap software (ver. 1.9; Roche NimbleGen).

Data analysis was performed by Dr Michael Buckland and Bjorn Espedido (The University of Sydney). Peak calls for adjacent probes were merged to represent whole CpG islands. Regions on 1p and 19q methylated in at least three out of seven tumours and no controls were identified and searched for candidate genes.

A secondary analysis of the data, with respect to the candidate genes identified by the gene expression microarray (Section 4.3.5) was conducted.

4.3 Results

4.3.1 Oligodendroglioma specimens analysed by exon array

Tumour specimens profiled by exon arrays included 5 grade II oligodendrogliomas with LOH 1p/19q, 2 grade II oligodendrogliomas without LOH 1p/19q, 5 grade III oligodendrogliomas with LOH 1p/19q, 5 grade III oligodendrogliomas without LOH 1p/19q. Oligodendroglioma specimens analysed by the exon array are summarised in Table 4.3.

Table 4.3 Oligodendroglioma specimens selected for exon array analysis

Diagnosis	LOH status	Brain tumour number	Gender	Age	RIN
OIII	LOH 1p/19q	3034	M	31	7.3
OIII	LOH 1p/19q	3122	F	31	8.7
OIII rec	LOH 1p/19q	3202	M	44	8.3
OIII	LOH 1p/19q	3283	M	34	7.4
OIII	LOH 1p/19q	3591	M	50	9
OIII	No LOH	3042	F	40	8.6
OIII	No LOH	3124	M	77	8.1
OIII	No LOH	3173	M	63	8.1
OIII	No LOH	3571	M	75	8.3
OIII	No LOH	3590	F	73	7.9
OII	LOH 1p/19q	3583	M	32	8.1
OII	LOH 1p/19q	3265	F	32	8.2
OII	LOH 1p/19q	3610	M	39	8.1
OII rec	LOH 1p/19q	3622	F	46	6.9
OII	LOH 1p/19q	3837	M	39	7.4
OII rec	No LOH	3506	M	36	7.1
OII	No LOH	3037	M	37	7.5

OII – Grade II Oligodendroglioma; OIII – Grade III Oligodendroglioma; rec – recurrent tumour; LOH – Loss of Heterozygosity; 1p/19q – Chromosomes 1p and 19q; M – Male; F – Female; RIN – RNA Integrity Number.

Analysis of the patient group selected for microarray analysis revealed some undesired bias in some of the genotype groups. Patients without LOH were significantly older than patients with LOH 1p/19q (p=0.035). This age difference was prominent in the grade III samples only (p=0.012).

In addition, gene expression profiling was also conducted on four normal brain and eight glioblastoma specimens (Table 4.4). The four commercially obtained normal brain RNA samples (Ambion) were included in the analysis as normal controls and consisted of one whole brain human reference RNA and three RNA specimens from specific regions of the brain where oligodendrogliomas occur most frequently - orbital frontal cortex, parietal cortex posterior and parietal cortex superior. The eight glioblastoma samples were included only in the global analysis of differential gene expression, providing a point of comparison.

Table 4.4 Additional RNA samples analysed by exon array

Sample number	Sample type	Age	RIN
NIWB	Normal brain	n/a	7.2
NOFC	Normal brain	n/a	7.3
NPCS	Normal brain	n/a	7.0
NPCP	Normal brain	n/a	7.6
3667	GBM	46	8.5
1379	GBM	51	8.7
R143	GBM	51	7.5
3743	GBM	54	7.7
3195	GBM	58	7.8
3166	GBM	48	8.7
3239	GBM	35	7.8
3146	GBM	34	7.7

NIWB – Normal Whole Brain; NOFC – Normal Orbital Frontal cortex; NPCS – Normal Parietal Cortex Superior; NPCP – Normal Parietal Cortex Posterior; GBM – Glioblastoma; RIN – RNA Integrity Number.

4.3.2 Overview of differential gene expression

Volcano plots were generated in GeneSpring to show differential gene expression between grade III oligodendrogliomas with LOH 1p/19q, grade III oligodendrogliomas without LOH 1p/19q and glioblastomas (Figure 4.1). Strikingly, only 58 genes were differentially expressed between glioblastoma and grade III oligodendroglioma without LOH 1p/19q, ten-fold fewer than were differentially expressed between grade III oligodendrogliomas with and without LOH 1p/19q (613 genes). The greatest number of differentially expressed genes was observed between grade III oligodendroglioma with LOH 1p/19q and glioblastoma (848 genes).

4.3.3 Differential gene expression in grade II and III Oligodendrogliomas

4.3.3.1 Principal Components Analysis

The four normal brain specimens grouped closely together when both two and three principal components were analysed (Figure 4.2). All grade III oligodendroglioma specimens and all but two grade II oligodendroglioma specimens aligned closely on principal component one. All but one of the grade III oligodendrogliomas with LOH 1p/19q clustered together on both two and three principal components. Little similarity was observed among any of the grade II oligodendrogliomas on either two or three principal components.

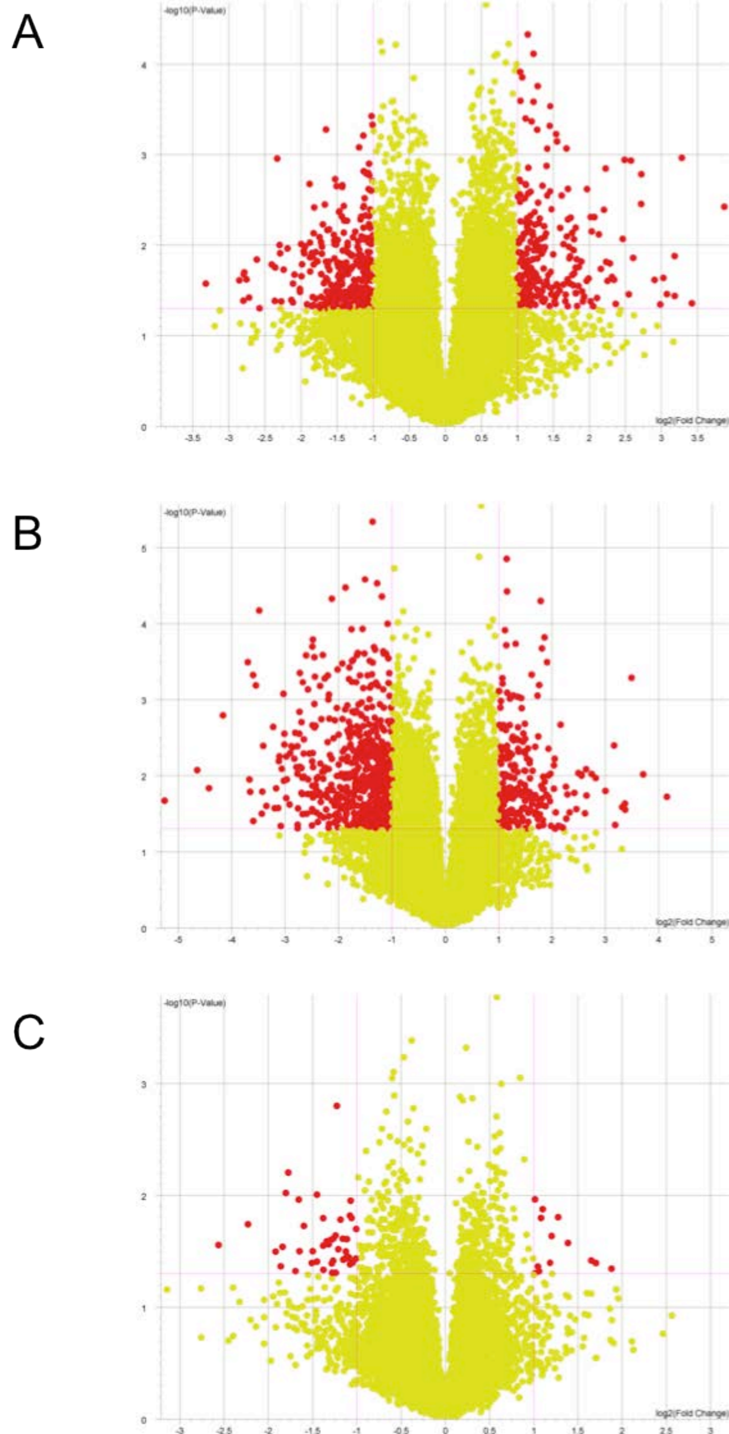
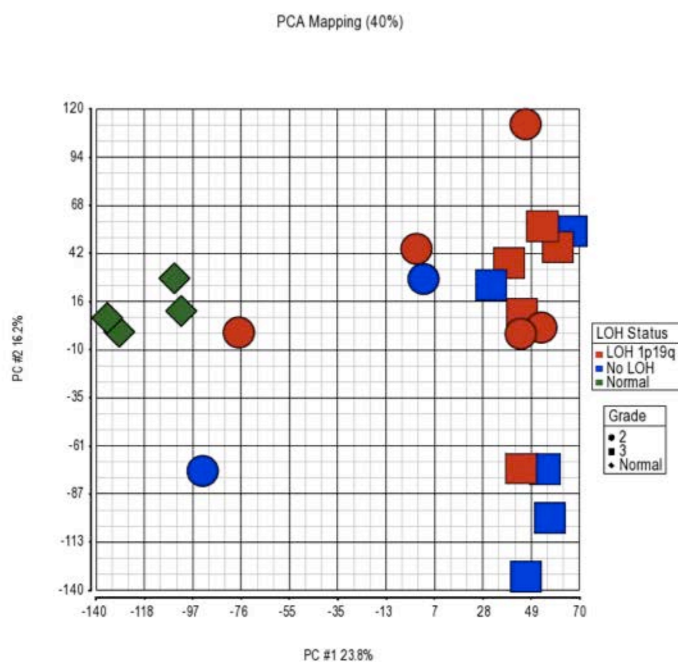


Figure 4.1 Volcano plots of differentially expressed genes between oligodendroglioma and glioblastoma.
[A] OIII with LOH 1p/19q and OIII with no LOH (613 genes) [B] OIII with LOH 1p/19q and GBM (848 genes) and [C] OIII without LOH and GBM (58 genes). Red dots represent genes that were differentially expressed with a fold change greater than 2 (or less than -2) and a p-value below 0.05. Yellow dots represent genes that were not significantly differentially expressed.

A



B

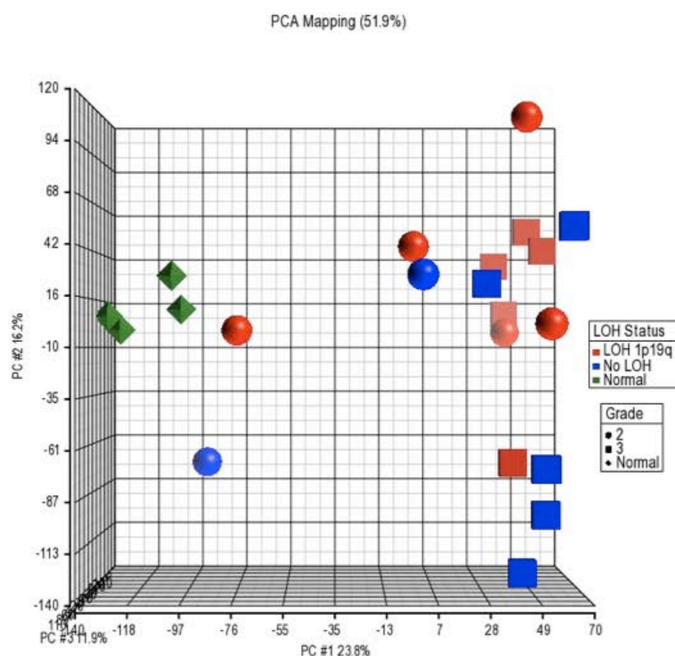


Figure 4.2 Principal Components Analysis.
4 normal, 10 grade III oligodendroglioma and 7 grade II oligodendroglioma tumour samples were analysed by [A] two or [B] three principal components. Red - Oligodendroglioma with LOH 1p/19q; blue - Oligodendroglioma without LOH; green - Normal brain sample. Circle/sphere - grade II tumour; Square/cube - grade III tumour; Diamond/dodecahedron – normal brain sample.

4.3.3.2 Genes differentially expressed in oligodendrogliomas separated by LOH status.

Gene expression analysis of 17 oligodendrogliomas, separated by LOH 1p/19q status, revealed 429 significantly differentially expressed genes. 21 genes without a gene assignment were excluded, resulting in a final list of 408 differentially expressed genes. 95 genes were expressed more highly in oligodendrogliomas with LOH 1p/19q and 313 genes were expressed more highly in oligodendrogliomas without LOH 1p/19q. The top ten over- and under-expressed genes are listed in Table 4.5. Of the 313 genes over-expressed in oligodendrogliomas without LOH 1p/19q, genes on chromosome 1p and 19q accounted for 10.54% and 3.88% of the genes, respectively. The 20 most differentially expressed genes on 1p and 19q are summarised in Table 4.6.

Hierarchical clustering invoked on the 17 oligodendroglioma and four normal samples showed that the four normal samples cluster closely together (Figure 4.3). Seven of the ten oligodendrogliomas with LOH 1p/19q formed a second tight cluster. Of the remaining samples, three with LOH 1p/19q and seven without LOH formed loose clusters, revealing outliers of the samples 3591 (OIII LOH 1p/19q) and 3622 (OII LOH 1p/19q) as they each clustered loosely with oligodendrogliomas without LOH 1p/19q. Sample 3583 (OII LOH 1p/19q) did not cluster with any samples.

Table 4.5 Top 20 differentially expressed genes in oligodendrogliomas with LOH 1p/19q

Gene Symbol	Gene Name, common alias'	Function	Locus	Fold Change	p-value	T-stat
Over-expressed genes						
SART3	Squamous cell carcinoma antigen recognized by T cells 3	Tumour rejection antigen		2.128	7.40E-05	5.105
MOSC1	MOCO sulphurase C-terminal domain containing 1	Reducing enzyme of N-hydroxylated compounds		2.449	0.00016	4.750
POLR2F	Polymerase (RNA) II (DNA directed) polypeptide F	RNA polymerase subunit		2.800	0.00018	4.675
FAM36A	Family with sequence similarity 36, member A	Putative protein		2.249	0.00019	4.670
LMBR1	Limb region 1 homolog (mouse)	Developmental gene, limb patterning		2.013	0.00045	4.664
MLLT3	Myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 3	Transcription factor		2.481	0.00019	4.273
KIF21B	Kinesin family member 21B	Microtubule-dependent motor protein		3.452	0.00060	4.147
THEM4	Thioesterase superfamily member 4	Inhibits PKB activity, commonly downregulated in GBM		2.046	0.00072	4.068
SEZ6L2	Seizure related 6 homolog (mouse)-like 2	Endoplasmic reticulum protein		2.110	0.00074	4.055
OR2L3	Olfactory receptor, family 2, subfamily L, member 3	Olfactory receptor		3.059	0.00157	3.719
Under expressed genes						
DHRS3	DHRS3	Oxidation and reduction	1p36.1	-2.207	4.73E-06	-6.430
ZNF436	Zinc finger protein 436	Transcription factor: negative regulator of MAPK signalling	1p36	-2.454	5.97E-06	-6.313
SRPX2	Sushi-repeat-containing protein, X-linked 2	Cell adhesion and motility	Xq21.33-q23	-3.843	9.21E-06	-6.099
ITGAX	Integrin, alpha X (complement component 3 receptor 4 subunit)	Cell adhesion with ECM and cytoskeleton	16p11.2	-2.159	1.93E-05	-5.741
C4B	Complement component 4B (Chido blood group)	Complement system	6p21.3	-4.206	4.29E-05	-5.360
LAMA2	Laminin, alpha 2	Embryonic cell attachment, adhesion and organisation	6q22-q23	-2.911	5.91E-05	-5.210
LDHA	Lactate dehydrogenase A	Muscle cell metabolism	11p15.4	-2.315	7.93E-05	-5.073
SH3BGRL3	SH3 domain binding glutamic acid-rich protein like 3	Regulator in all-trans retinoic acid-induced pathway.	1p35-p34.3	-2.131	9.64E-05	-4.983
PLAG1	Pleiomorphic adenoma gene 1	Transcription factor	8q12	-2.071	0.00011	-4.896
IGF2	Insulin-like growth factor 2 (somatomedin A)	Growth hormone	11p15.5	-4.824	0.00012	-4.853

Table 4.6 Top 20 genes on 1p and 19q most under-expressed (by fold change) in oligodendrogliomas with LOH 1p/19q

Location	Gene Symbol	Fold Change			
		OII	OIII	ALL	Average
1p13.3	*CHI3L2*		-4.382		-4.382
1p31.3	*GNG12*		-4.665	-3.161	-3.913
19q13.3	*EMP3*			-3.760	-3.760
1p21	*COL11A1*		-4.243	-3.037	-3.640
1p36.21	*PDPN*	-2.756	-4.056	-3.559	-3.457
1p34	*LAPTM5*		-3.738	-2.939	-3.338
1p13	*CD53*		-3.500	-2.889	-3.194
1p31.1	*IFI44L*	-3.975		-2.306	-3.141
1p22.2	*GBP5*		-3.666	-2.350	-3.008
1p36.2	*SLC2A5*		-3.450	-2.499	-2.974
19q13.3-q13.4	*C5AR1*		-3.429	-2.474	-2.951
1p35	*MAN1C1*		-3.215	-2.665	-2.940
1p36	*ZNF436*		-3.145	-2.454	-2.800
1p35-p34.3	*PTAFR*		-3.248	-2.327	-2.787
1p35.2	*SPOCD1*		-2.771		-2.771
19q13.33	*FTL*		-3.041	-2.450	-2.746
19q13.4	*LAIR1*		-2.977	-2.511	-2.744
1p36	*ERRFI1 (MIG-6)*		-3.185	-2.274	-2.730
1p13.3	*GSTM5*		-2.727		-2.727
1p13.2	*C1orf162*		-3.201	-2.197	-2.699

OII – Fold change by LOH status in grade II oligodendrogliomas only; OIII – Fold change by LOH status in grade III oligodendrogliomas only; ALL – Fold change by LOH status in all oligodendrogliomas.

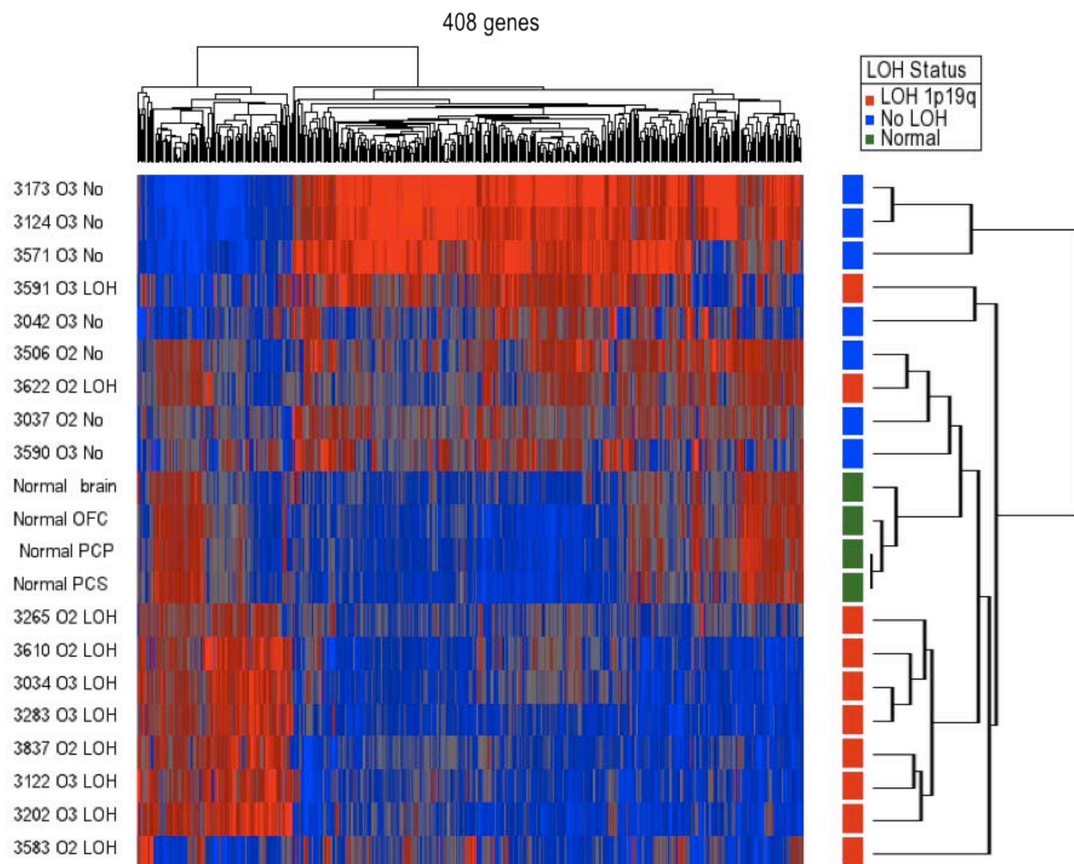


Figure 4.3 Hierarchical clustering of oligodendrogliomas.
Hierarchical clustering was invoked by 408 differentially expressed genes in 10 oligodendrogliomas with LOH 1p/19q, 7 oligodendrogliomas without LOH 1p/19q and 4 normal brain samples. Red – high expression; grey – no change in expression; blue – low expression.

4.3.4 Analysis of alternative splicing in Oligodendrogliomas

34 genes with a significant alternate splice p-value were identified when alternative splicing analysis was conducted on 17 oligodendrogliomas (10 with LOH 1p/19q: 7 without LOH) in Partek. The top ten alternately spliced genes are listed in Table 4.7. The *RUNX1* gene had the highest likelihood of alternative splicing between oligodendrogliomas with and without LOH 1p/19q, based on core probe sets (Figure 4.4). However it is unclear from the data, graphed by Partek, as to which exons are alternately spliced due to high expression variability of individual probes within each exon.

Table 4.7 Top ten alternately spliced genes

Gene Symbol	Gene name, Common Alias'	Function	Locus	Alt Splice p-value
RUNX1	Runt-related transcription factor 1	Transcription factor	21q22.3	9.51E-34
ITGAX	Integrin, alpha X (complement component 3 receptor 4 subunit)	Integrin chain protein	16p11.2	1.56E-20
SUPT4H1	Suppressor of Ty 4 homolog 1 (S. cerevisiae)	Transcriptional regulation	17q21-q22	6.14E-18
C4B	Complement component 4B (Chido blood group)	Complement system	6p21.3	4.60E-16
RHBDF2	Rhomboid 5 homolog 2 (Drosophila)	Unknown	17q.25.1	1.55E-13
ST3GAL3	ST3 beta-galactoside alpha-2,3-sialyltransferase 3	Sialyltransferase	1p	2.12E-13
SAMD4B	Sterile alpha motif domain containing 4B	Possible tumour suppressor	19q13.2	3.29E-12
ITK	IL2-inducible T-cell kinase	T-cell differentiation	5q31-q32	2.02E-10
AVIL	Advillin	Neuronal ganglia development	12q14.1	4.70E-10
PIK3R5	Phosphoinositide-3-kinase, regulatory subunit 5	Proliferation and cell survival	17p	8.89E-10

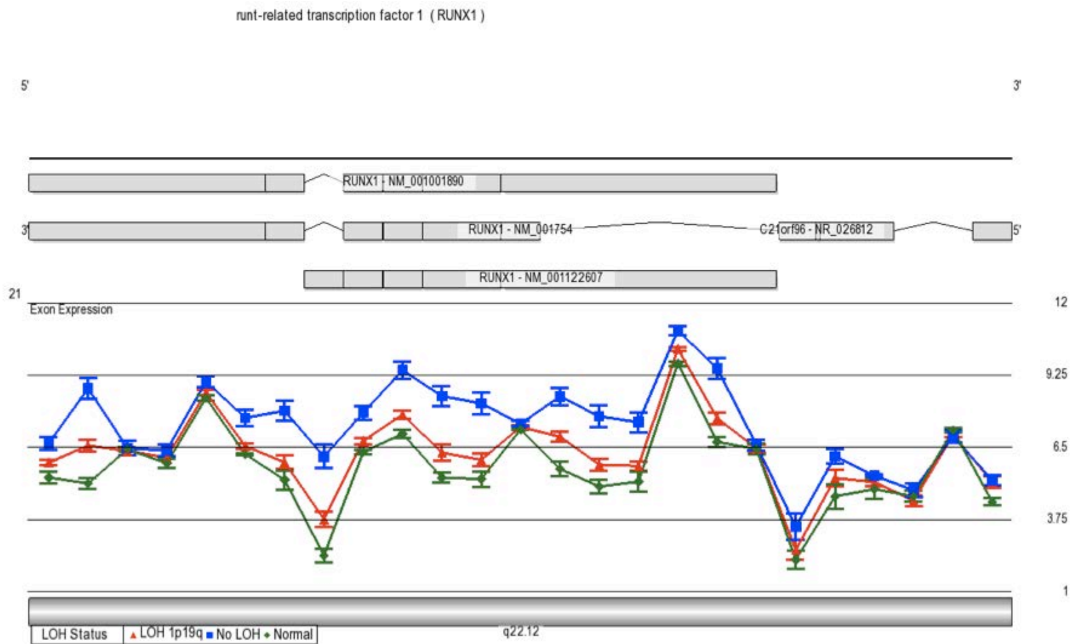


Figure 4.4 Alternate splice diagram of *RUNX1*.
RUNX1 the gene predicted to be most alternately spliced between oligodendrogliomas with and without LOH at 1p19q. Known variants of the gene are represented in the uppermost panel. The location of each probe throughout the transcript and its average expression in each sample group is shown in the lower panel.

4.3.5 Selection of candidate genes for further study

The large number of differentially expressed genes made it difficult to select just a few candidate genes to study further in this thesis. The list of 408 differentially expressed genes was reviewed for genes that have the potential to play a role in gliomagenesis of oligodendroglioma or mediation of a survival advantage to patients with LOH 1p/19q.

Six genes that were under-expressed in oligodendrogliomas with LOH 1p/19q were selected for further study in this thesis – Chitinase-3-like-1 (*CHI3L1*), Insulin-like growth factor 2 (*IGF2*), IQ motif containing GTPase activating protein 1 (*IQGAP1*), ERBB receptor feedback inhibitor 1 (*MIG-6*), Podoplanin (*PDPN*) and Pleiomorphic adenoma gene 1 (*PLAG1*). Each candidate gene displayed significantly higher expression in oligodendrogliomas without LOH 1p/19q. *PDPN* displayed significant over-expression among grade II oligodendrogliomas without LOH 1p/19q and *IGF2*, *IQGAP1*, *MIG-6*, *PDPN* and *PLAG1* were each significantly over-expressed in grade III oligodendrogliomas without LOH 1p/19q. The pattern of expression of these six genes in oligodendrogliomas with and without LOH 1p/19q as analysed by microarray is displayed in Figure 4.5 and summarised in Table 4.8.

Table 4.8 Expression of candidate genes in analyses of grade II, grade III and all oligodendrogliomas separated by LOH 1p/19q status

Locus	Gene	OII			OIII			All		
		p-value	Fold Change	T-stat	p-value	Fold Change	T-stat	p-value	Fold Change	T-stat
1q32.1	*CHI3L1*	0.102	**-8.136**	-1.996	0.213	**-6.803**	-1.352	**0.023**	**-7.722**	-2.489
1p36	*MIG-6*	0.353	-1.436	-1.023	**0.003**	**-3.185**	-4.221	**0.001**	**-2.274**	-3.909
11p15.5	*IGF2*	0.133	**-2.119**	-1.792	**0.004**	**-6.797**	-4.017	**0.0001**	**-4.824**	-4.853
15q26.1	*IQGAP1*	0.506	-1.440	-0.716	**0.016**	**-2.670**	-3.032	**0.005**	**-2.177**	-3.183
1p36.21	*PDPN*	**0.020**	**-2.756**	-3.386	**0.050**	**-4.056**	-2.308	**0.001**	**-3.559**	-3.803
8q12	*PLAG1*	**0.040**	-1.724	-2.765	**0.011**	**-2.295**	-3.299	**0.0001**	**-2.071**	-4.896

Fold Change was calculated change in expression from tumours without LOH 1p/19q to that in tumours with LOH 1p/19q.

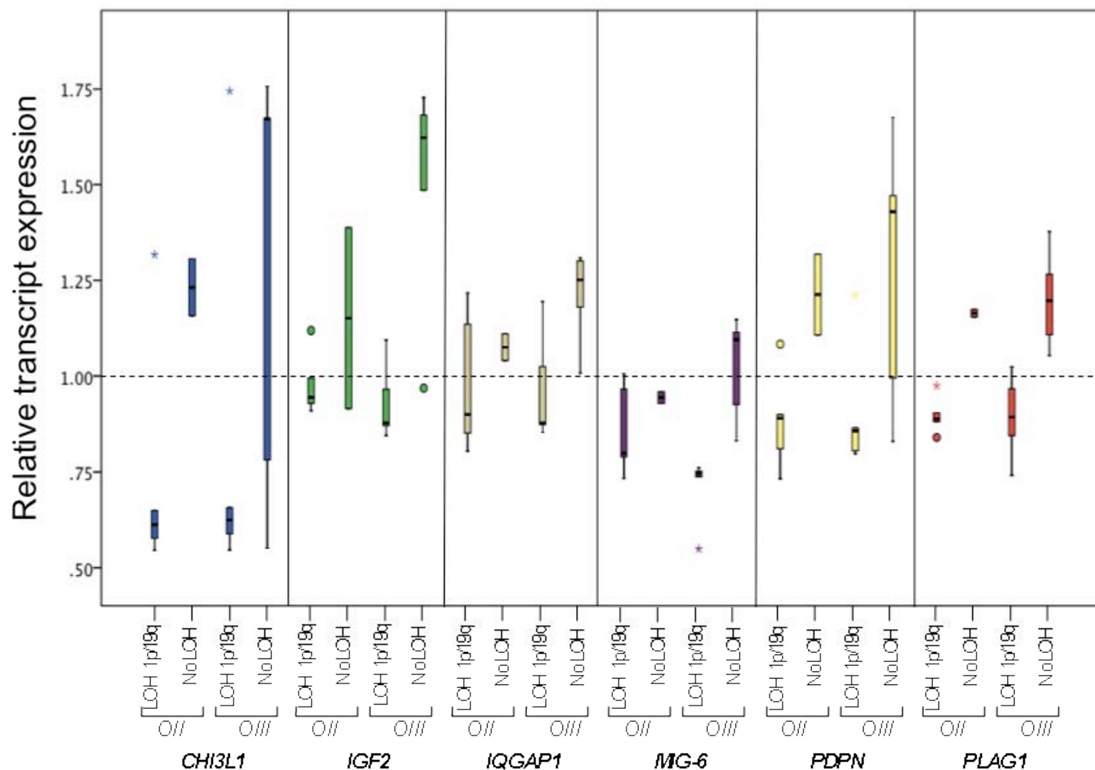


Figure 4.5 Relative transcript expression of candidate genes *CHI3L1*, *IGF2*, *IQGAP1*, *MIG-6*, *PDPN* and *PLAG1*.
Expression of each gene was examined in seven grade II oligodendrogliomas (5 with LOH 1p/19q, 2 without LOH) and ten grade III oligodendrogliomas (5 with LOH 1p/19q, 5 without LOH) by gene expression microarray. Expression for each tumour specimen was normalised to the baseline created by normal brain controls. Relative expression of each gene in normal brain is 1.00, represented by a dashed line.

4.3.6 Gene Set Enrichment Analysis

Gene Set Enrichment Analysis (GSEA) was conducted to identify chromosomal regions, published gene sets, motifs and gene ontology classes that were over represented in the expression profiles of oligodendroglioma specimens profiled here. To avoid bias due to tumour grade, analysis was conducted on the 10 grade III oligodendroglioma specimens, separated into two phenotype classes by LOH 1p/19q status.

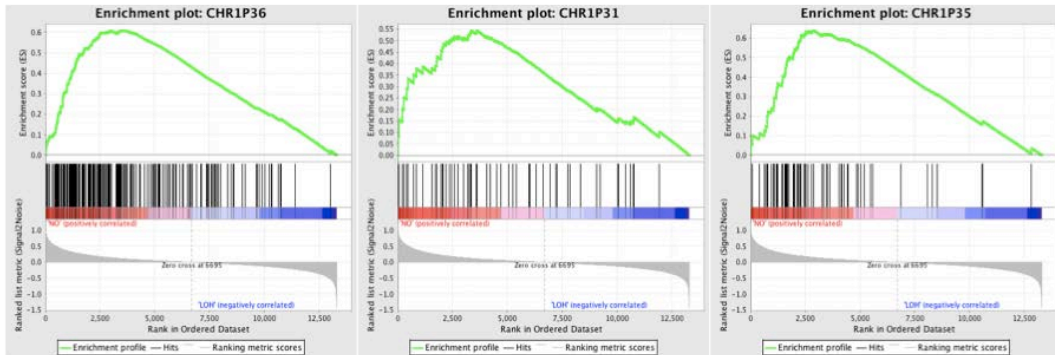
As expected, positional gene sets corresponding to chromosomes 1p and 19q were over represented in oligodendrogliomas without LOH 1p/19q (Figure 4.6). Micro RNA motifs were highly over represented among oligodendrogliomas with LOH 1p/19q, which had enrichment 67 miRNA gene sets. No miRNA motifs were significantly over represented in oligodendrogliomas without LOH.

Gene ontology (GO) grouped genes into three main organisational groups – Biological Process, Cellular Compartment and Molecular Function. The top 20 GO classes over represented in each group, categorised by LOH status in grade III oligodendrogliomas, are summarised in Table 4.9. GO classes over represented in grade III oligodendrogliomas with LOH 1p/19q included synapse biogenesis, nerve impulse (synaptic) transmission as well as methyltransferase activity. In oligodendrogliomas without LOH 1p/19q, over representation of GO classes related to immune response, wound healing, cell migration and extracellular matrix was observed.

Three gene sets representing proneural, mesenchymal and proliferative glioblastoma subgroups (Phillips et al., 2006) were analysed in this data set of grade III oligodendrogliomas. Enrichment of the proneural gene set was observed in oligodendrogliomas with LOH 1p/19q, whereas the proliferative and mesenchymal gene sets were over represented in oligodendrogliomas without LOH (Figure 4.6).

Positional gene sets

A NES= 2.557; p< 0.001 **B** NES= 2.450; p< 0.001 **C** NES= 2.251; p< 0.001



Glioblastoma phenotype gene sets

D NES= 2.920; p<0.001 **E** NES= 1.634; p=0.003 **F** NES= -2.807; p<0.001

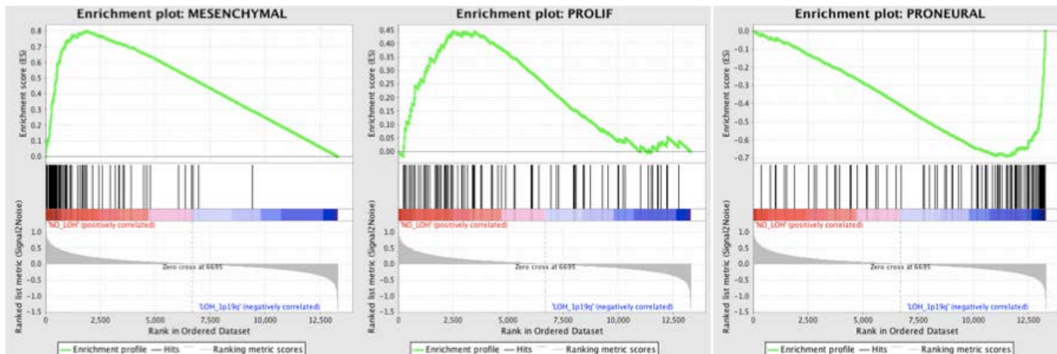


Figure 4.6 Positional gene sets for chromosomes 1p and 19q and glioblastoma phenotypes associated with poor survival are over represented in grade III oligodendrogliomas without LOH 1p/19q.
Gene Set Enrichment Analysis of 10 grade III oligodendroglioma samples revealed enrichment of [A] 1p36, [B] 1p33 and [C] 1p35 cytoband gene sets and the [D] Mesenchymal and [E] Proliferative phenotype gene sets in oligodendrogliomas without LOH 1p/19q. The Proneural gene set [F] is enriched in grade III oligodendrogliomas with LOH 1p/19q. NES – Normalised enrichment score.

Table 4.9 Top 20 Biological Process, Cellular Compartment and Molecular Function GO classes over represented in grade III oligodendrogliomas

LOH 1p/19q	No LOH
Biological Process	
Synaptic transmission	Response to external stimulus
Synaptogenesis	Response to wounding
Transmission of nerve impulse	Inflammatory response
Synapse organization and biogenesis	Defense response
Extracellular structure organization and biogenesis	Immune response
Establishment and or maintenance of chromatin architecture	Immune system process
Chromatin assembly or disassembly	Locomotory behaviour
Nervous system development	Coagulation
Regulation of mapkkk cascade	Blood coagulation
Chromatin modification	Humoral immune response
Potassium ion transport	Wound healing
Regulation of neurotransmitter levels	Positive regulation of cytokine biosynthetic process
Generation of a signal involved in cell cell signalling	Hemostasis
Central nervous system development	Regulation of body fluid levels
Exocytosis	Positive regulation of translation
Chromosome organization and biogenesis	Carbohydrate transport
Chromatin remodelling	Response to stress
Protein dna complex assembly	Angiogenesis
Secretory pathway	Vasculature development
Nuclear export	Cytokine biosynthetic process
Cellular Compartment	
Cortical cytoskeleton	Proteinaceous extracellular matrix
Synapse	Extracellular matrix
Chromatin	Extracellular region
Organelle inner membrane	Extracellular region part
Nucleolus	Integrin complex
Nuclear part^{ns}	Extracellular matrix part
Mitochondrial inner membrane^{ns}	Extracellular space
Nuclear lumen^{ns}	Receptor complex
Voltage gated potassium channel complex^{ns}	Lysosome
Membrane enclosed lumen^{ns}	ER golgi intermediate compartment
Ribonucleoprotein complex^{ns}	Lytic vacuole
Organelle lumen^{ns}	Basement membrane
Mitochondrial membrane^{ns}	Membrane fraction
Cell cortex part^{ns}	Cell fraction
Nucleoplasm^{ns}	Vacuole
Nucleoplasm part^{ns}	Lipid raft
Nuclear chromosome part^{ns}	Integral to plasma membrane
Centrosome^{ns}	Intrinsic to plasma membrane
Mitochondrion^{ns}	Basolateral plasma membrane
Cytoplasmic vesicle^{ns}	Plasma membrane
Molecular Function	
Methyltransferase activity	Integrin binding
Calcium channel activity	Extracellular matrix structural constituent
Extracellular ligand gated ion channel activity	Protease inhibitor activity
Excitatory extracellular ligand gated ion channel activity	Heparin binding
Transferase activity transferring one carbon groups	Hematopoietin interferon classd200 domain cytokine receptor activity
Cation channel activity	Cytokine activity
Glutamate receptor activity	Chemokine receptor binding
S adenosylmethionine dependent methyltransferase activity	Pattern binding
Gated channel activity	Hydrolase activity hydrolyzing o glycosyl compounds
Voltage gated cation channel activity	Protein complex binding
Ion channel activity	Phosphoric diester hydrolase activity
Metal ion transmembrane transporter activity	Cytokine binding
Microtubule binding	Hydrolase activity acting on glycosyl bonds
Ligand gated channel activity	Serine type endopeptidase inhibitor activity
Amine binding	Chemokine activity
Substrate specific channel activity	Glycosaminoglycan binding
Voltage gated channel activity	Polysaccharide binding
Neurotransmitter binding	Receptor binding
Neurotransmitter receptor activity	Hydrolase activity acting on ester bonds
Cation transmembrane transporter activity	G protein coupled receptor binding

^{ns} - not significant at FDR<0.25 and p≤0.05

4.3.7 Analysis of DNA methylation in oligodendrogliomas with LOH 1p/19q

Seven oligodendroglioma and four normal brain DNA samples were sent for methylation profiling by CpG island plus promoter array. Four grade II and three grade III oligodendrogliomas with LOH 1p/19q were selected for analysis, as this study was to focus on the methylation state of the remaining 1p and 19q chromosomes following loss of the other allele (Table 4.10).

Table 4.10 Oligodendroglioma patient specimens analysed by CpG island plus promoter array

Brain tumour number (MeDIP code)	Diagnosis	LOH Status	Gender	Age
3019 (4902)	OII	LOH 1p/19q	F	50
3265 (6602)	OII	LOH 1p/19q	F	32
3622 (73102)	OII rec	LOH 1p/19q	F	46
3837 (62002)	OII	LOH 1p/19q	M	39
3034 (5002)	OIII	LOH 1p/19q	M	31
3122 (5502)	OIII	LOH 1p/19q	F	31
3392 (73702)	OIII	LOH 1p/19q	F	45

rec – recurrent tumour;

CpG island plus promoter array data analysis was conducted by collaborator Dr. Michael Buckland. CpG islands with peak methylation calls in three or more oligodendroglioma samples and no peak methylation calls in normal brain samples were examined for associated genes. A list of candidate genes associated with methylated CpG islands on 1p and 19q was compiled (Table 4.11).

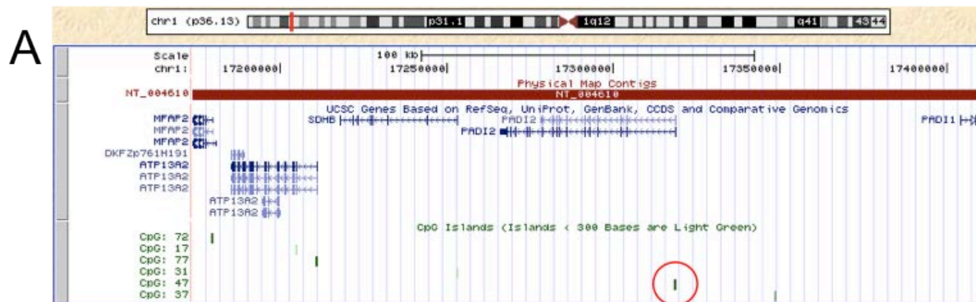
Twenty-one genes were identified by CpG island plus promoter array analysis as candidate genes due to their location near CpG islands that were methylated in oligodendroglioma with LOH 1p/19q and unmethylated in normal brain. Two genes from this list, *ALX3* and *PADI2*, were also selected for further study (Figure 4.7). DNA methylation data for each of the candidate genes selected in Section 4.3.5 is summarised in Table 4.12.

Table 4.11 Candidate genes near CpG islands methylated in oligodendrogliomas with LOH 1p/19q and unmethylated in normal brain specimens

Locus	Gene Symbol	Gene name	Tumours		Normals
			OII (n=4)	OIII (n=3)	(n=4)
1p					
1p36.13	*PADI2*	Peptidyl arginine deiminase, type II	0	3	0
1p36.13	*RCC2* *ARHGEF10L*	Regulator of chromosome condensation 2 Rho guanine nucleotide exchange factor (GEF) 10-like	2	3	0
1p36.11	*GALE*	UDP-galactose-4-epimerase	2	1	0
1p36.11	*TMEM50A*	Transmembrane protein 50A	2	1	4
1p36.11	*AIM1L*	Absent in melanoma 1-like	2	1	0
1p35.2	*MATN1* *LAPTM5*	Matrillin 1 cartilage matrix protein Lysosomal-associated multispanning membrane protein-5	2	3	1
1p34.2	*TMEM125*	Transmembrane protein 125	2	1	0
1p34.1	*VMD2L2* (*BEST4*)	Vitelliform macular dystrophy 2-like-2	3	1	0
1p31.3	*ROR1*	Receptor tyrosine kinase-like orphan receptor 1	2	2	0
1p13.3	*ALX3*	Aristaless-like homeobox 3	1	2	0
1p13.3	*KCNA3*	Potassium voltage-gated channel, shaker-related subfamily, member 3	4	2	1
19q					
19q13.11	*RHPN2*	Rhophilin, Rho GTPase binding protein 2	2	1	1
19q13.2	*SELV*	Selenoprotein V	2	2	1
19q13.2	*LYPD4* *DMRTC2*	LY6/PLAUR domain containing 4 DMRT-like family C2	2	1	0
19q13.32	*GLTSCR1*	Glioma tumor suppressor candidate region gene 1	2	2	1
19q13.33	*CD37*	CD37 molecule	1	2	0
19q13.33	*SLC17A7*	Solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter), member 7	2	2	1
19q13.42	*FIZ1*	Hypothetical protein FLJ14768	2	2	0

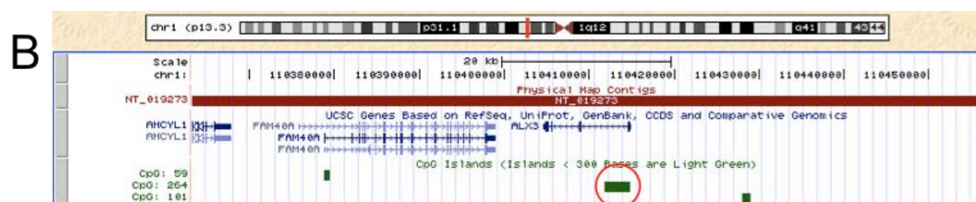
OII – grade II oligodendroglioma; OIII – grade III oligodendroglioma.

A



PADI2 CpG island

B



ALX3 CpG island

C

Specimen	Grade	*PADI2*	*ALX3*
Tumour 73702	OIII	red	green
Tumour 73102	OII	green	green
Tumour 62002	OII	green	red
Tumour 6602	OII	green	green
Tumour 5502	OIII	red	red
Tumour 5002	OIII	red	red
Tumour 4902	OII	green	green
Normal 4802		green	green
Normal 4502		green	green
Normal 4402		green	green
Normal 4302		green	green

Figure 4.7 *PADI2* and *ALX3* were methylated in oligodendrogliomas with LOH 1p/19q.
DNA methylation was observed in three out of seven oligodendrogliomas with LOH 1p/19q but not in normal brain specimens at the two CpG islands associated with the [A] *PADI2* and [B] *ALX3* promoter regions. [C] Red – methylation; Green – no methylation.

Table 4.12 DNA Methylation of candidate differentially expressed genes selected from gene expression microarray analysis

Locus	Gene	Gene Name	Tumours		Normals	Notes
			OII (n=4)	OIII (n=3)	(n=4)	
1q32.1	*CHI3L1*	Chitinase-3-like 1				No CpG island
11p15.5	*IGF2*	Insulin-like growth factor 2	3	1	2	
15q26.1	*IQGAP1*	IQ motif containing GTPase activating protein 1				No CpG island
1p36	*MIG-6*	ERBB receptor feedback inhibitor 1	2	1	2	
1p36.21	*PDPN*	Podoplanin	0	1	0	
8q12	*PLAG1*	Pleiomorphic adenoma gene 1				No CpG island

OII – grade II oligodendroglioma; OIII – grade III oligodendroglioma.

4.4 Discussion

A total of 408 differentially expressed genes were identified by microarray analysis of oligodendrogliomas separated by LOH status. 313 (76.7%) of those were under-expressed in oligodendrogliomas with LOH 1p/19q. Only 14.42% of differentially expressed genes were located on either 1p or 19q, which indicates that factors other than the loss of the 1p and 19q chromosome arms also play a role in tumour development following LOH 1p/19q.

Previous studies highlighted 21 genes as potential tumour suppressor genes or genes involved in chemosensitivity in oligodendrogliomas with LOH 1p/19q (Table 4.13). Of these 20 genes, only three genes, *INA*, *COL11A1* and *EMP3*, demonstrated significant differential expression between tumours separated by LOH 1p/19q in this analysis. The low reproducibility between this analysis and previous studies may be due to the small sample numbers in this and other studies, different proportions of grade II and grade III oligodendrogliomas and the use of different microarray platforms and analysis software.

Another plausible reason may be that these genes were not robust candidates for a role in either tumourigenesis or chemosensitivity. Despite its location on 1p32, differential expression of *CDKN2C* was not analysed by Husemann and colleagues, who reported just one case of *CDKN2C* mutation in oligodendroglioma, indicating that it is unlikely to be a strong candidate (Husemann et al. 1999). Tumour protein 73 (*TP73*) has sequence similarity to the tumour suppressor gene *TP53*, but appears to have a much more complex role in tumourigenesis as some evidence suggests that it may instead function as an oncogene (Kaghad et al. 1997; Melino et al. 2002). *TP73* has been investigated by numerous studies, but has not been confirmed as a robust candidate in oligodendroglioma (Dong et al. 2002; Watanabe et al. 2002). *CAMTA1* (Barbashina et al. 2005) was identified as a result of its location in a refined common region of loss on 1p36 in oligodendroglioma. Although differential expression was confirmed, no functional data for *CAMTA1* has been published. Wolf and colleagues (Wolf et al. 2003) demonstrated tumour suppressor activity for *p190RhoGAP* but did not examine its expression in oligodendrogliomas with LOH 1p/19q. The genes identified by microarray and

validated as by Tews et al, *MGC4339*, *RPL18* and *PLA2G4C*, have not been cited in any publications with regard to oligodendroglioma since (Tews et al. 2006). The sample set contained a higher number of oligoastrocytomas, which may have contributed to a skewed reference point for differential gene expression that was not comparable to this study. *Stathmin* (*AJAP1*) was identified following a screen of glioblastoma cell lines with and without 1p LOH, however it was Stathmin protein expression, rather than mRNA transcript expression, that correlated with 1p LOH in oligodendrogliomas (Ngo et al. 2007). While *Stathmin* may indeed confer resistance to nitrosoureas, there are no reports demonstrating differential gene expression in oligodendrogliomas with respect to LOH 1p/19q.

The evidence for a role for any of the above named genes in tumourigenesis or chemosensitivity in oligodendrogliomas with LOH 1p/19q is fairly weak. There remains a need to validate new candidate genes and investigate their function to find robust candidates for the biological differences between oligodendrogliomas with and without LOH 1p/19q.

Six previous studies in oligodendroglioma reported genes of interest on 1p or 19q that showed DNA methylation of the remaining allele (Table 4.13). The highest level of methylation observed in this study among these genes was at *TP73*, with three oligodendrogliomas and two normal specimens showing methylation. Neither the three CpG islands associated with *DIRAS3* nor the CpG island located within *EMP3* was interrogated by the CpG island plus promoter array, so these two genes could not be validated. The CpG island associated with the *MGMT* promoter was methylated in just one tumour and one normal specimen. This finding is discordant with the substantial literature on this gene, which reports strong *MGMT* methylation in oligodendrogliomas. These results suggest that methylated genes identified by both the CpG island plus promoter array and other methods should be carefully validated to ensure that they are robust candidates.

Table 4.13 Expression and methylation findings for oligodendroglioma genes identified in previous studies

Reference	Gene Symbol	Locus	Expression data			DNA Methylation data			
			FC: LOH vs NoLOH	p-value	T	Reported Methylation in Oligodendrogliomas	Tumours OII (n=4)	OIII (n=3)	Normals (n=4)
(Husemann et al., 1999)	*CDKN2C*	1p32	1.033	0.887	0.144	-	-	-	-
(Marie et al., 2001)	*OLIG2*	21q22	1.438	0.332	0.997	-	1	0	0
(Dong et al., 2002)	*TP73*	1p36.3	1.100	0.335	0.990	Hypermethylation of remaining 1p allele	1	2	2
(Mukasa et al., 2002)	***COL11A1***	1p21	**-3.037***	**0.003****	-3.370	-	-	-	-
	RBBP4	1p35.1	-1.053	0.814	-0.239	-	-	-	-
(Wolf et al., 2003)	*GRLF1 (p190RhoGAP)*	19q13.3	-1.107	0.361	-0.937	-	0	0	2
(Hong et al., 2003)	*ZNF296 (ZNF342)*	19q13.32	1.056	0.596	0.540	Hypermethylation of remaining 19q allele	1	1	0
(Trouillard et al., 2004)	*PEG3*	19q13.4	-1.275	0.634	-0.485	Methylation of remaining maternal 19q allele	0	0	1
(Barbashina et al., 2005)	*CAMTA1*	1p36	-1.143	0.674	-0.428	-	1	0	0
(Hegi et al., 2005)	*MGMT*	10q26.3	**1.**097	0.432	0.720	Methylated in temozolomide sensitive gliomas	1	0	1
(Alaminos et al., 2005; Kunitz et al., 2007)	***EMP3***	19q13.3	**-3.760***	**0.012****	-2.778	Hypermethylated in oligodendrogliomas with LOH 1p/19q	-	-	-
(McDonald et al., 2005a)	*DFFB*	1p36.3	-1.118	0.213	-1.292	-	1	1	0
(Tews et al., 2006)	***ICMT***	1p36.21	-1.432	**0.011****	-2.834	-	0	1	2
	MGC4399	1p36.22	-1.578	0.143	-1.533	-	1	0	0
	RPL18	19q13	-1.047	0.707	-0.382	-	-	-	-
	PLA2G4C	19q13.3	-1.028	0.856	-0.184	-	0	1	0
(McDonald et al., 2006)	*AJAP1*	1p36.32	1.126	0.712	0.375	-	0	1	2
(Tews et al., 2007)	*CITED4*	1p35-p34	-1.033	0.646	-0.467	Hypermethylated in oligodendrogliomas with LOH 1p/19q	1	0	1
(Ngo et al., 2007)	*STMN1*	1p36.11	-1.360	0.215	-1.287	-	1	1	1
(Riemenschneider et al., 2008)	***DIRAS3***	1p31	-1.936	**0.017****	-2.626	Hypermethylated in oligodendrogliomas	-	-	-
(Ducray et al., 2009)	***INA***	10q24.33	**5.185***	**0.035****	2.280	-	1	1	1

* Fold change above threshold (above 2 or below -2) ** P-value significant (below 0.05). Note - If no score is provided for methylation, either the promoter did not contain a CpG island, or the CpG island was not interrogated by the CpG island plus promoter array.

The selection of candidate genes from this study was difficult given the large number of differentially expressed genes that were identified by exon array analysis. A review of published literature was conducted to identify differentially expressed genes that may have the potential to biologically influence tumourigenesis or therapeutic sensitivity in oligodendrogliomas following LOH 1p/19q.

PLAG1, a gene over-expressed in oligodendrogliomas without LOH 1p/19q, is a transcription factor that is the target of a translocation in pleiomorphic adenomas of the salivary glands, where *PLAG1* behaves as an oncogene (Voz et al., 2000). A transcriptional target of *PLAG1*, *IGF2* was also over-expressed in oligodendrogliomas without LOH and is associated with tumour progression in endocrine cancers (Zatkova et al., 2004; Soon et al., 2009). While *PLAG1* has not previously been studied in glioma, *IGF2* is known to promote cell motility in astrocytoma (Rorive et al., 2008).

Candidate genes *CHI3L1* and *IQGAP1* were selected due to their known involvement in glioblastoma. *IQGAP1* is involved in glioma migration and its overexpression is associated with increased tumour aggression and shorter patient survival in glioma (McDonald et al., 2007). Located on 1q32.1, *CHI3L1* is overexpressed in a subset of aggressive glioblastomas and has been used as a diagnostic marker for subtypes of glioblastoma (Tanwar et al., 2002; Nutt et al., 2005). Furthermore, protein staining of CHI3L1 had higher discrimination power to distinguish glioblastoma from grade III oligodendroglioma than GFAP, a standard marker for astrocytes (Nutt et al., 2005).

Two differentially expressed genes selected for validation, *PDPN* and *MIG-6*, are located at the 1p36 locus, which is also lost in a subset of high grade astrocytoma and glioblastoma (Ichimura et al., 2008). *PDPN,* a marker of lymphangiogenesis, is involved in tumour invasion and metastasis and displayed high expression in glioblastoma and pilocytic astrocytoma (Breiteneder-Geleff et al., 1997; Shibahara et al., 2006; Wicki et al., 2006). *MIG-6* is a candidate tumour suppressor gene that normally functions as a negative feedback regulator of the EGFR and other ErbB family pathways (Zhang et al., 2007a; Ballarò et al., 2005; Anastasi et al., 2007). In

a mouse model, deficiency of MIG-6 led to tumour phenotypes in the lung, gall bladder and bile duct (Zhang et al., 2007a).

The PAD locus on 1p36.13, shown here to be methylated in oligodendrogliomas with LOH 1p/19q, contains the cluster of peptidylarginine deiminase (PAD) genes – *PADI1*, *PADI2*, *PADI3*, *PADI4* and *PADI6*. The PAD genes are posttranslational modification enzymes that citrullinate (deaminate) arginine residues and are expressed in a tissue specific manner, with *PADI2* the only isoform expressed in the brain (Ishigami and Maruyama, 2010). *PADI2* was chosen for further study due to its brain specific pattern of expression. *PADI2* also demonstrated lower expression in oligodendrogliomas with LOH 1p/19q (p=0.006, FC=-1.99). Another methylated gene, *ALX3*, is a developmental transcription factor and a promising candidate to explain the reduced aggression displayed by oligodendrogliomas with LOH 1p/19q. *ALX3* was also methylated in neuroblastoma (Wimmer et al., 2002), but was not shown here to be differentially expressed between oligodendrogliomas separated by LOH 1p/19q status.

Candidate genes *CHI3L1*, *IQGAP1* and *PDPN* are members of glioblastoma gene signatures identified in recent large scale analyses of gene expression in glioblastoma subgroups. High CHI3L1 protein expression was robustly associated with the mesenchymal glioblastoma phenotype, which represents a subset of glioblastomas with poor survival (Phillips et al., 2006). The Cancer Genome Atlas' (TCGA) publication identified *CHI3L1* and *PDPN* as negatively associated with the favourable proneural glioblastoma phenotype and *IQGAP1* as positively associated with the mesenchymal glioblastoma phenotype (Verhaak et al., 2010).

These glioblastoma gene signatures have also been used to characterise oligodendrogliomas with LOH 1p/19q. Ducray et al showed that when compared to glioblastomas with *EGFR* amplification, oligodendrogliomas with LOH 1p/19q demonstrated enrichment of the proneural gene signature (Ducray et al., 2008). A similar result was observed here, as the proneural gene signature was over-represented in grade III oligodendrogliomas with LOH 1p/19q and the proliferative and mesenchymal gene signatures were enriched in grade III tumours that lack LOH 1p/19q.

Of the other trends observed here in the grade III oligodendrogliomas, over-representation of genes associated with invasion, migration and immune response in tumours without LOH 1p/19q was expected, as these tumours are more aggressive. Gene ontology categories enriched in tumours without LOH were those associated with nerve impulse transmission and biogenesis and were confined to nuclear cell compartment, similar to those genes that define the proneural glioblastoma gene signature (Phillips et al., 2006). These findings are in line with those of Ferrer-Luna and colleagues, who note that the enriched gene ontology categories observed in grade II and III oligodendroglioma without LOH were those associated with cell motility, invasiveness and proliferation, whereas categories enriched in grade II and III oligodendrogliomas with LOH 1p/19q are those associated with neurogenesis (Ferrer-Luna et al., 2009). Interestingly, miRNA targets were over-represented in oligodendrogliomas with LOH 1p/19q, suggesting that miRNAs may demonstrate higher activity in less aggressive tumours. Just one previous study has included oligodendroglial tumours in an analysis of miRNAs, however no difference in miRNA expression was observed between tumours with and without LOH at chromosome 1p (Nelson et al., 2006).

Surprisingly, analysis of gene expression in grade III oligodendrogliomas and glioblastomas in GeneSpring revealed high similarity in gene expression patterns between grade III oligodendrogliomas without LOH and glioblastomas. More than ten times as many genes were differentially expressed between grade III oligodendrogliomas separated by LOH 1p/19q than between grade III oligodendrogliomas without LOH and glioblastomas. These results suggest that grade III oligodendrogliomas without LOH 1p/19q are more similar to glioblastoma than to classical oligodendrogliomas that have LOH 1p/19q.

4.4.1 Conclusion

The pattern of gene expression was shown here to be more similar between oligodendroglioma without LOH 1p/19q and glioblastoma, than between oligodendroglioma without LOH and oligodendroglioma with LOH 1p/19q, as indicated by the pattern of differentially expressed genes and enrichment of previously published gene sets.

Six genes, *PLAG1*, *IQGAP1*, *CHI3L1*, *PDPN*, *MIG-6* (*ERRFI1*) and *IGF2*, identified as differentially expressed and two genes, *PADI2* and *ALX3*, identified as methylated based on LOH status will be examined further in the remaining chapters of this thesis.

5 Validation and functional analysis of candidate genes in oligodendroglioma

5.1 Introduction

5.1.1 Overview

The previous chapter identified eight candidate genes - six differentially expressed genes and two methylated genes - in oligodendrogliomas with LOH 1p/19q. This chapter validates the differential expression of those candidate genes and their protein products using quantitative real-time PCR (qPCR) and immunohistochemistry on a larger cohort of oligodendroglioma samples.

A total of 408 differentially expressed and 22 methylated genes were reported in chapter four. Chitinase-3-like-1 (*CHI3L1*), ERBB receptor feedback inhibitor 1 (*MIG-6*), Insulin-like growth factor 2 (*IGF2*), IQ motif containing GTPase activating protein 1 (*IQGAP1*), Podoplanin (*PDPN*) and Pleiomorphic adenoma gene 1 (*PLAG1*) were identified by microarray analysis to be significantly under-expressed in oligodendrogliomas with LOH 1p/19q compared to those without LOH 1p/19q. These six genes were selected for further analysis, as they are of significant interest in tumour biology. Two candidate genes were located in the common region of loss: *MIG-6* (1p36.23) and *PDPN* (1p36.21). The remaining genes were located throughout the genome: *CHI3L1*, 1q32; *IGF2,* 11p15.5; *IQGAP1*, 15q26.1; *PLAG1*, 8q12.

Analysis of the CpG island plus promoter array identified the methylated genes; Homeobox protein aristaless-like 3 (*ALX3*) and Peptidyl arginine deiminase, type II (*PADI2*), both located on chromosome 1p, in oligodendrogliomas with LOH 1p/19q. Bisulfite sequencing, Combined Bisulfite Restriction Analysis (COBRA) and qPCR analysis were used to validate the occurrence of DNA methylation

associated with these genes and determine if the DNA methylation led to decreased mRNA expression.

MIG-6, identified by microarray analysis in chapter four to be under-expressed in oligodendrogliomas with LOH 1p/19q, is frequently deleted, mutated and down-regulated in breast cancer, lung cancer and glioblastoma (Anastasi et al., 2005; Ichimura et al., 2008; Ying et al., 2010). It is widely acknowledged that *MIG-6* is a tumour suppressor gene, active in numerous tissues throughout the body (Zhang et al., 2007a; Ying et al., 2010). I further investigated the impact of *MIG-6* on cell proliferation and migration in glioma cell lines.

5.1.2 Aims

1. To confirm differential expression of candidate genes of interest associated with LOH 1p/19q in grade II and III oligodendrogliomas using qPCR.

2. To validate methylation of genes at key CpG sites by bisulfite sequencing and COBRA.

3. To confirm differential expression of proteins associated with LOH 1p/19q in grade III oligodendrogliomas using immunohistochemistry.

4. To correlate mRNA transcript and protein expression levels with LOH 1p/19q and/or survival in grade III oligodendroglioma patients.

5. To assess the expression of *MIG-6* in glioblastoma and oligodendroglioma cell lines, to manipulate the *MIG-6* levels by gene over-expression or gene silencing and examine the effect of *MIG-6* on cell proliferation and migration proliferation.

5.2 Methods

5.2.1 Quantitative Real-Time PCR

cDNA synthesis and the qPCR reaction were performed as described in chapter two (cDNA synthesis – 2.4.8; qPCR –2.6.7).

18S ribosomal RNA was chosen as a housekeeping gene for qPCR experiments, as it was shown to be a reliable endogenous control gene for previous qPCR studies in brain and glioma tissues (McDonald et al., 2007; Payne et al., 2008).

Standard curves were generated (see section 2.6.7.1) for each TaqMan gene expression assay. A summary of efficiency values for each gene expression assay is provided in Table 5.1. The assay for the gene of interest was performed in the same tube as the 18S control gene (ie. multiplexed), with the exception of *ALX3* where 18S was performed in parallel in a separate tube.

Table 5.1 Calculated efficiency values for TaqMan gene expression assays

TaqMan Probe	Gene	Efficiency Value (between 0 and 1)	18S efficiency value
Hs00197798_m1	*ALX3*	0.80	N/A
Hs00609691_m1	*CHI3L1*	0.97	0.94
Hs01076092_m1	*EGFR*	0.88	0.85
HS01005964_g1	*MIG-6*	0.87	0.84
Hs00182622_m1	*IGF2*	0.95	0.88
Hs00219060_m1	*IQGAP1*	0.92	0.88
Hs00247108_m1	*PADI2*	0.90	0.84
Hs01089982_g1	*PDPN*	0.95	0.89
Hs00231236_m1	*PLAG1*	0.86	0.80

Relative expression was calculated with the Relative Expression Software Tool (REST 2008). The CT values for the gene of interest and for 18S were entered for the sample and for the normal control (pooled whole brain RNA).

qPCR reactions were run a minimum of two times (in triplicate) for each gene in each tumour specimen. The average expression value for each gene per specimen was used for subsequent calculations. p-values were obtained by log 10 transformation of relative expression values to normalise distribution, followed by a two-tailed t-test on the two groups of samples, conducted in SPSS Statistics (version 18.0, Chicago IL, U.S.A.).

5.2.2 Validation of DNA methylation

DNA methylation of the *ALX3* and *PADI2* genes in oligodendroglioma was validated by both methylation specific sequencing and COBRA. These assays were performed by our collaborators Dr Michael Buckland and Bjorn Espedido.

5.2.2.1 Bisulfite Sequencing

To assess the relative methylation of CpG islands in the selected genes, Bisulfite sequencing was conducted on DNA samples from the three oligodendroglioma specimens analysed by the CpG island plus promoter array array.

DNA was bisulfite converted using the Epitect Bisulphite kit (Qiagen) according to the manufacturers instructions. A representative selection of the CpG island within the *PADI2* promoter was amplified by PCR (Table 5.2). The amplicon from each sample was gel excised using the QIAquick Gel Extraction kit (Qiagen) and cloned into pGEM-T Easy Vector System II (Promega; Madison, WI). Plasmids from 12 individual clones from each sample were extracted using the QuickLyse Miniprep kit (QIAGEN) and sent to Macrogen (Seoul, KR) for sequencing using the pUC/M13-R primer. Sequencing results were analyzed using the BiQ Analyzer software (Bock et al., 2005) and identified the methylation state at 39 CpG dinucleotides within the PADI2 promoter.

5.2.2.2 COBRA

Validation of DNA methylation of the CpG island associated with the *PADI2* promoter was performed by COBRA on bisulfite treated DNA from 20 grade II and 23 grade III oligodendroglioma specimens with LOH 1p/19q (Xiong and Laird, 1997). A 96 base pair region of the *PADI2* promoter-associated CpG island was amplified by PCR (Table 5.2). Taq$^{\alpha}$I restriction enzyme (New England Biolabs, Ipswich MA, U.S.A.) was used to digest the resulting fragments. The Taq$^{\alpha}$I restriction site was preserved only in samples with DNA methylation at the CpG dinucleotide within the Taq$^{\alpha}$I site. Complete digestion of the fragment produced two bands of 39 and 57 base pairs, indicating DNA methylation.

Table 5.2 Primers used for the validation of *PADI2* methylation

Amplicon	**Forward (5' to 3')**	**Reverse (5' to 3')**	**Amplicon length (bp)**
Bisulfite seq (amplification)	PADI2-LF TGGAAAGCACAAAACTGGTCCCTGAT	PADI2-LR CACGACCCGTGGATGGAGACCTG	334
Bisulfite seq (sequencing)	pUC/M13-R CAGGAAACAGCTATGAC		
COBRA	PADI2-SF TGGAAAGTATAAAATTGGTTTTTGAT	PADI2-SR ATCCAAAAATAAATACCCAACAC	96

bp – Base pairs.

5.2.3 Immunohistochemistry

Sections (4μm) of paraffin embedded tumour tissue were placed onto Superfrost Ultra slides™, deparaffinised in xylene and rehydrated in graded ethanol. Sections were then heat retrieved in a boiling water bath. Staining of tumour sections was performed at room temperature, manually or on an automatic platform (DAKO, Autostainer Plus™ or Vision Biosystems BondmaXautostainer). Sections were quenched for endogenous peroxidase activity with 3% H_2O_2.

Primary, secondary and tertiary antibodies were incubated as per Table 5.3. Antibody complex was detected with '3,3-diaminobenzidine for 10 minutes at room temperature. Sections were counterstained using Harris haematoxylin, dehydrated in graded ethanol and cleared in xylene before mounting.

The Allred method, which takes into account the total area of viable staining (score of 0-5) and the intensity of staining (0-3), was used to score CHI3L1, MIG-6 and PDPN (Allred et al., 1993; Harvey et al., 1999). The two scores are added to give a total score of 0 or between 2 and 8. A score of 0-2 was considered negative, 3-4 was considered low, 5-6 intermediate and 7-8 high. A score of 3-8 was considered to be positive.

IGF2 staining was scored on a scale of 0 to 3. A section that showed no staining was given a score of 0. Staining in less than 10% of tumour cells with low intensity was given a score of 1. Intermediate staining in less than 50% of tumour cells was given a score of 2. Strong staining in more than 50% of tumour cells was given a score of 3.

IQGAP1 staining was scored as previously reported, on a scale of 0 to 4 as follows: 0, negative staining; 1, weak cytoplasmic staining in less than 5% of tumour cells; 2, moderate cytoplasmic staining in less than 20% of tumour cells; 3, moderate to strong cytoplasmic staining in less than 25% of tumour cells; 4, strong cytoplasmic staining in more than 25% of tumour cells (McDonald et al., 2007).

Table 5.3 Immunohistochemistry conditions

Protein	Antigen retrieval	Pre-treatment	Primary antibody	Antibody concentration, conditions	Secondary/ Tertiary antibodies
CHI3L1	pH 6.0, 100°C	serum-free protein block (10 minutes)	Human-anti-rabbit polyclonal YKL-40/CHI3L1 (Quidel Corporation)	1:400, overnight at room temperature (manual)	Polymer based secondary/ tertiary antibody (30 minutes)
IGF2	Enzyme based	-	Human anti mouse IGF2 - Clone S1F2 (Upstate Cell Signalling Solutions)	1:400, 60 minutes; (Vision Biosystems Bondma-Xautostainer)	-
IQGAP1	pH 9.0, 100°C	-	Human-anti-mouse monoclonal IQGAP1 (BD Biosciences)	1:300, 30 minutes (DAKO Autostainer Plus™)	Polymer based secondary/ tertiary (30 minutes)
MIG-6	pH 9.0, 100°C	-	Human-anti-rabbit polyclonal ERRFI1/MIG-6 (Proteintech Group, Inc)	1:100, 30 minutes (DAKO, Autostainer Plus™)	Polymer based secondary/ tertiary antibody (30 minutes)
PDPN	pH 6.0, 100°C	avidin (10 minutes); biotin blocking (10 minutes)	Human-anti-mouse monoclonal PDPN (ABCAM)	1:500, 60 minutes (DAKO, Autostainer Plus™)	Universal link secondary antibody (15 minutes); Streptavidin based tertiary antibody (15 minutes)

5.2.4 Analysis and survival statistics

Boxplots for qPCR and microarray data were generated using SPSS statistics (version 18.0, Chicago IL, U.S.A.) and t-tests were performed with SPSS statistics and Microsoft Excel.

Survival statistical analysis was performed. Kaplan Meier survival analysis was used to generate survival curves and estimates of median survival based on mRNA transcript expression of *CHI3L1*, *IGF2*, *IQGAP1*, *MIG-6*, *PDPN* and *PLAG1,* where specimens were divided into groups of high and low expression based on the median expression of each transcript, and immunohistochemistry scores for CHI3L1, IGF2, IQGAP1, MIG-6 and PDPN. The LogRank (Mantel-Cox) test was used to analyse survival times for samples based on protein expression scores (SPSS 18.0, Chicago IL, U.S.A.).

5.2.5 Cell lines

Glioblastoma cell lines U87MG and A172 were purchased from American Type Culture Collection (ATCC, Manassas VA, U.S.A.). Glioblastoma cell line H423 and oligodendroglioma cell lines TC620 and HOG were a gift from Dr. Hai Yan, Duke University (Durham NC, U.S.A). Oligodendroglioma cell line M03.13 was purchased from Cellutions Biosystems (Burlington, Canada).

Cell lines were maintained in a humidified incubator at 37°C with 5% CO_2. The U87MG cell line was maintained in Zinc Option (ZO) Media with 10% FCS. A172 and M03.13 cell lines were maintained in Dulbecco's modified Eagle Medium (DMEM, Gibco) with 10% FCS. TC620 and HOG cell lines were maintained in Modified Eagles Medium (MEM, Gibco) with 10% FCS.

1x ZO media was prepared from Gibco Improved MEM Zinc Option Media (5x Concentrate). Each litre of medium was supplemented with Hepes (10ml) and 5.5% $NaHCO_3$ (40ml). Cells were grown in media supplemented with 10% FCS (Gibco).

5.2.6 Plasmids

pCMV6-entry-*MIG6*, a mammalian expression plasmid containing full length human *MIG-6* cDNA was a gift from Chris Duncan, Duke University (Durham NC, U.S.A.). A map of this plasmid is provided in Appendix 5. pCMV6-entry (OriGene, Rockville MD, U.S.A.) was used as an empty vector negative control for all *MIG-6* over-expression experiments.

Four shRNA plasmids, shRNA-*MIG6*-1 to 4 (SureSilencing™ SABiosciences, Frederick MD, U.S.A.), containing sequences targeted to *MIG-6* were used to knockdown *MIG-6 in vitro*. A non-silencing shRNA plasmid, shRNA-neg (SABiosciences, Frederick MD, U.S.A.) was used as a negative control for all *MIG-6* knockdown experiments. Each of the shRNA plasmids contains a bacterial ampicillin resistance gene for plasmid amplification in bacteria and a GFP marker for sorting transfected cells by flow cytometry.

5.2.7 Transfections

Lipofectamine 2000 (Invitrogen, Carlsbad CA, U.S.A.) was used for all transfections, according to the manufacturer's instructions. Cells were plated at 90% confluence in 100 mm dishes the day before transfection. Plasmid (24 µg) was diluted in Opti-MEM I Medium (750 µL). Lipofectamine 2000 (30 µL) was diluted in Opti-MEM I Medium (750 µL), gently mixed by pipetting and incubated for 5 minutes at room temperature. The plasmid DNA and Lipofectamine 2000 Opti-MEM solutions were combined, gently mixed by pipetting and incubated for 20 minutes at room temperature. The solution was added drop wise while slowly swirling the culture plate. The culture plate was returned to the incubator. After 6 hours, the cell media was replaced. Cells were used for experiments 24 or 48 hours after transfection.

5.2.8 MTT assay

Cultured human glioma cells (H423, HOG, M03.13, TC620, U87MG) were transfected with pCMV6-entry-*MIG6*, pCMV6-entry, shRNA-*MIG6*-2 or shRNA-neg. After 24 hours, cells were seeded into 24-well plates, at densities between 10000

and 100000 cells per well in culture media (600 μL per well). Cells were incubated for time periods between 24 hours and 7 days. End timepoints for each experiment were set at either 24, 48 and 72 hours, or 3, 4, 5, 6 and 7 days. At each end timepoint, MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (Thiazolyl blue tetrazolium bromide)] was added to wells at a concentration of 330 μg/ml (40 μL of 5 mg/ml in DPBS). After four hours of incubation at 37°C, isopropanol (600 μL) containing 0.4% HCl was added to each well. Crystals were resuspended by pipetting. Samples (100 μL) from each well were added to wells of a 96-well plate in triplicate. Absorbance was read on a POLARstar Optima (BMG Labtech) plate reader (Optima Software version 2.0) at 520 nm.

Experiments were repeated at least twice for each cell line and condition. Results shown are for a single experiment, representative of the results obtained in each repeat. Charts were generated in SPSS (version 18.0, Chicago IL, U.S.A.).

5.2.9 Colony formation assay

Cells (H423, U87MG) were transfected with pCMV6-entry-*MIG6*, pCMV6-entry, shRNA-*MIG6*-2 or shRNA-neg. Cells were seeded into 6-well plates at low density (1000 cells/well). Cells were incubated at 37°C for 10 to 14 days to allow formation of visible colonies. Following incubation, cell media was removed and colonies were washed twice in water and stained with crystal violet (0.2% in DPBS with 20% ethanol) for 60 minutes. Colonies were photographed and counted. ANOVA was conducted and charts were generated in SPSS (version 18.0, Chicago IL, U.S.A.).

5.2.10 Wound healing assay

Cells (H423, U87MG) were transfected with pCMV6-entry-*MIG6*, pCMV6-entry, shRNA-*MIG6*-2 or shRNA-neg. The following day, cells were seeded into 6-well plates at 90% confluence. 24 hours after seeding, a wound was created in the cell monolayer with a p1000 pipette tip. Wound closure by cell migration was

monitored hourly under an inverted light microscope. Photographs of wound healing progress were taken at 0, 6 and 24 hours.

5.2.11 Trans-well migration assay

Trans-well inserts (24-well format, 8 µm pore size, polycarbonate membrane) were coated with collagen (25 mg/ml) overnight at 4°C. Delipidised Bovine Serum Albumin (BSA) was dissolved in sterile DBPS at 25 mg/ml and heat inactivated in a 65°C water bath for one hour.

Cells (H423, U87MG) were transfected with pCMV6-entry-*MIG6*, pCMV6-entry, shRNA-*MIG6*-2 or shRNA-neg. At 24 hours following transfection, 10 000 cells were washed in ZO media containing 250 µg/ml Heat Inactivated BSA (HI BSA/ZO), containing no FCS, then seeded into trans-well inserts. The lower chamber of each well was filled with HI BSA/ZO with 5% FCS as a chemo-attractant (600 µL). After 24 hours, media was removed from trans-well inserts and cells remaining in the upper chamber were swabbed away with a cotton tip. Cells that had migrated to the lower surface of the trans-well insert were fixed in methanol (10 minutes) and stained in 0.2% Crystal violet in 2% ethanol (10 minutes). Trans-well inserts were washed twice in water and dried before visualisation. Four fields of view for each stained trans-well insert were visualised and photographed with an inverted light microscope. Stained cells were counted from triplicate trans-well inserts to determine the average number of migrated cells. Boxplots and an ANOVA to analyse statistical significance were conducted in SPSS (version 18.0, Chicago IL, U.S.A.).

5.3 Results

5.3.1 qPCR validation of differential gene expression in oligodendroglioma

qPCR was conducted on RNA extracted from 46 grade II and III oligodendroglioma specimens (Table 5.4). A full list of specimens used for qPCR is listed in Appendix 3. One normal brain RNA sample (Ambion), consisting of RNA from pooled whole brain specimens was used as a control.

Table 5.4 Oligodendroglioma RNA samples used for qPCR

	LOH 1p/19q	LOH 1p only	LOH 19q only	1p/19q intact	Total
Grade II Oligodendroglioma	10	2	0	4	**16**
Grade III Oligodendroglioma	15	1	1	13	**30**
Total	**25**	**3**	**1**	**17**	**46**

qPCR analysis confirmed the genes *CHI3L1, IGF2*, *IQGAP1*, *MIG-6*, *PDPN, PLAG1* and *PADI2* to be significantly over-expressed in oligodendrogliomas without LOH 1p/19q (n=17) compared to oligodendrogliomas with LOH 1p/19q (n=29) (Table 5.5).

When oligodendrogliomas were analysed separately by grade, expression of genes *CHI3L1*, *IQGAP1*, *PDPN* and *PADI2* were significantly higher in grade II oligodendrogliomas without LOH (n=4), compared to grade II oligodendrogliomas with LOH 1p/19q (n=12) (Figure 5.1-3).

Significant over-expression of genes *CHI3L1*, *IQGAP1*, *MIG-6*, *PDPN*, *PLAG1* and *PADI2* were observed in grade III oligodendrogliomas without LOH (n=13) compared to grade III oligodendrogliomas with LOH 1p/19q (n=17) (Figure 5.1-3).

Differential expression of *EGFR* was not observed between tumours separated by LOH status. No genes examined by qPCR were significantly differentially expressed between grade II and III oligodendrogliomas (irrespective of LOH status).

Table 5.5 Mean expression, Fold change and p-value for *CHI3L1, IGF2*, *IQGAP1*, *MIG-6, EGFR*, *PDPN, PLAG1*, *ALX3* and *PADI2* analysed by qPCR in oligodendrogliomas

	All Oligodendrogliomas				All Oligodendrogliomas					Grade II Oligodendrogliomas				Grade III oligodendrogliomas			
	No LOH (n=17)	LOH 1p/19q (n=29)	FC (No LOH/ LOH 1p19q)	p-value	Grade II (n=16)	Grade III (n=30)	FC (Grade II/ III)	FC (Grade III/II)	p-value	No LOH (n=4)	LOH 1p/19q (n=12)	FC (No LOH/ LOH 1p19q)	p-value	No LOH (n=13)	LOH 1p/19q (n=17)	FC (No LOH/ LOH 1p19q)	p-value
CHI3L1	18.579	2.529	7.347	**0.000***	9.434	7.941	1.188	0.842	0.089	36.805	0.310	118.726	**0.007***	12.971	4.095	3.168	**0.024***
IGF2	8.517	2.840	2.999	**0.041***	2.066	6.470	0.319	3.132	0.120	3.825	1.479	2.586	0.257	9.961	3.801	2.620	0.155
IQGAP1	39.733	5.083	7.817	**0.000***	13.172	20.404	0.646	1.549	0.486	39.775	4.304	9.241	**0.017***	39.720	5.633	7.051	**0.001***
MIG-6	1.135	0.557	2.040	**0.007***	0.919	0.691	1.329	0.752	0.299	1.668	0.669	2.492	0.100	0.972	0.477	2.037	**0.016***
EGFR	47.876	24.813	1.929	0.903	20.472	40.197	0.509	1.964	0.680	9.620	24.089	0.399	0.538	59.647	25.324	2.355	0.709
PDPN	6.719	0.564	11.910	**0.000***	0.956	3.843	0.249	4.018	0.219	2.640	0.395	6.684	**0.015***	7.974	0.684	11.666	**0.000***
PLAG1	0.396	0.175	2.263	**0.010***	0.272	0.249	1.092	0.916	0.390	0.400	0.229	1.745	0.191	0.395	0.137	2.885	**0.016***
ALX3	2.636	1.409	1.871	0.166	2.253	1.654	1.362	0.734	1.000	5.064	1.316	3.847	0.096	1.890	1.474	1.282	0.607
PADI2	1.084	0.439	2.466	**0.002***	0.890	0.564	1.577	0.634	0.507	2.191	0.456	4.804	**0.010***	0.743	0.428	1.737	**0.036***

FC – Fold Change; * - significant ($p \leq 0.05$)

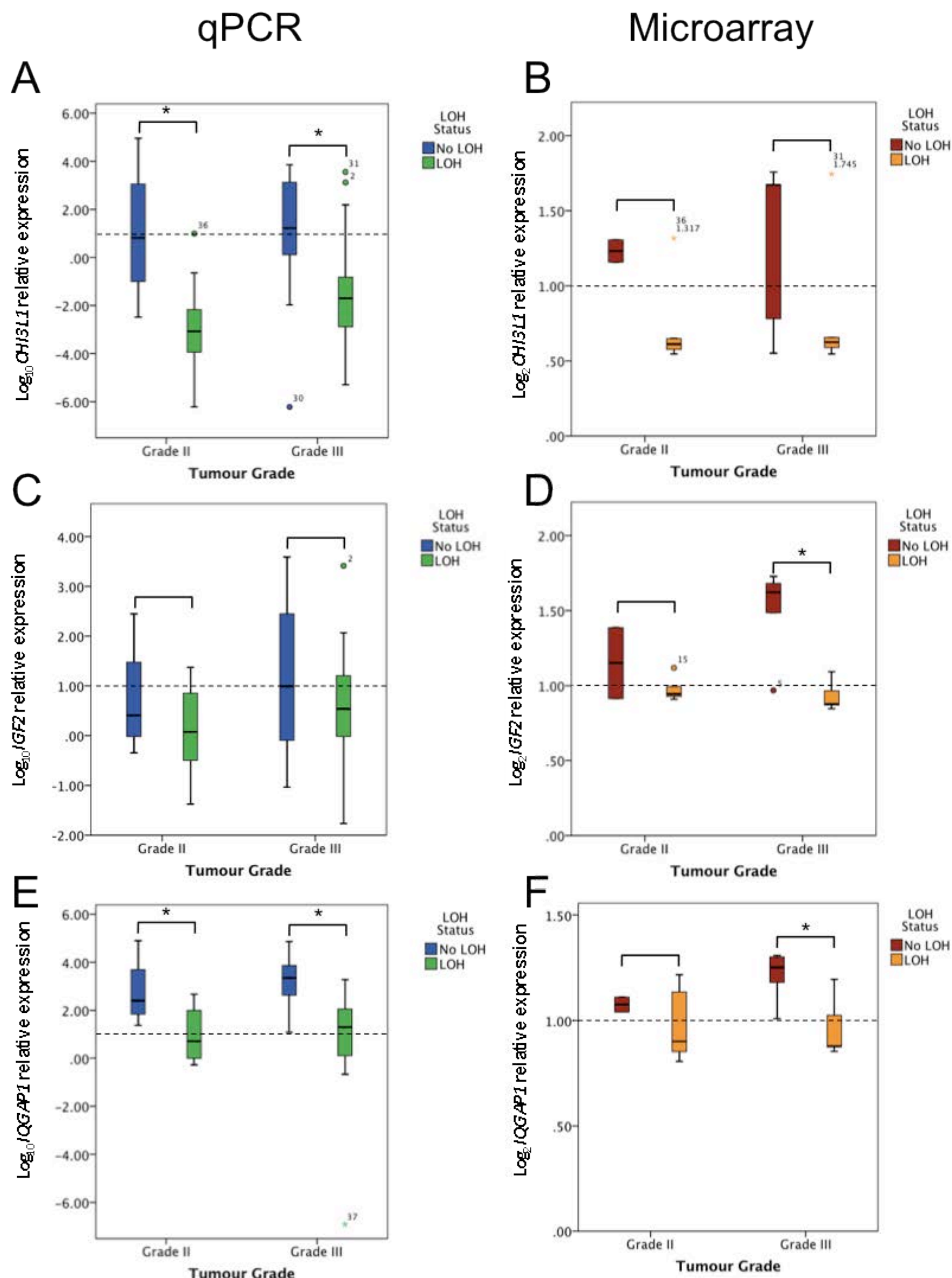


Figure 5.1 *CHI3L1*, *IGF2* and *IQGAP1* expression in oligodendrogliomas. *CHI3L1* and *IQGAP1* but not *IGF2* had significantly higher expression in oligodendrogliomas without LOH 1p/19q in cohorts of both grade II and grade III tumours. Expression of [A] *CHI3L1,* [C] *IGF2* and [E] *IQGAP1* genes was analysed by qPCR in OII with LOH 1p/19q (n=12), OII without LOH (n=4), OIII with LOH 1p/19q (n=17) and OIII without LOH (n=13), relative to normal brain control (relative expression of each gene in normal brain is 1.00). Gene expression assessed by microarray in OII with LOH 1p/19q (n=5), OII without LOH (n=2), OIII with LOH 1p/19q (n=5) and OIII without LOH (n=5) for [B] *CHI3L1,* [D] *IGF2* and [F] *IQGAP1* normalised to normal brain control is shown for comparison. * significant ($p < 0.05$).

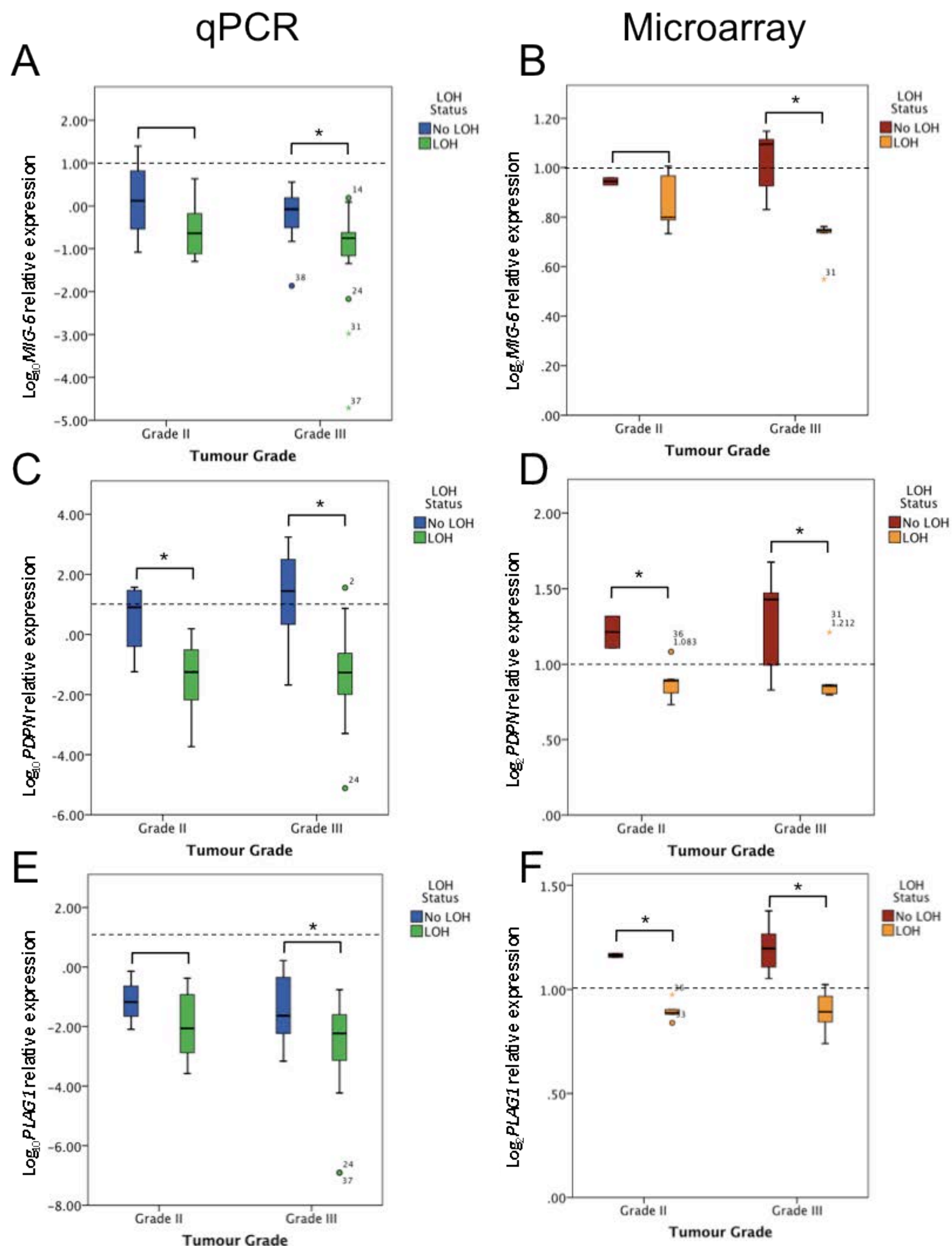


Figure 5.2 *MIG-6*, *PDPN* and *PLAG1* expression in oligodendrogliomas. *PDPN* but not *MIG-6* or *PLAG1* were differentially expressed between grade II oligodendrogliomas separated by LOH status. *MIG-6*, *PDPN* and *PLAG1* had significantly higher expression in grade III oligodendrogliomas without LOH 1p/19q, compared to those with LOH 1p/19q. Expression of genes [A] *MIG-6*, [C] *PDPN* and [E] *PLAG1* was analysed by qPCR in OII with LOH 1p/19q (n=12), OII without LOH (n=4), OIII with LOH 1p/19q (n=17) and OIII without LOH (n=13), relative to normal brain control (relative expression of each gene in normal brain is 1.00). Gene expression assessed by microarray in OII with LOH 1p/19q (n=5), OII without LOH (n=2), OIII with LOH 1p/19q (n=5) and OIII without LOH (n=5) for [B] *MIG-6,* [D] *PDPN* and [F] *PLAG1* normalised to normal brain control is shown for comparison. * significant ($p < 0.05$).

Figure 5.3 *EGFR*, *ALX3* and *PADI2* expression in oligodendrogliomas. *PADI2* but not *EGFR* or *ALX3* had significantly higher expression in both grade II and grade III oligodendrogliomas without LOH 1p/19q, compared to oligodendrogliomas with LOH 1p/19q. Expression of the genes [A] *EGFR* [C] *ALX3* and [E] *PADI2* were analysed by qPCR in OII with LOH 1p/19q (n=12), OII without LOH (n=4), OIII with LOH 1p/19q (n=17) and OIII without LOH (n=13), relative to normal brain control (relative expression of each gene in normal brain is 1.00). Gene expression assessed by microarray in OII with LOH 1p/19q (n=5), OII without LOH (n=2), OIII with LOH 1p/19q (n=5) and OIII without LOH (n=5) for [B] *EGFR,* [D] *ALX3* and [F] *PADI2* normalised to normal brain control is shown for comparison. * significant ($p < 0.05$).

5.3.1.1 Validation of DNA methylation

Allelic bisulfite sequencing confirmed DNA methylation at the *ALX3* locus in three normal brain and also three oligodendroglioma specimens, which had previously shown no methylation when analysed by the CpG island plus promoter array. qPCR revealed that the mRNA expression of *ALX3* was not differentially expressed in oligodendrogliomas when characterised by tumour grade (p=0.649) or LOH status (p=0.247). Additionally, *ALX3* was not differentially expressed between normal and LOH (p=0.847) or No LOH (p=0.529).

Allelic bisulfite sequencing confirmed high levels of methylation at the PADI2 locus in the three oligodendrogliomas with LOH 1p/19q previously analysed by the CpG array (Figure 5.4). Very low level methylation was detected in normal brain specimens. Analysis of additional oligodendroglioma specimens by COBRA revealed methylation in 17/20 grade II and 17/23 grade III oligodendrogliomas with LOH 1p/19q (Figure 5.5).

qPCR revealed that *PADI2* expression was 2.12 fold higher in oligodendrogliomas without LOH, compared to oligodendrogliomas with LOH 1p/19q (p=0.041, Figure 5.3). Expression in oligodendrogliomas without LOH was not significantly different to expression in normal brain (fold change=0.86, p=0.204). However expression in oligodendrogliomas with LOH 1p/19q was significantly lower than in normal brain (fold change=0.41, p=0.015).

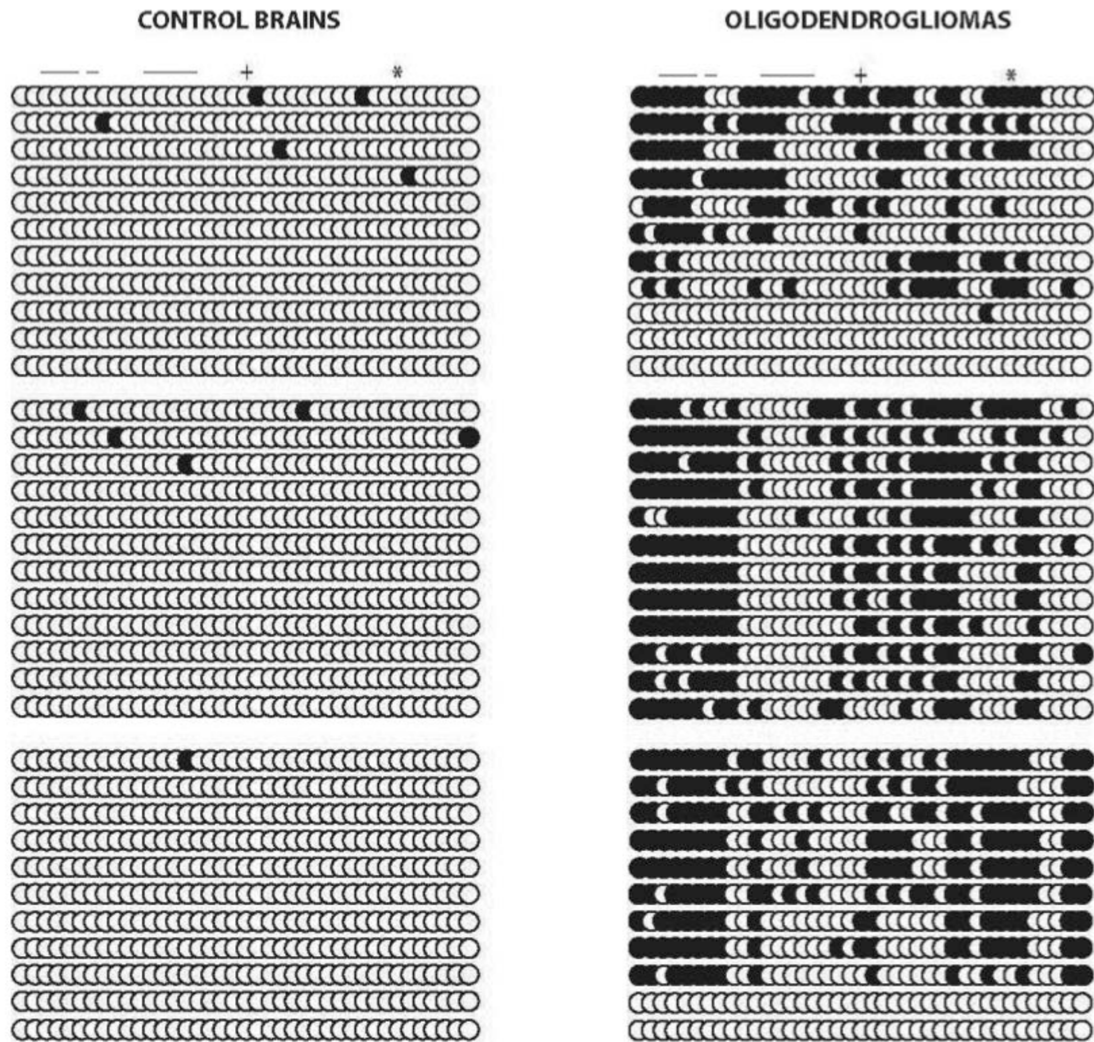


Figure 5.4 Bisulfite sequencing confirmed methylation of *PADI2* in three oligodendrogliomas with LOH 1p/19q
No methylation was observed in normal brain specimens. Each allele is represented by a horizontal line and each CpG dinucleotide is represented by a circle. Methylated CpG's are shown in black. SP1/3 transcription factor binding site (grey line), Transcription start site (+) and COBRA Taq1 site (*) are shown.

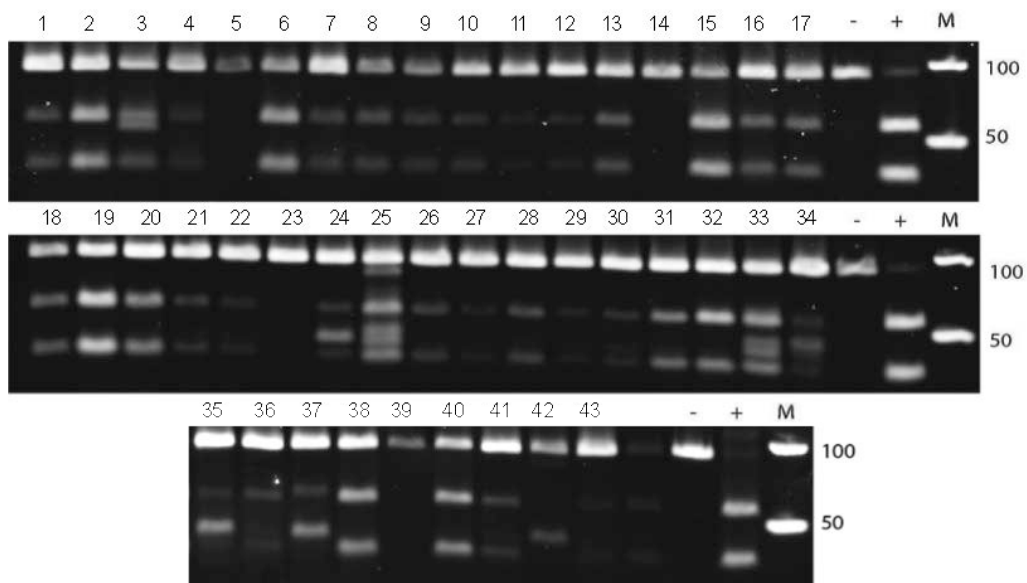


Figure 5.5 Methylation of *PADI2* confirmed by COBRA.
COBRA revealed methylation of the *PADI2* locus in 17/20 grade II oligodendrogliomas with LOH 1p/19q (samples 1-20) and 17/23 grade III oligodendrogliomas with LOH 1p/19q (samples 21-43). The negative control (–), normal brain DNA, shows a single, undigested band of 96 bp. The positive control (+), U251 cell line DNA, shows two bands of 57 bp and 39 bp as a result of complete digestion. Molecular marker (M) bands at 100 bp and 50 bp.

5.3.2 Expression of CHI3L1, IGF2, IQGAP1, MIG-6 and PDPN proteins in oligodendroglioma

Immunohistochemistry was conducted on grade III oligodendrogliomas only. A full list of specimens analysed by immunohistochemistry is presented in Appendix 3A. A breakdown of the LOH status of oligodendroglioma specimens stained by each antibody is provided in Table 5.6.

Table 5.6 Specimens analysed by immunohistochemistry

Antibody	Specimen	LOH 1p/19q	1p/19q intact	Total
CHI3L1	Grade III Oligodendroglioma	11	13	**24**
IGF2	Grade III Oligodendroglioma	14	12	**26**
IQGAP1	Grade III Oligodendroglioma	13	16	**29**
MIG-6	Grade III Oligodendroglioma	13	15	**28**
PDPN	Grade III Oligodendroglioma	13	15	**28**

Immunohistochemistry for the assessment of the CHI3L1 was performed on 24 grade III oligodendroglioma specimens. Staining for CHI3L1 was specific to the cytoplasmic areas of tumour cells (Figure 5.6). Positive immunostaining (score ≥3) for CHI3L1 was observed more frequently in grade III oligodendrogliomas without LOH than those with LOH 1p/19q (Table 5.7; Fisher's exact test, p=0.021).

IGF2 protein expression was analysed by immunohistochemistry in 26 grade III oligodendrogliomas. Images for IGF2 staining were unfortunately not available, however the assessment of the staining was provided by a pathologist. Immunostaining for IGF2 did not correlate with LOH 1p/19q status among grade III oligodendrogliomas (Table 5.8; Fisher's exact test, one sided, p=0.348).

IQGAP1 immunohistochemistry was performed on 29 grade III oligodendroglioma. Staining was observed in the cytoplasm. Representative staining for each score is shown in Figure 5.7. Positive staining (score >0) for IQGAP1 was significantly higher in grade III oligodendrogliomas without LOH 1p/19q compared to those with LOH (Table 5.9; Fisher's exact test, one sided, p=0.023).

MIG-6 staining was analysed in 28 grade III oligodendroglioma specimens. Cytoplasmic and nuclear MIG-6 staining was observed, however staining was generally weak with few areas of intensity (Figure 5.8). Immunostaining scores for MIG-6 did not correlate with LOH 1p/19q status among grade III oligodendrogliomas (Table 5.10; Fisher's exact test, one sided, p=0.140).

Expression of PDPN was assessed by immunohistochemistry in 28 grade III oligodendroglioma specimens. Nuclear, cytoplasmic and cell surface staining of PDPN was observed (Figure 5.9). Immunostaining for PDPN did not correlate with LOH 1p/19q status among grade III oligodendrogliomas (Table 5.11; Fisher's exact test, one sided, p=0.6).

Table 5.7 Protein expression scores for CHI3L1 in grade III oligodendrogliomas, classified by LOH 1p/19q

CHI3L1 staining score	LOH 1p/19q	No LOH
0	4 (36.4%)	2 (15.4%)
2	3 (27.3%)	
3	2 (18.2%)	8 (61.5%)
4	1 (9.1%)	2 (15.4%)
5	1 (9.1%)	1 (7.7%)
	11	**13**

Table 5.8 Protein expression scores for IGF2 in grade III oligodendrogliomas, classified by LOH 1p/19q

IGF2 staining score	LOH 1p/19q	No LOH
0	1 (7.1%)	1 (8.3%)
1	5 (35.7%)	6 (50%)
2	7 (50%)	5 (41.7%)
3	1 (7.1%)	
	14	**12**

Table 5.9 Protein expression scores for IQGAP1 in grade III oligodendrogliomas, classified by LOH 1p/19q status

IQGAP1 staining score	LOH 1p/19q	No LOH
0	8 (61.5%)	3 (18.75%)
1	4 (30.8%)	6 (37.5%)
2	1 (7.7%)	5 (31.25%)
3		2 (12.5%)
	13	**16**

Table 5.10 Protein expression scores for MIG-6 in grade III oligodendrogliomas, classified by LOH 1p/19q status

MIG-6 staining score	LOH 1p/19q	No LOH
0	5 (38.5%)	8 (53.3%)
2	1 (7.7%)	3 (20%)
3	4 (30.8%)	1 (6.7%)
4	1 (7.7%)	
5	1 (7.7%)	3 (20%)
6	1 (7.7%)	
	13	**15**

Table 5.11 Protein expression scores for PDPN in grade III oligodendrogliomas, classified by LOH 1p/19q status

PDPN staining score	LOH 1p/19q	No LOH
0	1 (7.7%)	3 (20%)
2	2 (15.4%)	
3	4 (30.8)	2 (13.3%)
4	3 (23.1%)	2 (13.3%)
5	2 (15.4%)	6 (40%)
6	1 (7.7%)	2 (13.3%)
	13	**15**

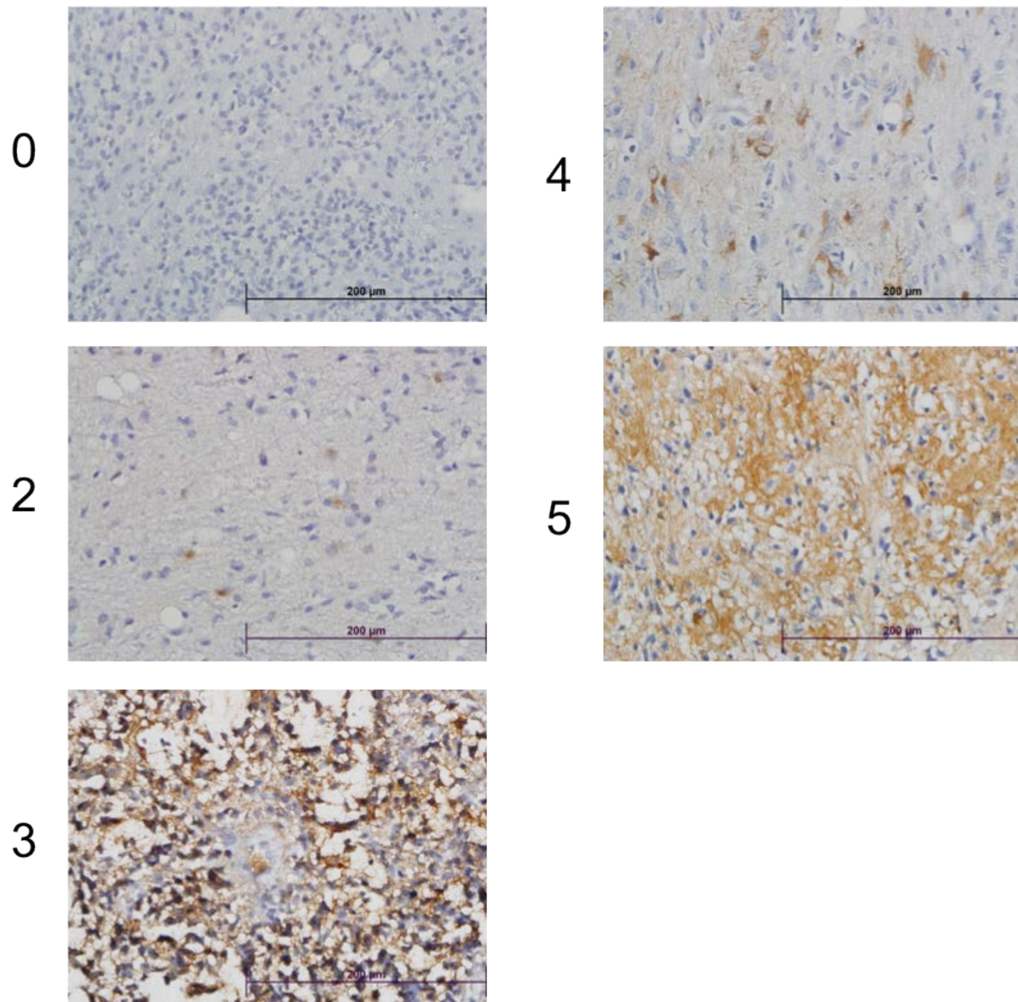


Figure 5.6 Expression and localisation of CHI3L1 in grade III oligodendrogliomas. Images are representative of Allred scores 0 to 5. At score 5, cytoplasmic staining is observed in over 75% of cells. At score 2, cytoplasmic staining is observed in less than 25% of cells.

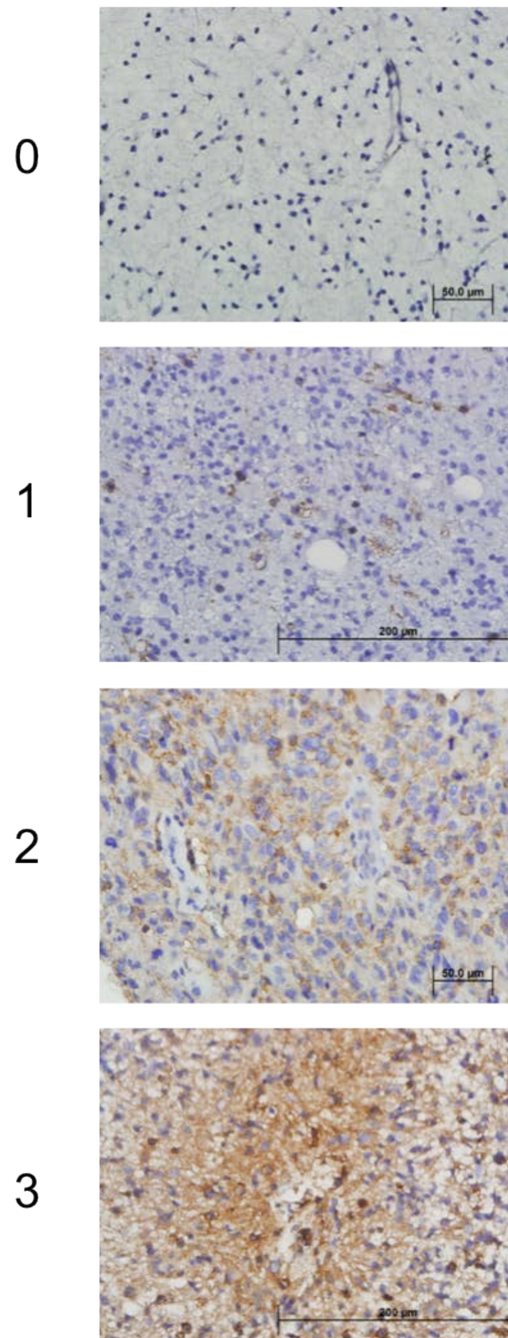


Figure 5.7 IQGAP1 staining in grade III oligodendrogliomas. Images shown here are representative of staining for scores 0 to 3.

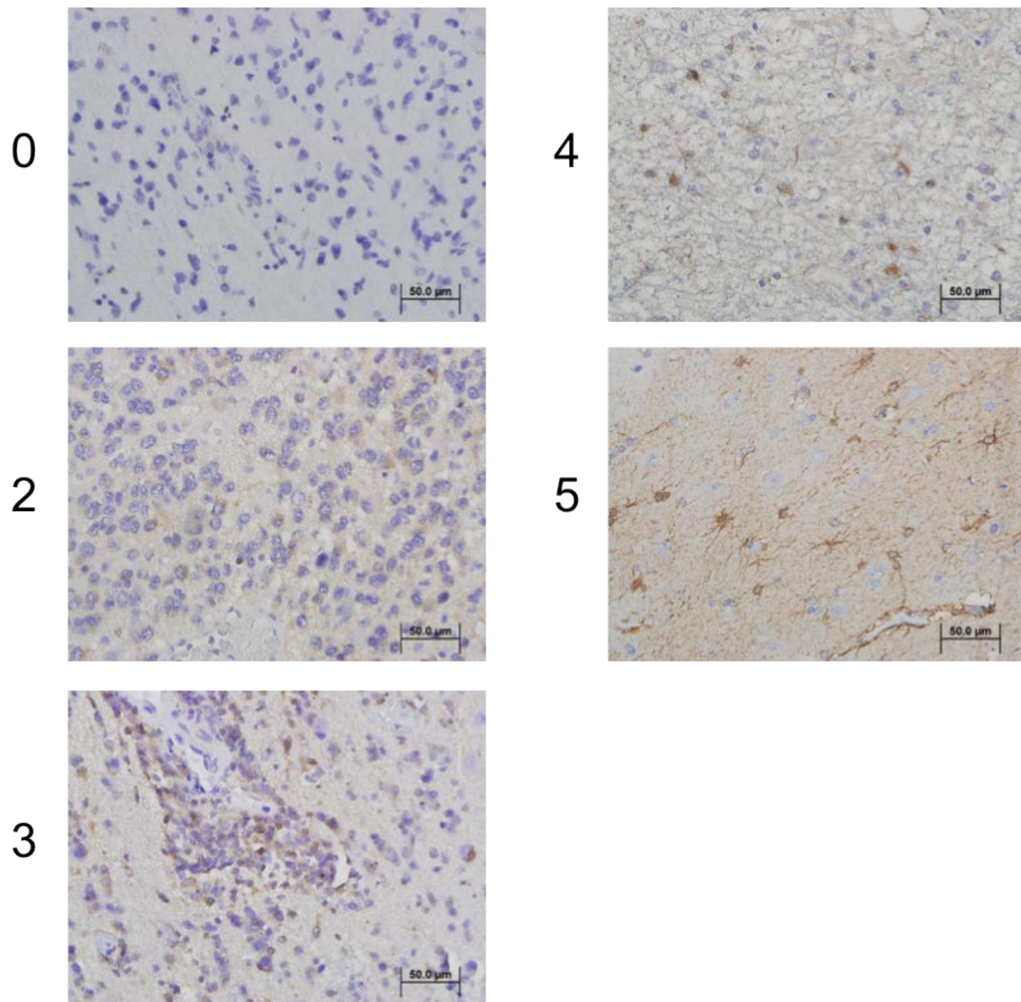


Figure 5.8 MIG-6 staining in grade III oligodendrogliomas.
Images shown here are representative of staining for scores 0 to 5. Sections were scored using the Allred method. MIG-6 staining was observed in cytoplasm.

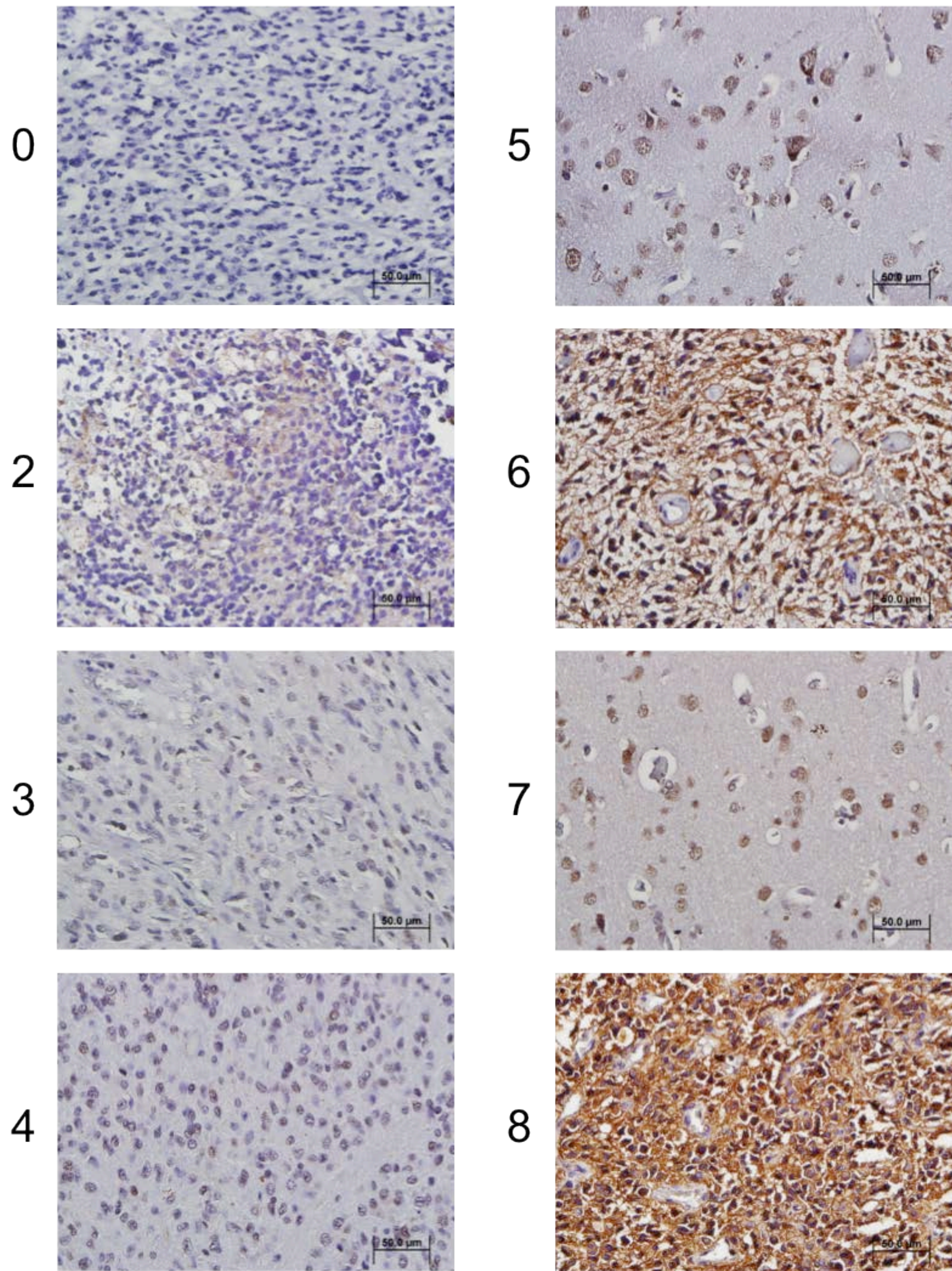


Figure 5.9 PDPN staining in grade III oligodendrogliomas.
Images shown here are representative of staining for scores 0 to 8. Sections were scored according to the Allred method. Staining is observed in nuclear areas at low scores (2-5) and in both nuclear and cytoplasmic areas at high scores (6-8).

5.3.2.1 Survival analysis

Univariate analysis (LogRank test) was conducted to examine the prognostic significance of mRNA expression of *CHI3L1*, *IGF2*, *IQGAP1*, *MIG-6*, *PDPN* and *PLAG1* and protein staining positivity for CHI3L1, IGF2, IQGAP1, MIG-6 and PDPN in grade III oligodendroglioma specimens. Kaplan Meier curves illustrating the relationship between each expression marker and survival of grade III oligodendroglioma patients are shown in Figure 5.10-11. Median survival of patients categorised by transcript expression and protein staining positivity is summarised in Table 5.12. Significance was calculated by the LogRank test (Mantel-Cox).

High *PDPN* transcript expression in grade III oligodendrogliomas was significantly associated with shorter survival. Similar trends were observed for *CHI3L1* and *MIG-6*, however these were not significant. Expression of the IGF2, IQGAP1 and PLAG1 transcripts did not demonstrate any correlation with survival in grade III oligodendrogliomas.

Grade III oligodendroglioma patients that stained positive for CHI3L1 showed a trend toward shorter survival, but this was not significant. Neither IGF2 nor PDPN staining scores in oligodendroglioma correlated with survival. Patients that stained positive for IQGAP1 or MIG-6 demonstrated slightly longer survival, however the relationship was not significant for either protein.

Table 5.12 Survival among grade III oligodendroglioma patients

	Median survival (months)		p-value
mRNA expression	**High expression (95% CI)**	**Low expression (95% CI)**	
CHI3L1	19.10 (0.78 – 37.42)	89.13 (N/A)**	0.052
IGF2	47.52 (0 – 182.26)	20.07 (5.04 – 83.71)	0.591
IQGAP1	23.90 (14.78 – 33.02)	89.13 (N/A)**	0.128
MIG-6	6.73 (5.91 – 32.29)	47.70 (0 – 182.61)	0.069
PDPN	9.35 (0.78 – 37.42)	48.98 (0 – 185.12)	**0.005***
PLAG1	25.90 (14.93 – 36.97)	89.13 (N/A)**	0.405
Protein expression	**Positive (95% CI)**	**Negative (95% CI)**	
CHI3L1	14.77 (5.00 – 24.54)	23.77 (19.12 – 28.42)	0.262
IGF2	24.33 (N/A)**	23.77 (0 – 59.96)	0.564
IQGAP1	23.67 (2.93 – 26.60)	14.77 (15.46 – 31.88)	0.356
MIG-6	26.67 (0 – 93.31)	21.27 (8.57 – 33.76)	0.325
PDPN	21.37 (14.22 – 28.52)	23.77 (0 – 58.55)	0.625

* Significant, p<0.05; ** Median survival reached, remaining cases censored.

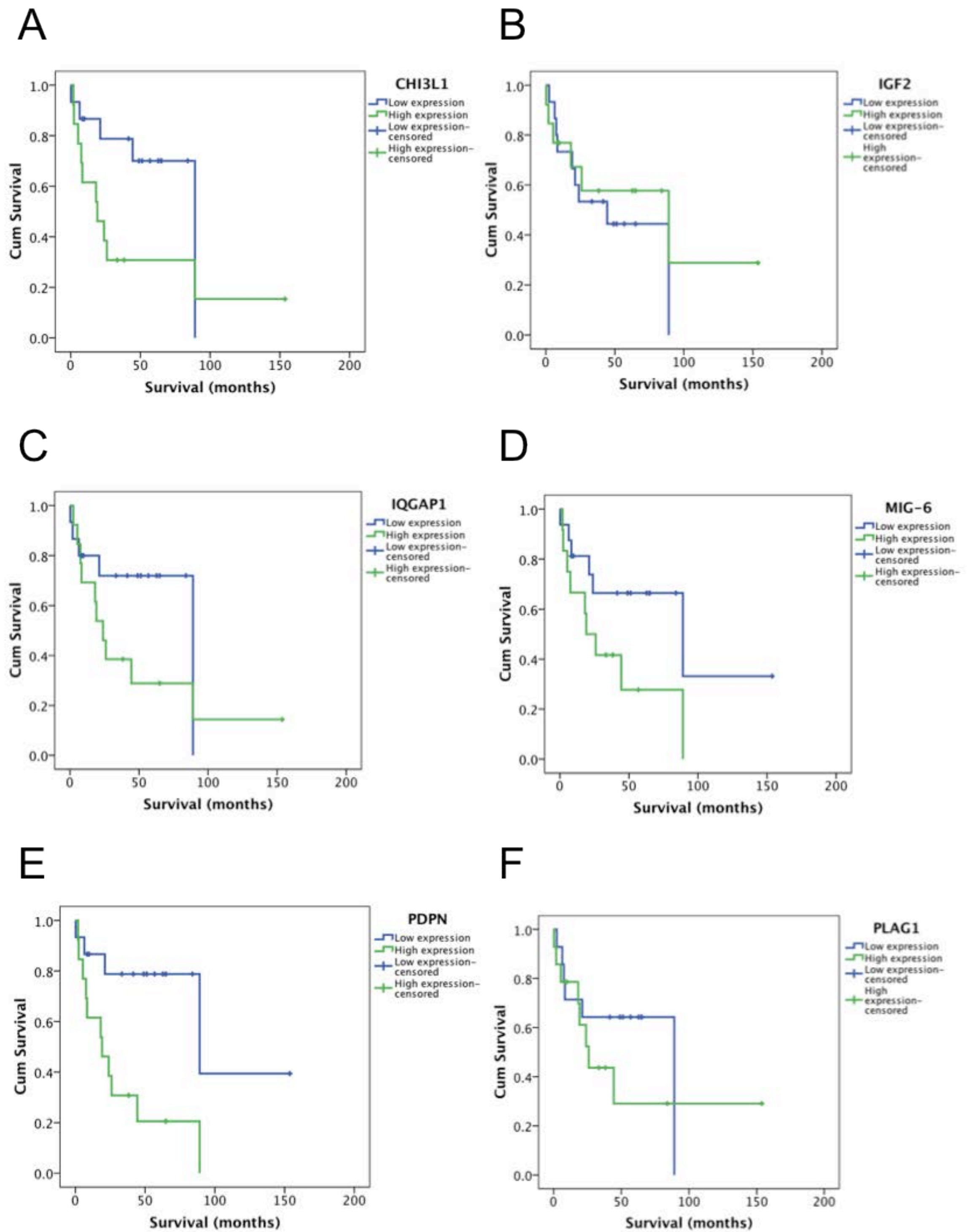


Figure 5.10 Kaplan-Meier survival curves show the prognostic value of *CHI3L1*, *IGF2*, *IQGAP1*, *MIG-6*, *PDPN* and *PLAG1* mRNA expression
Gene expression was analysed by qPCR in grade III oligodendroglioma patients. [A] *CHI3L1* (p=0.052) [B] *IGF2* (p=0.591) [C] *IQGAP1* (p=0.128) [D] *MIG-6* (p=0.069) [E] *PDPN* (p=0.005) [F] *PLAG1* (p=0.405). Significance calculated by Log Rank (Mantel-Cox).

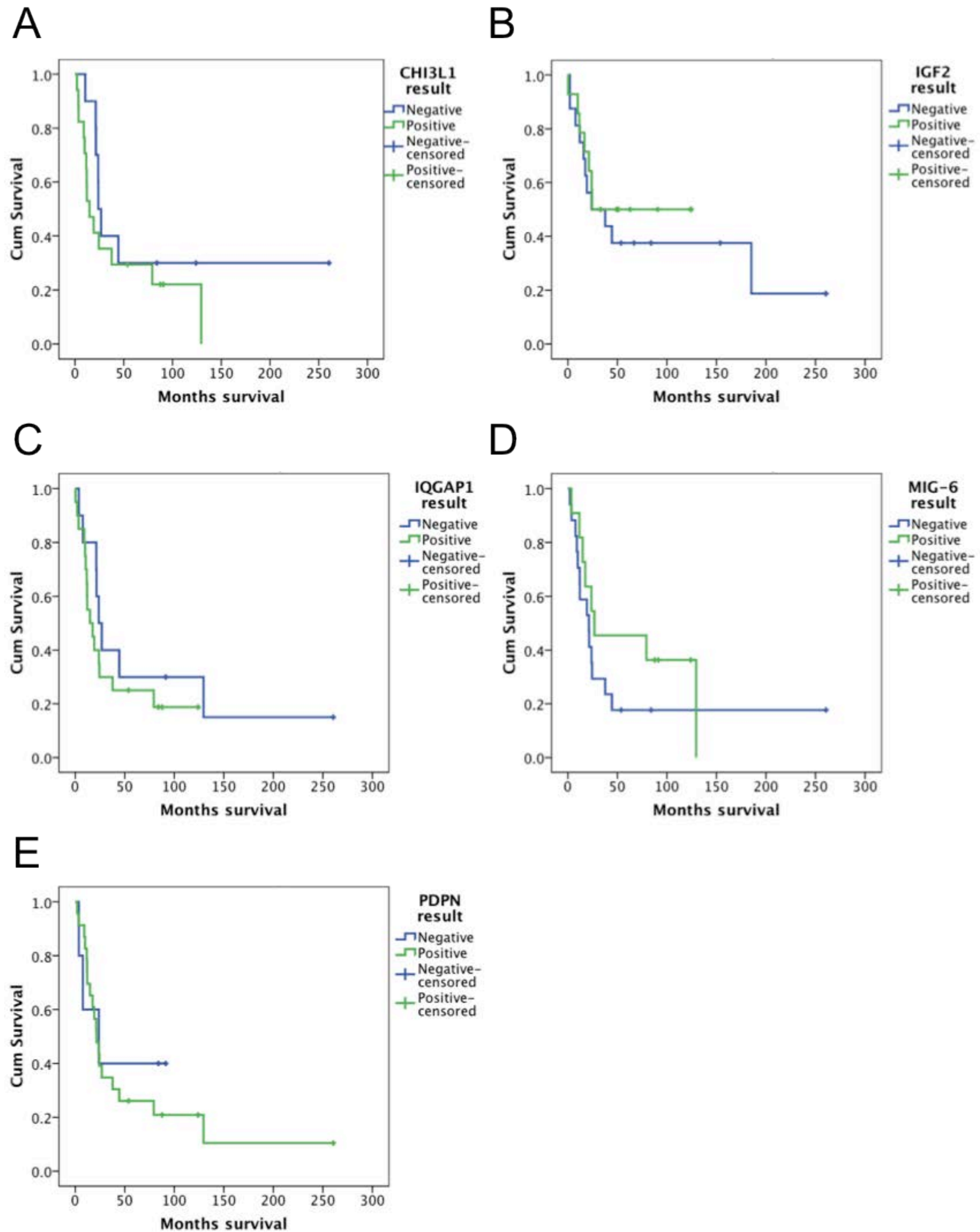


Figure 5.11 Kaplan-Meier survival curves show prognostic value of CHI3L1, IGF2, IQGAP1, MIG-6 and PDPN protein staining in grade III oligodendroglioma patients [A] CHI3L1 (n=24 ;p=0.262) [B] IGF2 (n=26; p=0.564) [C] IQGAP1 (n=29; p=0.356) [D] MIG-6 (n=28; p=0.325) [E] PDPN (n=28; p=0.652). Significance calculated by Log Rank (Mantel-Cox).

5.3.3 Functional analysis of MIG-6 in glioma cell lines

5.3.3.1 Endogenous MIG-6 expression

Endogenous expression of *MIG-6* mRNA transcript in three oligodendroglioma and three glioblastoma cell lines, relative to normal brain, was determined by qPCR. All three oligodendroglioma cell lines expressed lower than normal levels of *MIG-6*, ranging from 1.39 to 2.67 fold below normal. Glioblastoma cell lines expressed higher than normal levels of both MIG-6, with the exception of H423 (Figure 5.12, Table 5.13). The H423 cell line does not express MIG-6, due to a homozygous deletion of the *MIG-6* locus on chromosome 1p (Duncan et al., 2010). Analysis of copy number data from 267 glioblastoma specimens in The Cancer Genome Atlas (TCGA) database revealed a subset of glioblastomas with similar focal deletions of 1p36 including the *MIG-6* locus (Figure 5.13). Homozygous loss of the *MIG-6* locus occurred in 2.25% and heterozygous loss occurred in 10.49% of glioblastomas.

Table 5.13 Expression of *MIG-6* in Oligodendroglioma and glioblastoma cell lines

Cell Line	*MIG-6* expression (compared to normal human brain, 1)
Oligodendroglioma cell lines	
HOG	0.675
MO3.13	0.717
TC620	0.374
Glioblastoma cell lines	
A172	1.311
H423 *	0
U87MG	10.407

* The H423 cell line has a homozygous deletion of the MIG-6 locus.

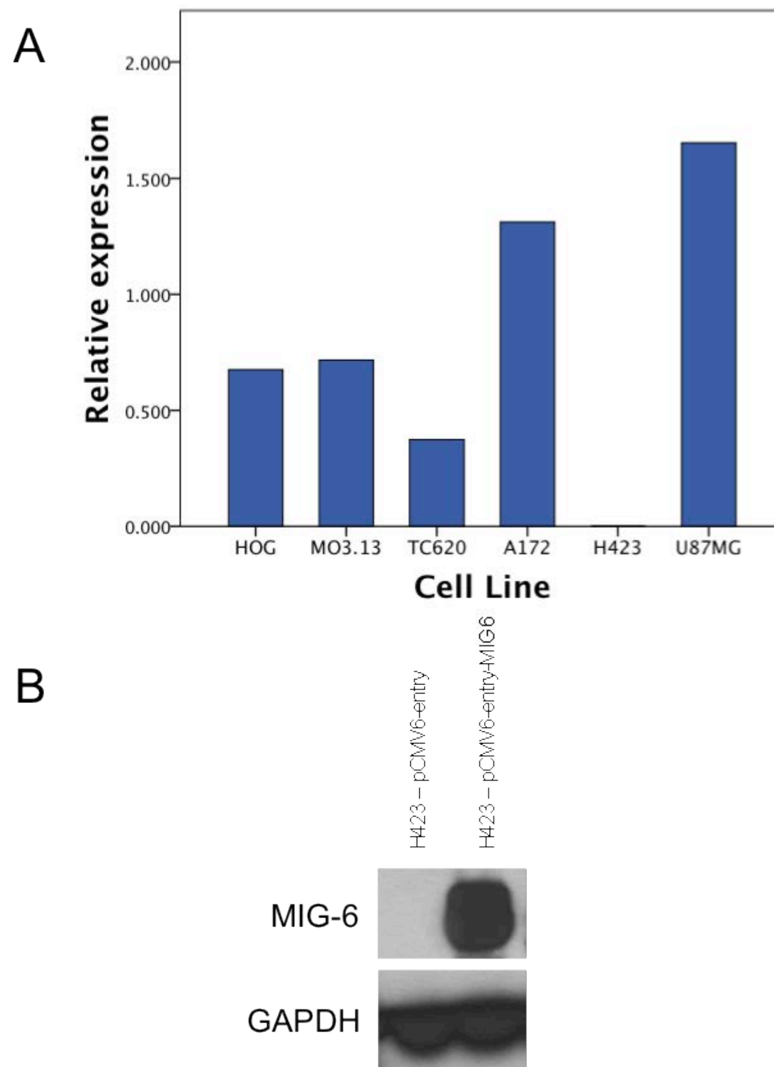


Figure 5.12 Endogenous expression of *MIG-6* in oligodendroglioma and glioblastoma cell lines.
[A] *MIG-6* mRNA expression was assessed by qPCR in three oligodendroglioma (HOG, MO3.13, TC620) and three glioblastoma (A172, H423, U87MG) cell lines and is shown relative to normal brain (1.00). [B] Expression of MIG-6 protein in H423 cells transfected with empty vector (pCMV6-entry) or MIG-6 overexpression vector (pCMV6-entry-MIG-6), analysed by western blot.

Figure 5.13 Heatmap of *MIG-6* copy number data in TCGA glioblastoma samples. 267 glioblastoma samples were analysed from the TCGA glioblastoma data set. 6 samples show homozygous deletion of *MIG-6*. 28 samples show heterozygous deletion of *MIG-6*. All events shown here are due to small, focal deletions at 1p36. Copy number shown is the average of data collected from three centres (Broad MIT, Genome_Wide_SNP_6; Harvard, HG-CGH-244A; MSKCC, HG-CGH-244A). TCGA data is available online (http://cancergenome.nih.gov/).

5.3.3.2 Over-expression and knockdown of MIG-6 in glioma cell lines

To over-express MIG-6 in cells, full-length human *MIG-6* cDNA was cloned into the mammalian expression vector pCMV6-entry, which is designed for the transient transfection of the cloned gene in cultured cells (*MIG-6* cloning was conducted by Chris Duncan, Duke University). pCMV6-entry-*MIG6* or pCMV6-entry was transfected into H423 glioma cells and protein expression was assessed after 24 hours by western blot. MIG-6 was present in abundance in H423 cells transfected with pCMV6-Entry-MIG-6. No MIG-6 expression was observed in H423 cells transfected with pCMV6-entry (empty vector) (Figure 5.12).

To knock down *MIG-6* in cells, a set of four *MIG-6* targeted shRNA plasmids were purchased. The knockdown capacity of the four *MIG-6* targeted shRNA plasmids was assessed by qPCR 48 hours after transfection. Plasmid shRNA-MIG-6 (2) caused the greatest knockdown of *MIG-6*, resulting in a 34.6% reduction in *MIG-6* mRNA. The optimal incubation time following transfection for maximum *MIG-6* knockdown by shRNA-MIG-6 (2) was assessed by qPCR. Inhibition of *MIG-6* mRNA was most pronounced after 48 hours (34.4% knockdown). The knockdown of MIG-6 by shRNA-MIG-6 (2) did not translate to an observable reduction in *MIG-6* protein expression when assessed by western blot (Figure 5.14).

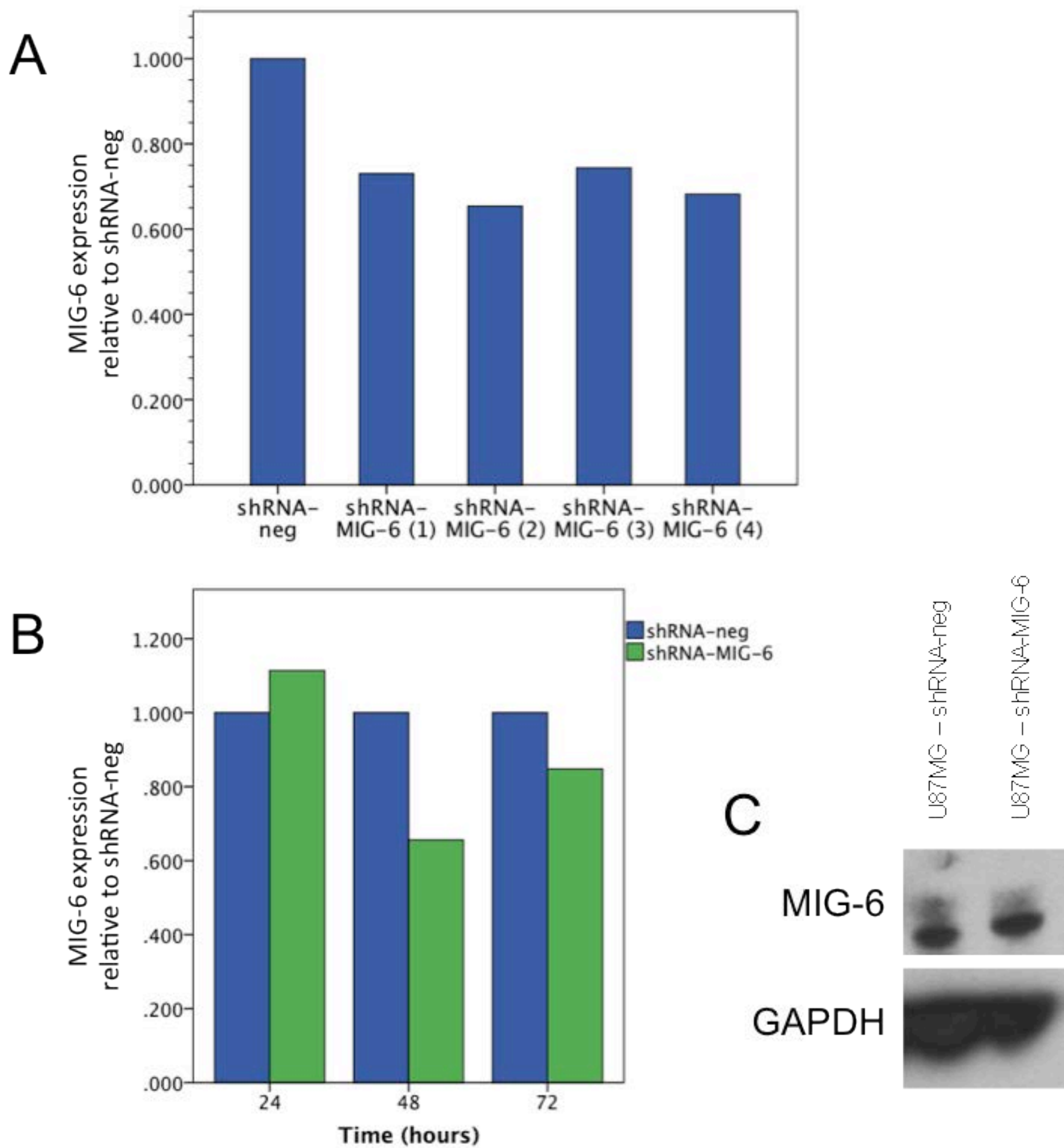


Figure 5.14 MIG-6 knockdown in U87MG glioblastoma cells.
[A] Transfection of U87MG cells with shRNA plasmids (targeted to *MIG-6*) was effective in reducing expression of *MIG-6* as assessed by qPCR. shRNA plasmid 2 was determined to be the most effective and was used for all subsequent experiments. [B] MIG-6 expression in U87MG cells, 24-72 hours following transfection with shRNA plasmids. 48 hours was found to be the optimal time period for knockdown. [C] MIG-6 knockdown in U87MG cells did not produce a visible result by western blot.

5.3.3.3 MIG-6 and cell proliferation

An MTT assay was used to determine the effect of MIG-6 over-expression and knockdown on cell proliferation in two oligodendroglioma and two glioblastoma cell lines. No change in cell viability was observed in H423 glioblastoma cells, HOG or TC620 oligodendroglioma cells with the over-expression of MIG-6 (Figure 5.15-16). Knockdown of MIG-6 in U87MG glioblastoma cells did not alter cell proliferation as assessed by MTT assay (Figure 5.17).

A colony formation assay was conducted to examine the effect of MIG-6 over-expression and knockdown on the ability of glioblastoma cells to form colonies *in vitro* as a measure of their proliferative ability. Over-expression or knockdown of MIG-6 did not change the colony formation ability of glioblastoma cells, as no significant difference in the number of colonies was detected (Figure 5.18-19).

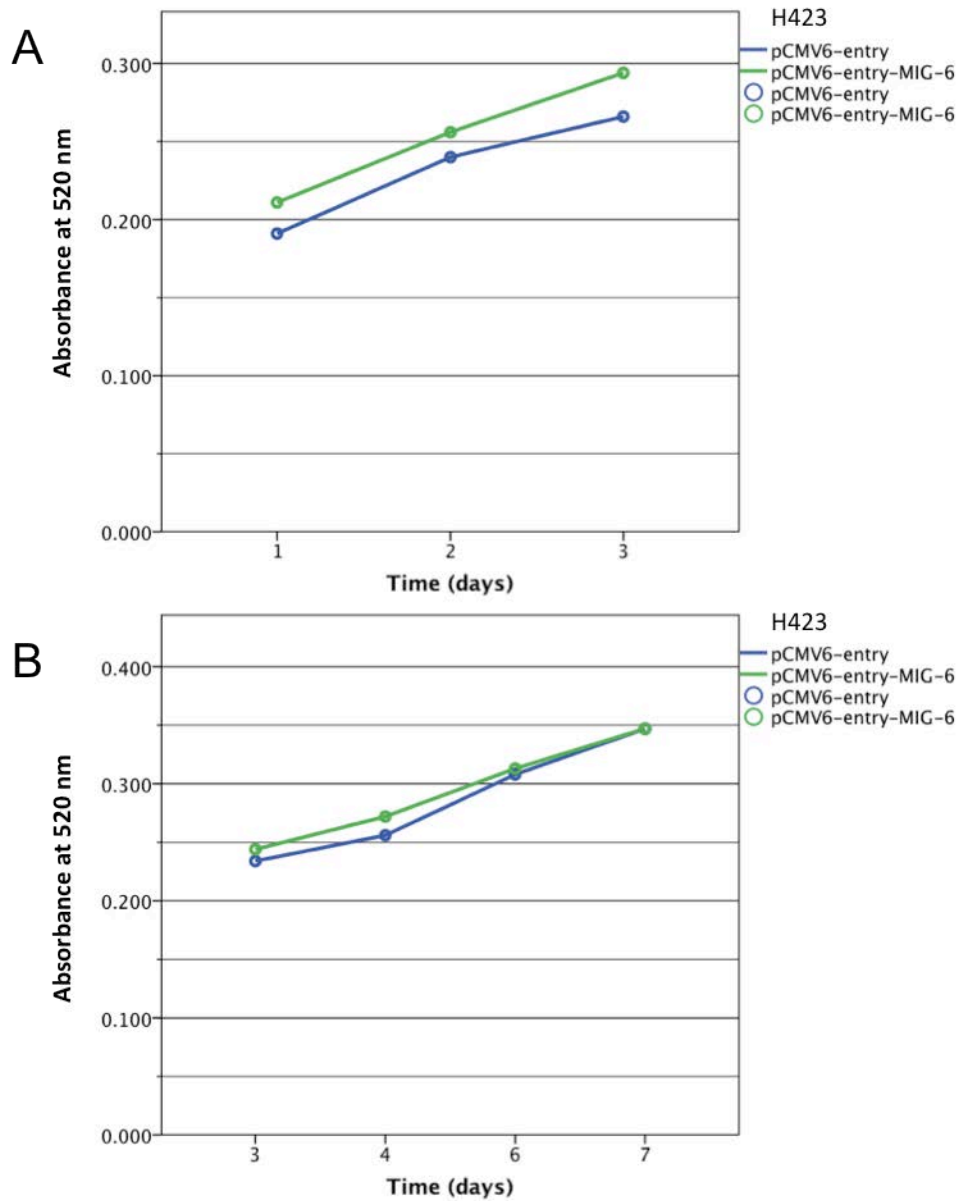


Figure 5.15 Restoration of *MIG-6* expression did not affect cell growth rate in H423 glioblastoma cells.
Growth of H423 cells transfected with empty pCMV6-entry or pCMV6-entry-MIG-6 was assessed by MTT assay over [A] one to three and [B] three to seven days. Results shown are representative of two experiments (50 000 cells/well).

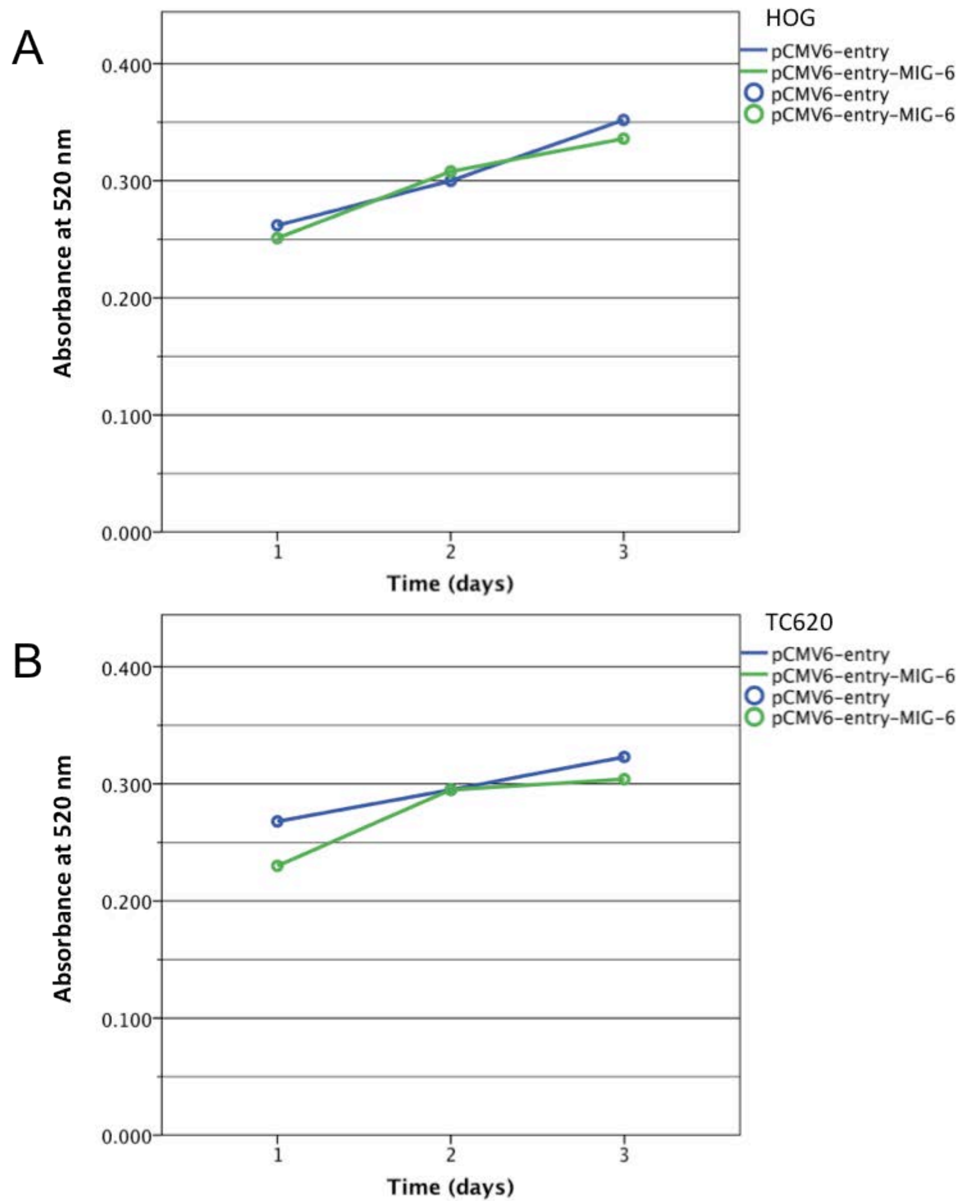


Figure 5.16 Over-expression of *MIG-6* did not affect cell growth rate in HOG or TC620 oligodendroglioma cells.
Growth of [A] HOG (50,000 cells/well) and [B] TC620 cells (20,000 cells/well) transfected with either pCMV6-entry or pCMV6-entry-MIG-6 was assessed by MTT assay over three days. Results shown are representative of two experiments.

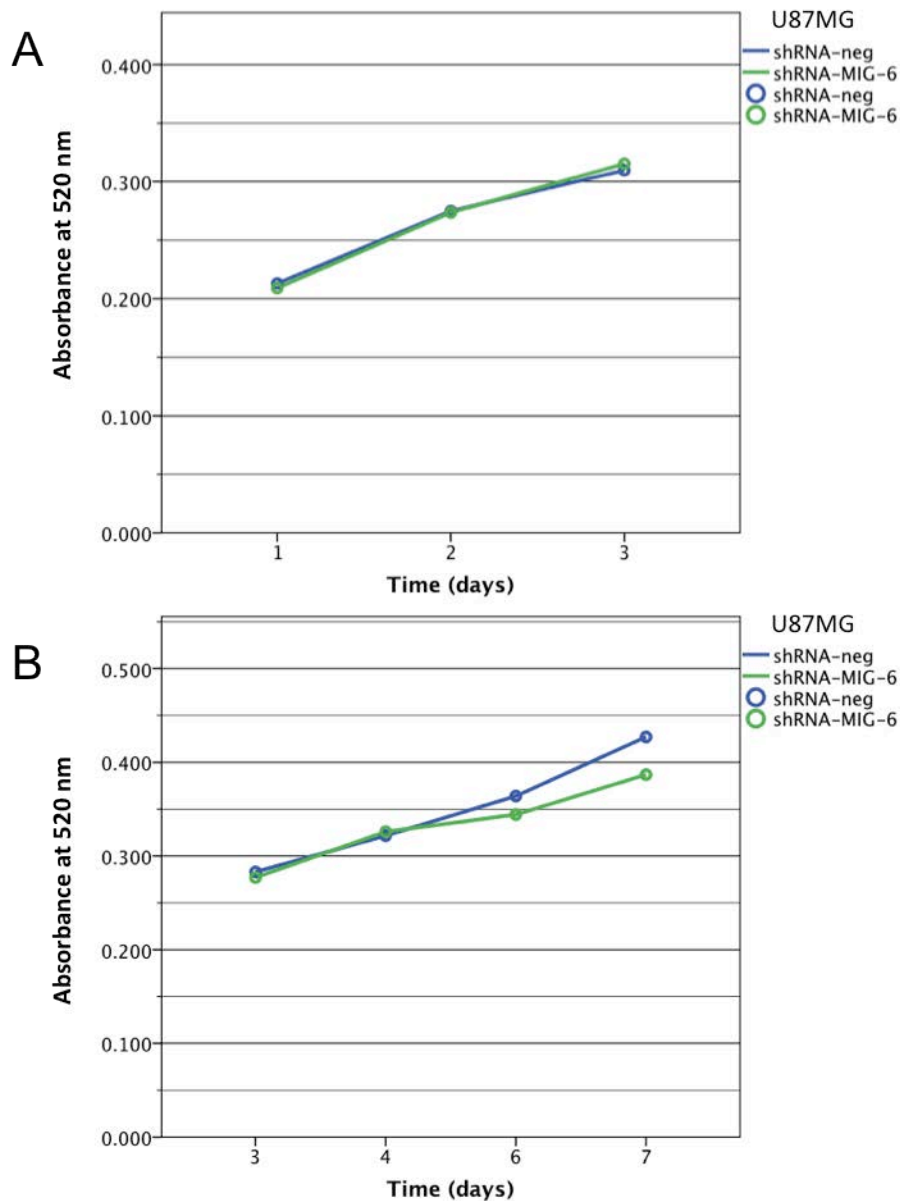


Figure 5.17 Knockdown of *MIG-6* did not affect cell growth rate in U87MG glioblastoma cells.

Growth of U87MG cells transfected with either neg shRNA or shRNA MIG6 plasmid was assessed by MTT assay over [A] one to three (50,000 cells/well) and [B] three to seven days (10,000 cells/well). Results shown are representative of two experiments.

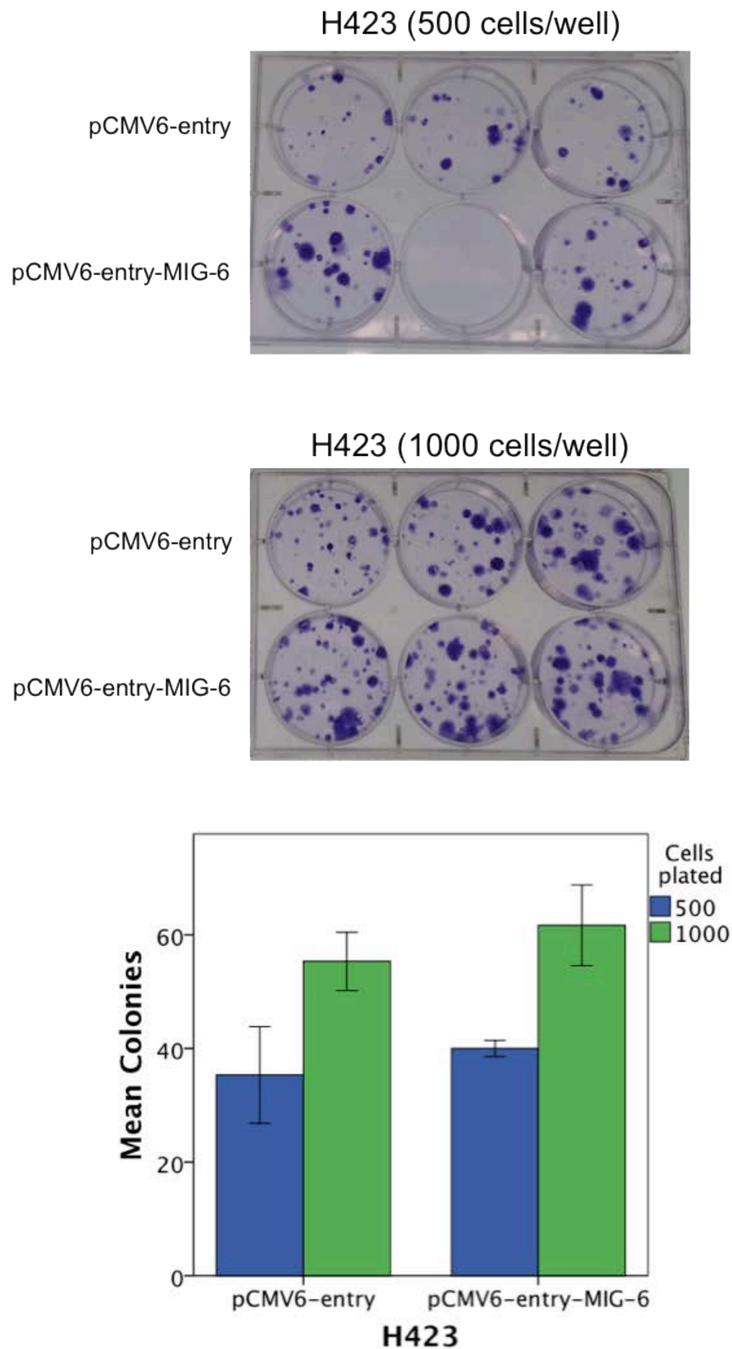


Figure 5.18 Overexpression of *MIG-6* did not alter colony formation in H423 glioblastoma cells.
Images show colonies formed over 18 days by H423 cells transfected with pCMV6-entry or pCMV6-entry-MIG-6, stained with crystal violet (0.2% in DPBS with 20% ethanol) (500 cells, p=0.518; 1000 cells, p=0.279; error bars: +/- 1 standard deviation). No cells were seeded middle well of H423 (500 cells/well) pCMV6-entry-MIG-6 – this well was not used in data analysis.

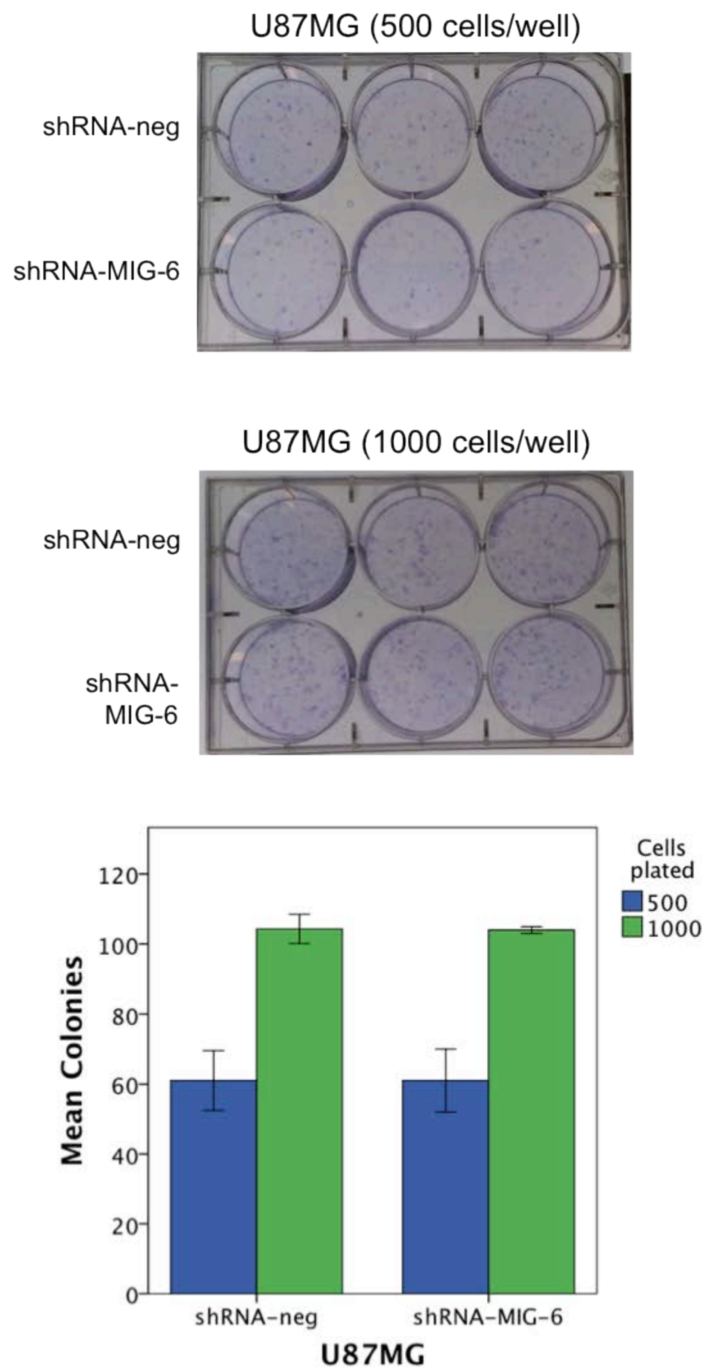


Figure 5.19 Knockdown of *MIG-6* did not alter colony formation in U87MG glioblastoma cells.
Images show colonies formed over 14 days by U87MG cells transfected with shRNA-neg or shRNA-MIG-6, stained with crystal violet (0.2% in DPBS with 20% ethanol) (500 cells, p=1.00; 1000 cells, p=0.899; error bars: +/- 1 standard deviation).

5.3.3.4 MIG-6 and cell migration

The effect of MIG-6 over-expression and knockdown on cell migration in glioblastoma cells was assessed by a wound healing assay. Wound closure of H423 cells was retarded in cells that overexpressed MIG-6 compared to control cells (Figure 5.20 A). MIG-6 knockdown in U87MG cells led to visibly faster wound closure, compared to control cells (Figure 5.20 B)

A trans-well assay was used to quantitatively investigate the trends observed in cell migration due to over-expression or knockdown of MIG-6 in glioblastoma cells. MIG-6 over-expression significantly reduced the number of H423 cells that migrated through the trans-well membrane over 24 hours (p=0.001; Figure 5.21 A). MIG-6 knockdown in U87MG cells trended towards an increase in the number of cells that migrated through to the lower membrane, but this was not significant (p=0.285; Figure 5.21 B).

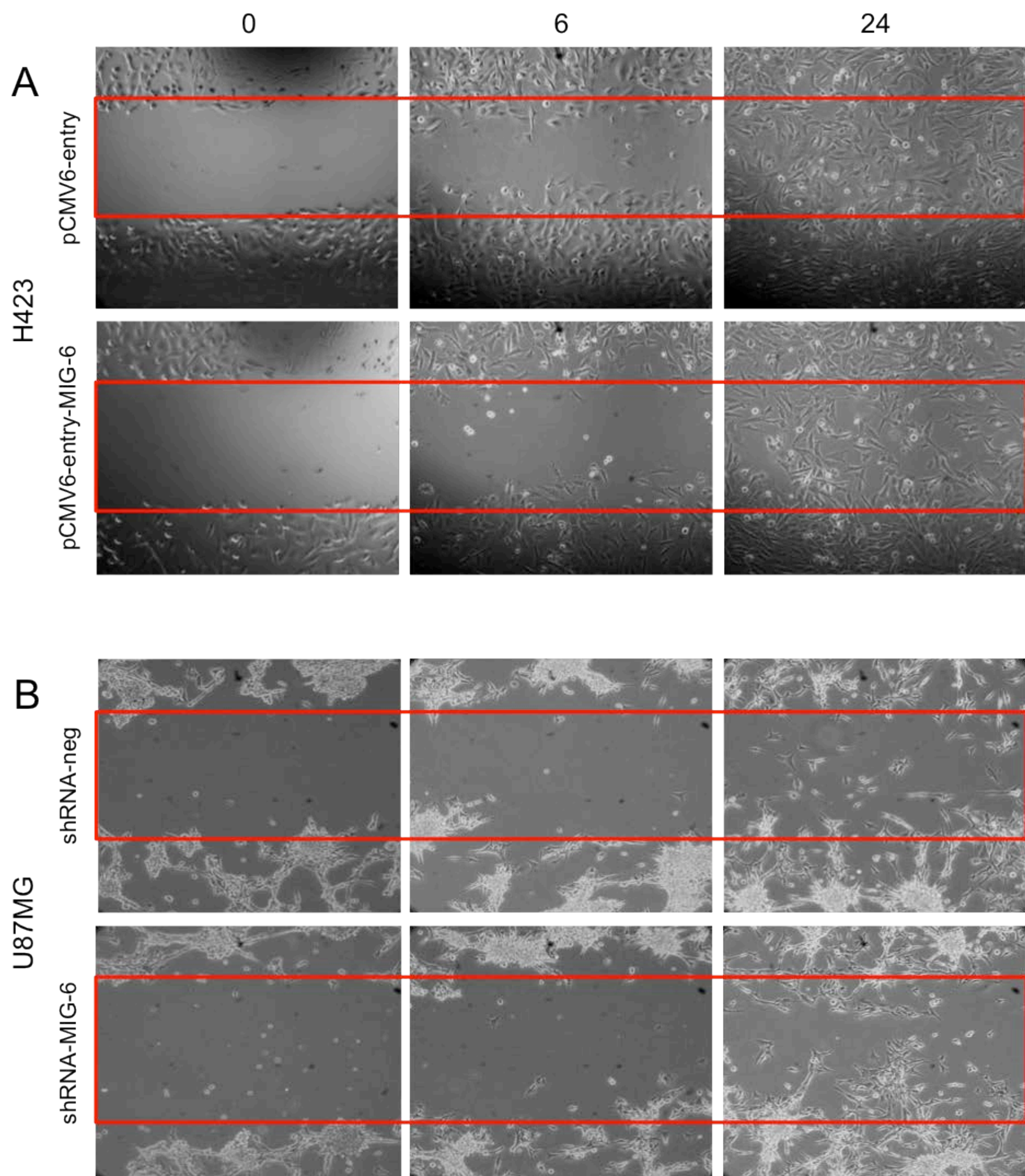


Figure 5.20 *MIG-6* expression alters wound healing in glioma cell lines.
[A] Restoration of *MIG-6* expression in H423 cells inhibited wound healing. Images show wound healing progress of H423 cells transfected with pCMV6-entry or pCMV6-entry-MIG-6 after 0, 6 and 24 hours. [B] Knockdown of *MIG-6* increased wound healing in U87MG cells. Images show wound healing progress of U87MG cells transfected with shRNA-neg or shRNA-MIG-6 at 0, 6 and 24 hours.

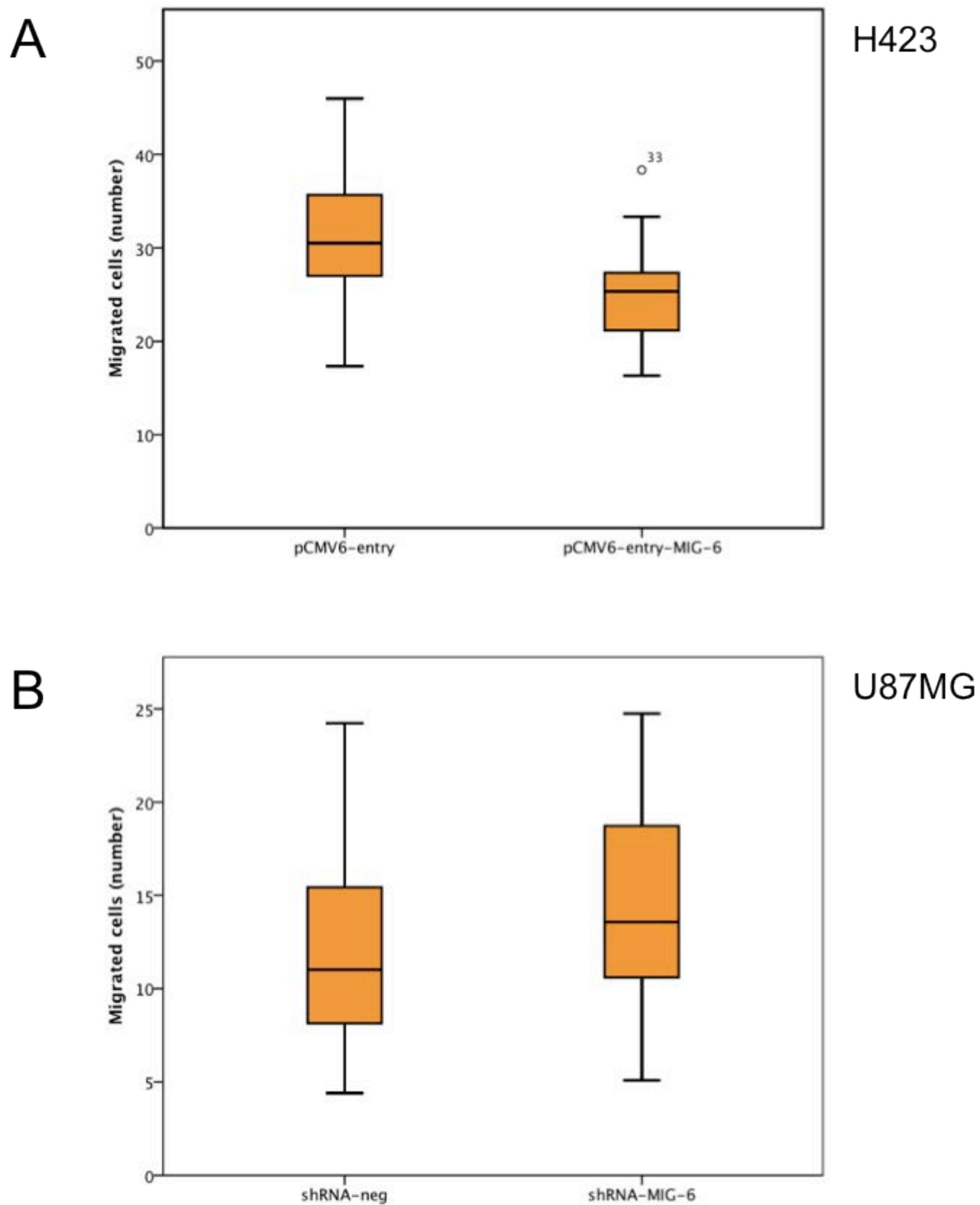


Figure 5.21 *MIG-6* alters cell migration in glioma cells.
[A] Overexpression of *MIG-6* reduced trans-well migration in H423 cells (p=0.001). Results shown are representative of two experiments. [B] Knockdown of *MIG-6* in U87MG cells by shRNA resulted in a trend toward increased trans-well migration (not significant, p=0.285). Results shown are representative of two experiments.

5.4 Discussion

Validation methods used in this chapter were successful in determining candidate genes that showed robust differential expression or methylation in oligodendrogliomas with and without LOH 1p/19q. In the larger cohort of 46 oligodendrogliomas, differential expression of all candidate genes was confirmed by qPCR. The mRNA expression of *CHI3L1*, *IGF2*, *IQGAP1*, *MIG-6*, *PDPN*, *PLAG1* and *PADI2* gene transcripts were significantly higher in oligodendrogliomas without LOH 1p/19q. Only *CHI3L1*, *IQGAP1*, *PDPN* and *PADI2* mRNA transcripts were over-expressed in tumours without LOH 1p/19q when the analysis was restricted to grade II oligodendrogliomas only. These four genes, with the addition of *MIG-6* and *PLAG1* were significantly over-expressed in oligodendrogliomas without LOH 1p/19q when just grade III tumours were examined. Among grade III oligodendrogliomas, high *PDPN* expression was significantly associated with shorter overall survival. Higher CHI3L1 and IQGAP1 protein expression was confirmed by immunohistochemistry in grade III oligodendrogliomas without LOH compared to grade III oligodendrogliomas with LOH 1p/19q. Despite this, none of the genes identified proved to be a robust immunohistochemical marker for survival in grade III oligodendroglioma.

Validation of DNA methylation confirmed *PADI2* as an interesting candidate gene, demonstrating heavy methylation of the entire promoter region and methylation at a single site in over 80% of oligodendrogliomas with LOH 1p/19q. *ALX3* was rejected as a candidate gene, as bisulfite sequencing showed methylation of the gene in both oligodendroglioma and normal brain samples.

The importance of validating microarray findings was demonstrated in the qPCR analysis of the *PLAG1* gene. While *PLAG1* was significantly over-expressed in oligodendrogliomas without LOH 1p/19q compared to oligodendrogliomas with LOH 1p/19q when analysed by microarray, qPCR analysis of gene expression revealed a lower magnitude of differential expression. While qPCR confirmed differential expression of all candidate genes, the rate of validation by IHC at the protein expression level was much lower, validating just two genes as significant discriminators of LOH 1p/19q among grade III oligodendrogliomas. The reasons

for a lower immunohistochemistry validation rate for the genes studied are twofold. First, the sample number was lower than expected as grade II oligodendrogliomas demonstrated negative staining for all antibodies, which led to their exclusion from the sample set. Second, changes in mRNA transcript level are not always translated to observable changes at the protein expression level. Immunohistochemical detection of PLAG1 could not be conducted as all antibodies tested gave negative staining results in oligodendroglioma (grade II and III tumours) and normal brain specimens.

The confirmation that *CHI3L1*, *IQGAP1* and *PDPN* display stronger expression in oligodendrogliomas without LOH 1p/19q is in concordance with findings in glioblastoma, as each of these genes has been associated with increased tumour aggression (Nutt et al., 2005; Pelloski et al., 2005; McDonald et al., 2007; Mishima et al., 2006). These genes have been used as markers of malignancy in glioblastoma, but further testing should be conducted on a larger sample cohort before they are used as immunohistochemical markers in oligodendroglioma.

Validation of *MIG-6* expression by qPCR and functional experiments in glioma cell lines demonstrated that the loss of *MIG-6* is implicated in glioma biology, both at a genetic and a functional level. An EGFR inhibitor and tumour suppressor gene, *MIG-6* is located on 1p36 and may be the elusive tumour suppressor gene involved in early tumourigenesis in oligodendroglioma following LOH 1p/19q.

Compared to mRNA levels in normal brain, oligodendroglioma cell lines displayed low levels of *MIG-6* expression. *MIG-6* expression in grade III oligodendrogliomas with 1p/19q loss was significantly lower than in grade III oligodendrogliomas without the loss of heterozygosity. Although derived from oligodendroglial cells, none of the oligodendroglioma cell lines analysed in this chapter harboured loss of heterozygosity of 1p/19q, so the low expression of MIG-6 must be due to some other factor.

MIG-6 was not expressed in the H423 glioblastoma cell line due to its homozygous deletion of chromosome 1p36 at the *MIG-6* locus, resulting in a complete lack of *MIG-6* expression (Duncan et al., 2010). Similar deletions at 1p36 resulting in homozygous loss of *MIG-6* were observed in a subset of TCGA glioblastoma

samples. A172 cells are known to have loss of heterozygosity on chromosome 1p from 1p35 to the p-terminal and U87MG cells have a small region of loss of heterozygosity at 1p36 (Law et al., 2005), however the expression of MIG-6 in each cell line was above that of normal brain.

For functional analysis of *MIG-6*, a strategy using shRNA was designed to knockdown *MIG-6* expression in the glioma cell line that had the highest endogenous level of *MIG-6* expression – in this case, U87MG. It is possible that the low levels of *MIG-6* present in the oligodendroglioma cell lines HOG, TC620 and MO3.13 may account for some tumour suppressor activity in these cell lines. An shRNA approach was selected over siRNA due to greater stability of shRNA plasmids compared to siRNAs and flexibility, including the option of sorting transfected cells by FACS.

Attempts to fully inhibit *MIG-6* in U87MG cells were to no avail. The mRNA transcript was reduced by just a third following transfection with shRNA plasmids targeted to *MIG-6*. Due to the low level of *MIG-6* knockdown achieved in U87MG cells, an attempt was made to develop a stable U87MG cell line and transfected cells were sorted based on GFP fluorescence by flow cytometry, however the cells died within seven days of sorting. For this reason, functional experiments were instead carried out on transiently transfected U87MG cells. To see a functional consequence, much higher suppression of *MIG-6* is most likely needed. Despite low *MIG-6* knockdown, the experiments were included here to investigate whether the limited knockdown achieved in U87MG cells had any effect on cell proliferation, colony formation or cell migration. A recent report showed that knockdown of *MIG-6* by lentiviral mediated shRNA in U87MG cells resulted in significantly increased cell invasion (Ying et al., 2010). Replication of this experiment here showed a similar trend, but to a less striking degree, most likely as a result of insufficient knockdown of *MIG-6* in U87MG cells. The inverse experiment was successful, as *MIG-6* over-expression resulted in slowed cell migration, as demonstrated in H423 glioblastoma cells. A 2005 study showed that MIG-6 inhibits Hepatocyte Growth Factor and Met induced cell migration (Pantè et al., 2005). The mechanism proposed by Pante et al. was that MIG-6 inhibition of MET signalling led to a reduction in cell migration. Alternatively, Ying et al. demonstrated that

MIG-6 was involved in the recruitment of EGFR to late endosomes for degradation, which caused an overall reduction in malignant potential (Ying et al., 2010). The fact that each study used a different cell model – a mouse derived liver epithelial cell line (MLP29) was used by Pante et al., whereas Ying et al. used human glioblastoma cell lines – indicates that the tissue type may dictate the cellular pathways in which MIG-6 is involved.

It was shown in this chapter that *MIG-6* over-expression or knockdown did not affect cell proliferation, as measured by MTT and colony formation assays. Contrary to these findings, Ying et al. showed that over-expression of MIG-6 dramatically decreased cell proliferation in two GBM cell lines (Ying et al., 2010). The disparity in the findings shown here and those shown by Ying et al could be due to the cell lines selected for experiments. The cell lines used by Ying et al. (LN319 and LN464 glioblastoma cell lines) are different cell lines than were used here (H423 and U87MG glioblastoma cell lines). Although each of the three cell lines are derived from glioblastoma and lack *MIG-6* expression, the EGFR and other cellular pathways may behave differently, as glioblastoma is a highly heterogeneous disease.

In addition, the lack of effect from the over-expression of *MIG-6* may be due to the low transfection efficiency of glioma cells used in this chapter. While significant *MIG-6* expression was induced by transient transfection of *MIG-6* plasmid into H423 cells, it is possible that fewer than 50% of cells over-expressed *MIG-6* due to low transfection efficiency. Transient, rather than stable, *MIG-6* over-expression may limit the duration of *MIG-6* expression so as not to reach the seven days tested by the MTT assay (Figure 5.15) or the 18 days tested by the colony formation assay (Figure 5.18). These issues together likely contributed to the lack of effect on cell proliferation and colony formation observed in the cell lines examined here.

5.4.1 Conclusion

Differential expression of candidate genes *CHI3L1*, *IGF2*, *IQGAP1*, *MIG-6*, *PDPN*, *PLAG1* and *PADI2* was confirmed by qPCR in a larger cohort of oligodendroglioma specimens as over-expressed in tumours without LOH 1p/19q compared to those

with LOH 1p/19q. The genes CHI3L1 and IQGAP1 were confirmed by immunohistochemistry to encode higher protein expression in grade III oligodendrogliomas without LOH compared to grade III oligodendrogliomas with LOH 1p/19q. With the exception of *ALX3*, each of the candidate genes investigated here may have a role in tumourigenesis or therapeutic sensitivity of oligodendroglioma with LOH 1p/19q.

MIG-6 was under-expressed in oligodendrogliomas with LOH 1p/19q. Here we found that MIG-6 had no effect on cell proliferation, but over-expression of MIG-6 reduced cell migration and invasion. A review of the literature reveals that over-expression of *MIG-6* reduced cell proliferation and knockdown of MIG-6 permitted increased cell proliferation, migration and invasion in glioblastoma cells. These findings suggest that *MIG-6* has the potential to be the elusive 1p/19q tumour suppressor gene responsible for the initiation of oligodendroglioma tumourigenesis following LOH 1p/19q. Further studies may help to elucidate its role in the process.

6 Primary culture of oligodendrogliomas

6.1 Introduction

6.1.1 Overview

Human cell lines are vital for cancer research as they provide a model for examining cancer cell function at the physiological and genetic levels, and for developing novel cancer treatments. The culture of human cancer cells has been a core component of cancer research for over 50 years (Zimmerman, 1955; Alberts et al., 2002).

The most commonly used and widely available glioma cell lines are derived from glioblastoma because these tumours proliferate rapidly in cell culture conditions. Cell lines derived from the benign WHO grade II gliomas and malignant WHO grade III oligodendroglioma or astrocytoma are scarce due to lower incidence and slower rate of proliferation *in vitro*. While between 50% and 70% of oligodendrogliomas have LOH 1p/19q, no oligodendroglioma cell lines available for research harbour this deletion and consequently do not exhibit the correct genetic characteristics of the primary tumour. Experiments such as those conducted in chapter five of this thesis would carry greater significance for the biology of oligodendroglioma if they were carried out on a cell line with LOH 1p/19q.

The oligodendroglioma cell lines obtained for use in chapter five were TC620, HOG and MO3.13. The TC620 cell line was established from the primary culture of an oligodendroglioma in 1968 (Manuelidis et al., 1977). The HOG (Human Oligodendroglioma) cell line was established from a surgically removed oligodendroglioma in 1991 (Post and Dawson, 1992). The Human Glial (Oligodendrocytic) Hybrid Cell Line (MO3.13) was created by fusing a 6-thioguanine-resistant mutant of the human rhabdomyosarcoma RD with adult

human oligodendrocytes and is available commercially from Cellutions (Canada). While these three cell lines retain the morphological characteristics of an oligodendroglioma, a significant limitation for this research is that none of them harbour the loss of heterozygosity of 1p/19q (McLendon, R. and Yan, H. pers. comm.). In addition, the literature describes two other cell lines available for purchase – KG-1C and HS683 – as being of oligodendroglial origin.

Oligodendroglioma derived cell lines HOG, MO3.13 and KG-1C were investigated for their oligodendrocytic characteristics (Buntinx et al., 2003). MO3.13 and HOG were shown to be indicative of de-differentiated oligodendrocytes, whereas KG-1C cells could not be induced to grow reliably or proliferate in cell culture. Additionally, the KG-1C cell line was established from an oligodendroglioma excised from a 13-year-old patient, which is not considered to be representative of the adult spectrum of disease (age 18-80) (Miyake, 1979).

The HS683 cell line was reported in 2002 to have LOH 1p/19q, which led to its being classed as an oligodendroglioma cell line (Branle et al., 2002), but more recently it was shown to have no chromosomal losses on 1p or 19q (Law et al., 2005). The ambiguity of its 1p/19q status and the lack of information available about the tumour of origin means that HS683 is unsuitable as a cell line model of oligodendroglioma.

A 2009 report revealed that the *IDH1* and *IDH2* genes are frequently mutated in WHO grade II and III oligodendrogliomas and astrocytomas (Yan et al., 2009). Additionally, *IDH1* and *2* mutations in oligodendrogliomas correlated strongly with LOH 1p/19q. Consequently, all available oligodendroglioma lines have also been tested for the genetic mutation at R132 (*IDH1*) and R172 (*IDH2*), but no available cell line has been found to harbour either of these mutations (Yan, H. Jin, G. Pers. comm.).

6.1.2 Aims

1 – To culture primary oligodendroglioma specimens to generate oligodendroglioma cell lines that harbour LOH 1p/19q.

2 – To culture primary grade II and III astrocytoma, oligodendroglioma and oligoastrocytoma specimens to generate cell lines that harbour a mutation of *IDH1* or *IDH2*.

6.2 Methods

6.2.1 Ethics

Ethics approval was granted for use of brain tumour specimens and patient data for patients at Duke Hospital as described in Chapter 2.3.2.

6.2.2 Dissociation and culture of primary tumours

Primary tumour specimens were transported from the Neurosurgical Operating Suite (Duke Hospital) in transportation media (Table 6.1). Dissociation of primary tumours and handling of primary cells was conducted in a biosafety cabinet. Tumour specimens (100 to 400mg) were placed on a sterile plastic petri dish and dissociated manually with sterile surgical scissors, then transferred to a sterile flask and dissociated enzymatically for 15 minutes with 20ml Liberase Enzyme (100 mg/mL, Roche) in a 37°C water bath. Enzymatic dissociation was stopped by the addition of a one tenth volume of Human Serum Albumin (Albumin Bioscience). Cells were separated from undigested tissue by filtration through a sterile metal sieve, washed twice with DPBS, then resuspended in the desired culture media (ZO media with 10% FCS or stem cell media) and placed in a T25 flask. Primary cell cultures were incubated at 37°C in a humidified incubator with 5% carbon dioxide.

6.2.3 Cell maintenance and recording

Primary cells were maintained in ZO media with 10% FCS, stem cell (SC) media or SD media. SC media is a chemically defined media without FCS that permits cell growth without promoting differentiation (Table 6.2). SD media is a mixture of SC media and DMEM, with 10% FCS, used here to enhance cell growth and promote differentiation (Table 6.3). Cells were observed and photographed at frequent intervals from the time of initial culture of the specimen for up to six months.

Slow growing cell cultures in SC media and SD (1-4% FCS) media were passaged 1 in 2 when 50% confluence was reached (between 4 and 8 weeks). Fast growing cell cultures in SD media (10% FCS) and ZO media were passaged 1 in 8 when 100% confluence was reached (between 5 and 14 days).

Following passage of an SC cell line (1:2), FCS was added to SC or SD media in one of the two flasks at concentrations between 2% and 10% to encourage faster growth in slow growing cell lines.

Table 6.1 Composition of Transportation media

Component	Concentration
MEM/F12 low osmolality w/o L-glutamine	Base media (100%)
Gentamicin	50 mg/L
Ciproflaxin	10 mg/L

MEM/F12 – Modified Eagle's Medium/F12.

Table 6.2 Composition of Stem cell (SC) media

Component	Concentration
MEM/F12 low osmolality w/o L-glutamine	Base Media (100%)
Gluta-max I	1x
Non-essential Amino Acids	1x
B-27 w/o vitamin A supplement	10 mL
Epithelial Growth Factor (EGF)	40 µg/L
Beta-Fibroblast Growth Factor (b-FGF)	50 µg/L
Leukaemia Inhibitory Factor (LIF)	10 µg/L
Heparin	0.2%
Sodium Bicarbonate	5.5%

B-27® without vitamin A supplement (Invitrogen).

Table 6.3 Composition of SD media

Component	Concentration
SC media	45%
DMEM (Gibco)	45%
FCS	10%

DMEM – Dulbecco's Modified Eagle's Medium; FCS – Fetal Calf Serum.

6.2.4 DNA extraction, PCR and sequencing

The Qiagen DNeasy kit was used for the extraction of DNA from cultured cells, blood cells and primary tumour specimens. Details of this method are described in chapter two (sections 2.5.1.2, 2.5.2 and2.5.4).

DNA was used at a concentration of 5 ng/µl for PCR. Sequencing was performed as described in chapter two (section 2.6.1 and 2.6.4).

6.2.5 LOH screening of cell lines

Paraffin embedded tumour specimens were tested for LOH 1p/19q at Duke University Medical Center by FISH (see chapter 2.7.1).

Primary cell cultures were tested for LOH 1p/19q by a PCR based assay. DNA from blood, primary tumour and all cell lines derived from the original tumour was used. Microsatellites D1S552 (1p34), D1S3728 (1p31), D1S1191 (1p11), D1S1625 (1q22), D1S1278 (1q25), D1S547 (1q47), D19S1034 (19p13.2), D19S1165 (19p13.1), D19S1037 (19p12), D19S587 (19q12), D19S1167 (19q13.1) and D19S601 (19q13.3) were analysed for changes in size.

Each forward primer was labelled with a fluorescent primer for ease of detection following PCR amplification (Table 6.4).

Table 6.4 Primer pairs for amplification of microsatellite markers on chromosomes 1 and 19

Human Map Pair	Region	Rxn #	Dye	Forward Primer Sequence (labelled)	Reverse Primer Sequence	Size range (bp)
D1S552	1p34	2	FAM	TTCATGCAGCATCATCCC	TGTGGGCAGGTGTAAAGAGT	244-260
D1S3728	1p31	4	NED	GTAACCTTGCCCAAAACAGA	AAGAGGTTAAGAATAAAGGCTGC	244-268
D1S1191	1p11	4	NED	CTTTTTCAATCACTGCTAGTT	CTTTGTTCACTGTTATACCC	111-135
D1S1625	1q22	4	FAM	AAAAGAACTCCCAAAGCCAC	GGTCTGCCAGTTCACAAATT	177-209
D1S1278	1q25	3	HEX	ACTTTCTCACATGACCACAGG	CAGCGAGACTCTGTCAAAAA	297-313
D1S547	1q47	3	FAM	CTGAAGTGGGAGGATTGCTT	AATTCAGGGGAGTTCCAGAG	282-308
D19S1034	19p13.2	2	NED	AGGCTGTGGTGAGCTATGAC	GTGTCCCTAGCACCTAGCAA	222-242
D19S1165	19p13.1	1	NED	AAGCTATGATGGGTGCCAAT	ATCACTCTTCATTATGGCTTCA	139-161
D19S1037	19p12	1	HEX	CTGCGGAGTCAGAAAACAGT	ATGCAGCTATCCCTCATTCA	110-146
D19S587	19q12	1	FAM	TCCATTGTATATTTTGTAAAACACA	TCTGTCAATGGATGAATGGA	139-156
D19S1167	19q13.1	3	FAM	CTGAGGGAACAGCAAGGTAA	AGAGCAAGACTCTGACTCTATAAAT	340-376
D19S601	19q13.3	2	HEX	CAATGTGAGGCTGGTCTCTT	ATCATATGGCCTTCAGTGGA	191-215

Each DNA sample was used as the template in four PCR reactions, each amplifying three microsatellite markers.

Table 6.5 Components of PCR reactions for detection of LOH 1p/19q

Component	Volume (µL)	Final Concentration
5X PCR Buffer	2	1x
dNTP (10 mM, 2.5 mM each)	0.8	0.8 mM (0.2 mM each)
Marker 1 F (8 µM)	0.25	0.2 µM
Marker 1 R (8 µM)	0.25	0.2 µM
Marker 2 F (8 µM)	0.25	0.2 µM
Marker 2 R (8 µM)	0.25	0.2 µM
Marker 3 F (8 µM)	0.25	0.2 µM
Marker 3 R (8 µM)	0.25	0.2 µM
Water	1.155	
KAPA *Taq* (5 U/µL)	0.045	0.225 U/rxn
DNA (5 ng/µL)	2	5 ng/rxn
Total	**10**	

A touchdown PCR thermocycling program was used to amplify the microsatellite markers (full description in chapter 2.6.1).

6.2.5.1 Visualisation and detection of LOH

ROX HiDi Mastermix was prepared by adding GeneScan® 500 ROX size standard (5 µL) to HiDi formamide (1 mL). PCR products (1 µL) were mixed with ROX HiDi MasterMix (19 µL) in a 96-well PCR plate, centrifuged briefly and denatured at 95°C for 5 minutes. The plate was loaded into the 3100 Genetic Analyzer (Applied Biosystems). The Run Module GeneScan50_POP6DefaultModule and Analysis Module GS500Analysis.gsp were selected before starting the 3100 analyzer. Data were collected automatically during electrophoresis and then analysed with the Gene Scan software (Applied Biosystems). LOH was determined when one of the two peaks in the tumour or cell line sample was reduced by more than 50% compared to the corresponding peak in the blood sample.

6.3 Results

6.3.1 Primary tumour specimens

Primary tumour specimens (n=46) were obtained fresh from surgery for primary culture. The following primary tumour specimens were cultured: 10 grade II oligodendrogliomas, 8 grade III oligodendrogliomas, 4 grade II oligoastrocytomas, 3 grade III oligoastrocytomas, 5 grade II astrocytomas, 6 grade III astrocytomas and 3 glioblastomas, 3 pilocytic astrocytomas, 2 ependymomas, 1 central neurocytoma and 1 craniopharyngioma.

Tumour specimens were collected at the time of surgery and cultured within 24 hours. As the confirmed diagnosis of the patient was available 7 days after surgery, several undesired tumour types were cultured initially. Four undesired primary cell cultures (two ependymomas, one craniopharyngioma and one neurocytoma) were discarded after the diagnosis was made available.

6.3.2 Primary cell culture of tumour specimens

The culture of 35 primary tumour specimens resulted in at least one viable cell line. 22 primary tumour specimens were successfully cultured in more than one type of culture medium. Details of the specimens cultured and the resulting cell lines are summarised in Table 6.6.

Primary cells cultured in SC media grew slowly (4 to 8 weeks between passages) and senesced quickly (median passage number at senescence was 3). Cells cultured in SD media grew more quickly (5 to 14 days between passages) and did not senesce as early as those in SC media (median passage number at senescence was 11). Primary cells in ZO media also grew rapidly (7 to 14 days between passages) and senesced later than the SC cell cultures, but earlier than SD cell lines (median passage number at senescence was 9).

SC cell cultures displayed two morphological types, either neurospheres floating in the culture media, or fibroblast-like cells adherent to the culture surface. SD and

ZO cell cultures most often displayed fibroblast-like morphology, appearing as elongated cells adherent to the culture media with few extensions or processes. Occasionally, an SD cell culture displayed more complex morphology, as large adherent cells with elongated extensions (Figure 6.1).

Table 6.6 Biorepository number, diagnosis and cell lines established form primary culture of tumour specimens

Tumour Biorepository number	Diagnosis	Cell lines - passage number at senescence		
		SC	SD	ZO
09-0096	Grade III astrocytoma	8*	22*	17*
09-0114	Grade II astrocytoma	-	-	7*
09-0174	Grade II oligoastrocytoma	-	-	4*
09-0225	Grade II oligoastrocytoma	-	-	15
09-0230 **	Grade III gemistocytic astrocytoma	-	-	-
09-0241	Pilocytic astrocytoma	-	-	13
09-0261	Grade II oligodendroglioma	2	2	6*
09-0281 **	Grade II oligodendroglioma	-	-	-
09-0390	Grade II oligodendroglioma	-	-	4*
09-0405	Grade II astrocytoma	5	-	8
09-0437	Grade III astrocytoma	4	-	4*
09-0442	Grade II astrocytoma	6*	-	7*
09-0463	Grade II oligodendroglioma	-	-	4*
09-0466	Grade II oligodendroglioma	-	-	3*
09-0468	Grade II astrocytoma	3	-	-
09-0475 **	Grade III astrocytoma	-	-	-
09-0481	Grade III oligodendroglioma	-	-	4*
09-0482	Grade II oligodendroglioma	2	8	3*
09-0516 ***	Central neurocytoma	-	-	-
09-0519 ***	Ependymoma (grade II)	-	-	-
09-0520	Grade II oligodendroglioma	5	11*	-
09-0533	Gemistocytic astrocytoma (grade II)	-	-	3*
09-0601	Grade III astrocytoma	3	3	4*
09-0603 **	Grade III oligodendroglioma	-	-	-
09-0607	Pilocytic astrocytoma	4	-	5*
09-0609 **	Grade II oligoastrocytoma	-	-	-
09-0630	Grade III oligodendroglioma	6	-	4*
09-0632	Grade III oligodendroglioma	3	-	5*
09-0637	Pilocytic astrocytoma	2	9*	6
09-0644	Grade II astrocytoma	4	6*	4*
09-0645	Grade II oligoastrocytoma	3	11	7*
09-0652	Grade III oligoastrocytoma	4	5*	9
09-0653 ***	Craniopharyngioma	-	-	-
09-0655	Grade III oligodendroglioma	3	4*	4*
10-0015	Glioblastoma	-	-	2*
10-0041 **	Glioblastoma	-	-	-
10-0045	Grade III oligodendroglioma	-	-	4
10-0055	Glioblastoma	8*	-	-
10-0059	Grade II oligodendroglioma	3	-	3*
10-0061	Grade III oligoastrocytoma	5	-	3*
10-0101 **	Grade II oligodendroglioma	-	-	-
10-0102 ***	Grade III clear cell ependymoma	-	-	-
10-0104	Grade III oligodendroglioma	2	-	3*
10-0131	Grade III astrocytoma	3	-	3*
10-0215	Grade II oligodendroglioma	3*	7*	-
10-0251	Grade III oligoastrocytoma	2	3*	3*

SC – Cell line in SC Media; SD – cell line in SD media (10%) FCS; ZO – cell line in ZO media; Passage number – the number of times a cell line was passaged before senescence; * - highest passage reached, cell line did not undergo senescence; ** - no cell line was established; *** - cultured cells were discarded due to unwanted diagnosis.

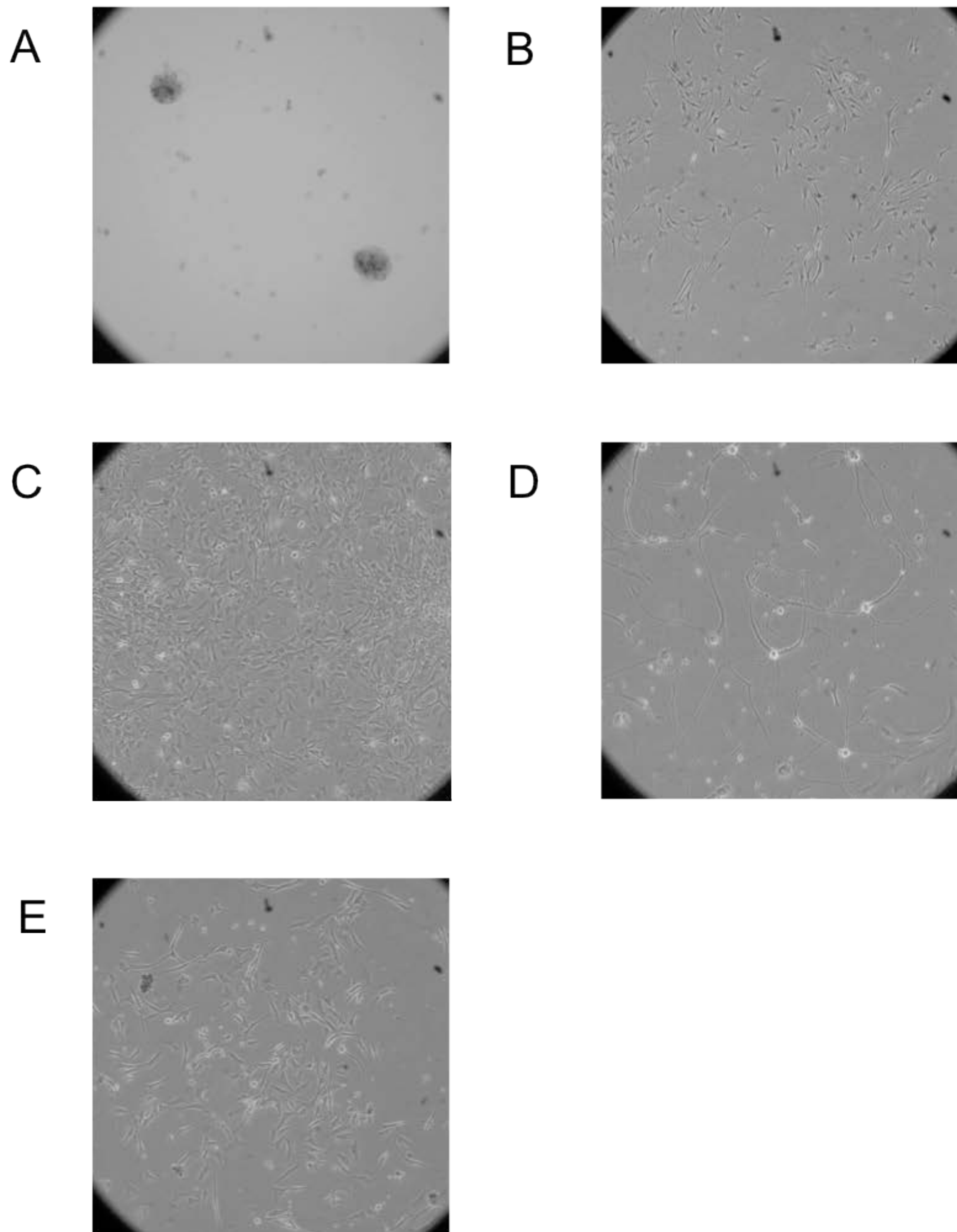


Figure 6.1 Morphologies observed in SC, SD and ZO primary cell cultures.
SC cell cultures as [A] neurospheres (TB09-0644 SC) or [B] fibroblast-like cells (TB09-0520 SC). SD (10% FCS) cell cultures as [C] fibroblast-like cells (TB09-0520 SD) or [D] complex cells (TB09-0652 SD). [E] ZO cells as fibroblast-like cells (TB10-0059 ZO).

6.3.3 *IDH1* and *TP53* mutations in primary tumours and cell cultures

21 primary tumours (4 x OII, 5 x OIII, 1 x OAII, 3 x OAIII, 2 x AII, 3 x AIII, 1 x GBM, 2 x AI) were analysed for mutations of *IDH1* and *IDH2*. 16 primary tumours displayed the R132H *IDH1* mutation (3 x OII, 4 x OIII, 1 x OAII, 2 x OAIII, 2 x AII, 3 x AIII, 1 x AI), while the remaining 5 primary tumours had wild type *IDH1* (1 x OII, 1 x OIII, 1 x OAIII, 1 x GBM, 1 x AI) (Table 6.7). None of the 21 tumours had an *IDH2* mutation.

Of the 16 primary tumours that had an *IDH1* R132H mutation, 13 formed a cell line when cultured in SC media. Of these primary cell lines, 9 maintained the *IDH1* mutation and 4 had wild type *IDH1*. Of the 9 primary cultures in SC media, just one primary cell culture, TB09-0096 (grade III astrocytoma) maintained the *IDH1* mutation when subcultured in SD (10% FCS) media. No cell line retained the *IDH1* mutation when cultured in ZO media.

Photographs were available for 10 of the 13 SC cell lines that formed from *IDH1* mutated tumours. Of the 7 photographed SC cell lines that maintained the *IDH1* mutation from the primary tumour, all formed neurospheres in cell culture, which attached to the culture surface in two cases after at least 12 weeks in culture. Each of the three photographed SC cell lines that did not maintain the *IDH1* mutation adhered to the culture surface and displayed fibroblast-like morphology (Figure 6.2).

Seven out of 16 primary tumour specimens had nonsense or missense mutations of the *TP53* gene, including one oligodendroglioma (OIII), three oligoastrocytomas (1 x OAII, 2 x OAIII) and three astrocytomas (1 x AII, 2 x AIII). Additionally, one grade II oligodendroglioma was found to have a silent mutation. Primary cells for 6 out of 7 *TP53* mutated primary tumours grew when cultured in SC media and the mutation was conserved in 5 of these cell lines. The only SC cell line that did not maintain the *TP53* mutation from the original tumour sample was derived from a grade III oligodendroglioma (TB09-0630). Four of the 7 *TP53* mutated tumours formed cell lines in SD media, but the mutation was only conserved in one sample, a grade III astrocytoma (TB09-0096). All 7 primary tumours with *TP53* mutations

formed a cell line when cultured in ZO media, but no *TP53* mutation was retained in the ZO cell line. Full details of the cell lines and mutations are listed in Table 6.7.

Table 6.7 Summary of *IDH1* and *TP53* mutations in primary tumours and primary cell cultures

TB #	Diagnosis	***IDH1* Tumour**	***IDH1* SC**	***IDH1* SD**	***IDH1* ZO**	***TP53* Tumour**	***TP53* SC**	***TP53* SD**	***TP53* ZO**
09-0096	AIII	R132H	R132H	R132H	WT	G245V	G235V	G245V	WT
09-0468	AII	R132H	R132H						
09-0482	OII	R132H	R132H	WT					
09-0520	OII	R132H	WT	WT		nt639 A$\rightarrow$AG	nt639 A$\rightarrow$AG	nt639 A$\rightarrow$AG	
09-0601	AIII	R132H	R132H	WT	WT	WT	WT	WT	WT
09-0607	AI	R132H	WT		WT				
09-0630	OIII	R132H	WT		WT	R273C	WT		WT
09-0632	OIII	WT	WT		WT	WT	WT		WT
09-0637	AI	WT		WT	WT	WT		WT	WT
09-0644	AII	R132H	R132H	WT	WT	G244N	G244N	WT	WT
09-0645	OAII	R132H		WT	WT	Y220C		WT	WT
09-0652	OAIII	R132H	R132H	WT	WT	H179R	H179R	WT	WT
09-0655	OIII	R132H	R132H	WT	WT	WT	WT	WT	WT
10-0045	OIII	R132H			WT	WT			WT
10-0055	GBM	WT	WT			WT	WT		
10-0059	OII	R132H	WT		WT	WT	WT		WT
10-0061	OAIII	R132H	R132H		WT	R273C	R273C		WT
10-0104	OIII	R132H			WT	WT			WT
10-0131	AIII	R132H	R132H		WT	Y220C	Y220C		WT
10-0215	OII	WT		WT		WT		WT	
10-0251	OAIII	WT	WT	WT	WT	WT	WT	WT	WT

TB – Tumour Biorepository; AI – pilocytic astrocytoma (WHO grade I); AII – grade II astrocytoma; AIII – grade III astrocytoma; GBM – glioblastoma (WHO grade IV); OII – grade II oligodendroglioma; OIII – grade III oligodendroglioma; OAII – grade II oligoastrocytoma; OAIII – grade III oligoastrocytoma; WT – Wild Type.

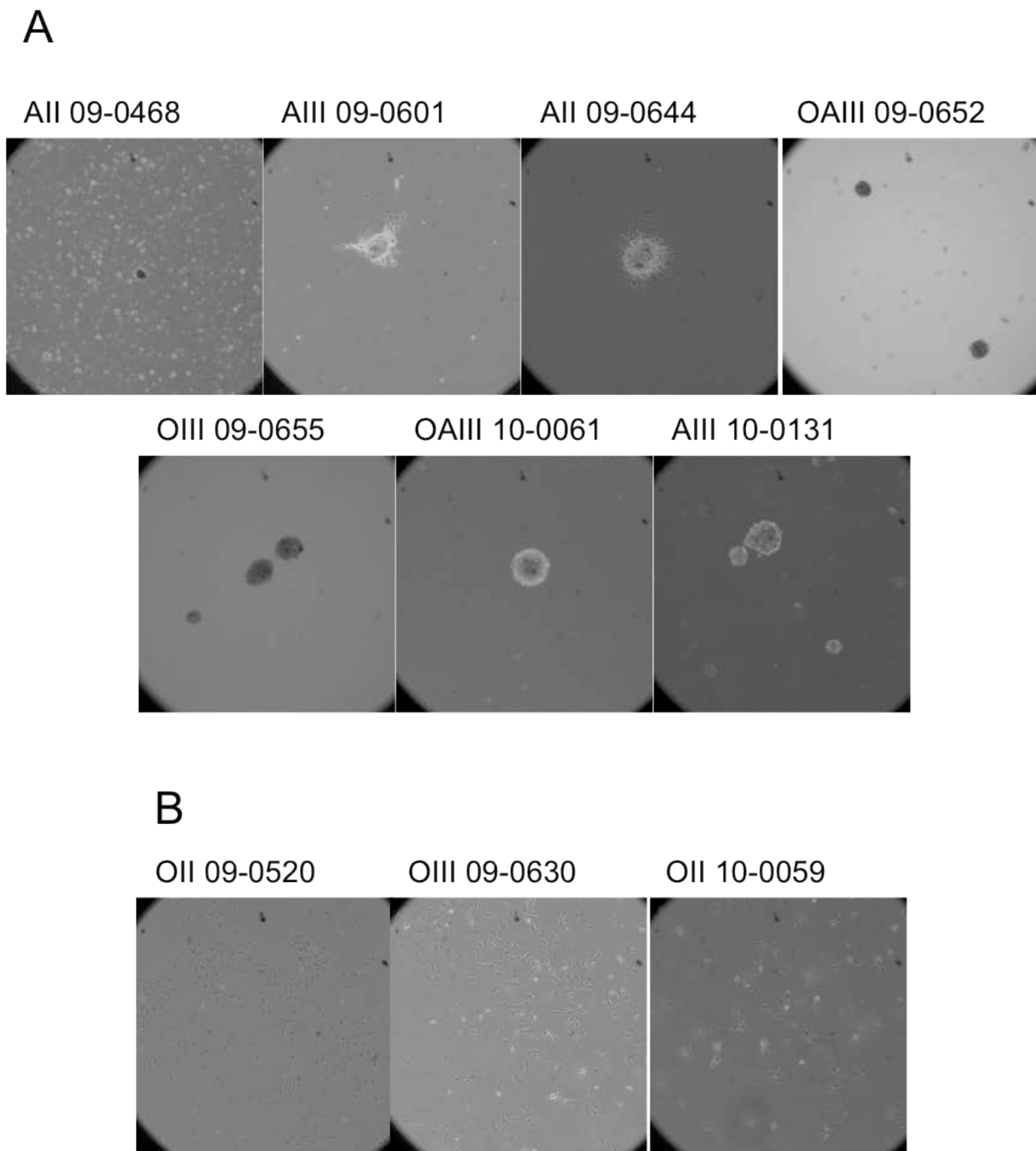


Figure 6.2 Morphology of SC cell lines derived from tumours harbouring an *IDH1* mutation.
[A] Seven lines maintaining the *IDH1* mutation: AII 09-0468, AIII 09-0601, AII 09-0644, OAIII 09-0652, OIII 09-0655, OAIII 10-0061, AIII 10-0131. [B] Three lines not maintaining the *IDH1* mutation: OII 09-0520, OIII 09-0630, OII 10-0059.

6.3.4 LOH 1p/19q in primary tumours and cell cultures

23 primary tumours (9 x OII, 7 x OIII, 4 x OAII, 3 x OAIII) were tested for LOH 1p/19q by FISH at Duke University Medical Center (see chapter 2.7.1). 13 tumours (8 x OII, 4 x OIII, OAIII) showed LOH for both 1p and 19q, four tumours had loss of 1p only (OII, OIII, 2 x OAIII), four tumours had loss of 19q only (4 x OAII), one oligodendroglioma (OIII) had no loss and one oligoastrocytoma (OAIII) showed polysomy for all chromosomes, including 1p and 19q (Table 6.8).

Seven primary tumours (2 x OII, 2 x OIII, OAII, 2 x OAIII) that showed LOH 1p/19q or loss of either 1p or 19q were successfully cultured to produce primary cell lines in SC, SD or ZO media. All primary cell lines (n=17) derived from these seven tumours were tested for LOH 1p/19q by PCR of microsatellite markers. No 1p or 19q chromosomal losses were detected in any of the 17 cell lines derived from the seven primary tumours, indicating that LOH 1p/19q was not maintained in any primary cell line (Table 6.8).

Table 6.8 LOH 1p/19q in primary oligodendroglial tumours and cell lines

TB #	Diagnosis	Tumour 1p36	Tumour 19q13	SC 1p36	SC 19q13	SD 1p36	SD 19q13	ZO 1p36	ZO 19q13
09-0174	OAII	No loss	LOH						
09-0225	OAII	No loss	LOH						
09-0261	OII	LOH	LOH						
09-0281	OII	LOH	LOH						
09-0390	OII	LOH	No loss						
09-0463	OII	LOH	LOH						
09-0466	OII	LOH	LOH						
09-0481	OIII	LOH	LOH						
09-0482	OII	LOH	LOH			No loss	No loss	No loss	No loss
09-0520	OII	LOH	LOH	No loss	No loss	No loss	No loss		
09-0603	OIII	LOH	LOH						
09-0609	OAII	No loss	LOH						
09-0630	OIII	LOH	No loss	No loss	No loss			No loss	No loss
09-0632	OIII	No loss	No loss						
09-0645	OAII	No loss	LOH			No loss	No loss	No loss	No loss
09-0652	OAIII	LOH	No loss	No loss	No loss	No loss	No loss	No loss	No loss
09-0655	OIII	LOH	LOH	No loss	No loss	No loss	No loss	No loss	No loss
10-0045	OAIII	LOH	LOH						
10-0061	OAIII	Polysomy	Polysomy						
10-0101	OII	LOH	LOH						
10-0104	OIII	LOH	LOH						
10-0215	OII	LOH	LOH						
10-0251	OAIII	LOH	No loss	No loss	No loss	No loss	No loss	No loss	No loss

OII – grade II oligodendroglioma; OIII – grade III oligodendroglioma; OAII – grade II oligoastrocytoma; OAIII – grade III oligoastrocytoma; SC – cell line grown in SC media; SD – cell line grown in SD media; ZO – cell line grown in ZO media.

6.3.5 Primary cell culture of a grade III oligodendroglioma (TB09-0655) – Detailed notes on a representative specimen and its derivative primary cell cultures

Grade III oligodendroglioma sample TB09-0655 was cultured in SC, SD and ZO media and first passaged 4 weeks after initial culture. Cells were photographed after 14 days of cell growth following the first passage. TB09-0655 cells in SC media grew as neurospheres. Cells in SD (10% FCS) displayed a variety of morphologies, including neurospheres, cell clumps adhered to the culture flask, adherent epithelial-like-cells with one to six processes. Cells in ZO media were observed as adherent, rapidly dividing, elongated cells with no processes (Figure 6.3).

The primary tumour and cells cultured in SC media each had an R132H mutation of *IDH1*, which was not maintained in SD or ZO cell cultures. LOH 1p/19q was detected in the primary tumour but not in any of the resulting cell lines.

TB09-0655 cells in SC media ceased to divide following the third passage, twelve weeks after initial culture. To induce growth, one third of the cells were transferred to a new flask containing SD (2% FCS). Following subculture of TB09-0655 cells in SD (2% FCS) media, a change in morphology was observed from floating neurospheres to adherent clumps of cells. A small number of cells appeared to spread out slightly from the cell clump, but no increase in size or number of cell clumps was observed (Figure 6.4).

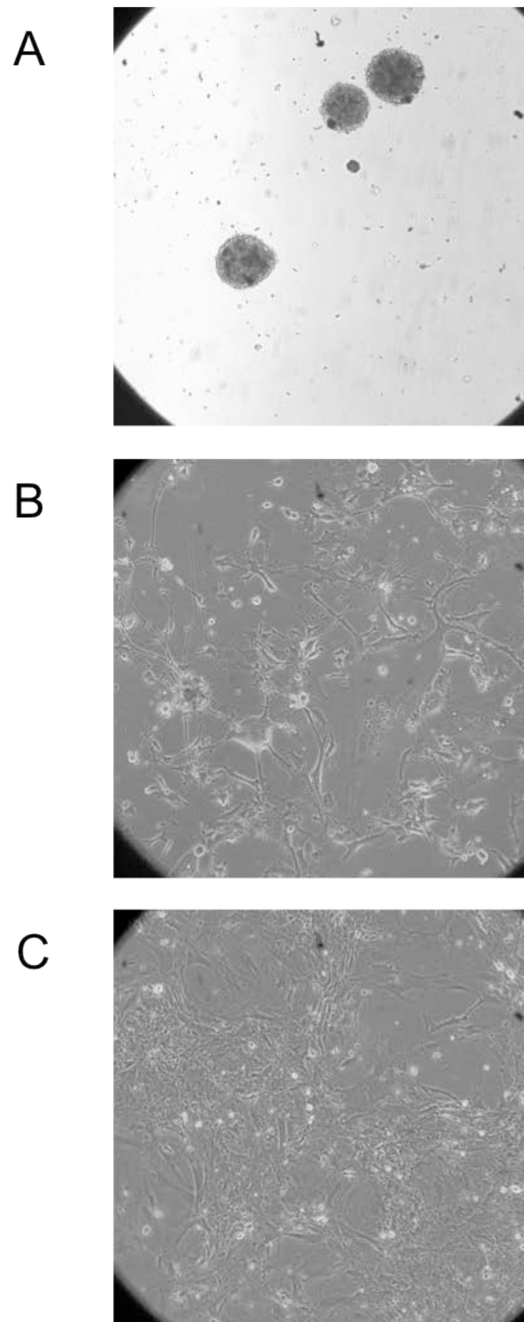


Figure 6.3 Morphology of grade III oligodendroglioma (TB09-0655) cells under different culture conditions.
Specimen TB09-0655 was cultured for six weeks in [A] SC media (no FCS), [B] SD media (10% FCS) and [C] ZO media (10% FCS).

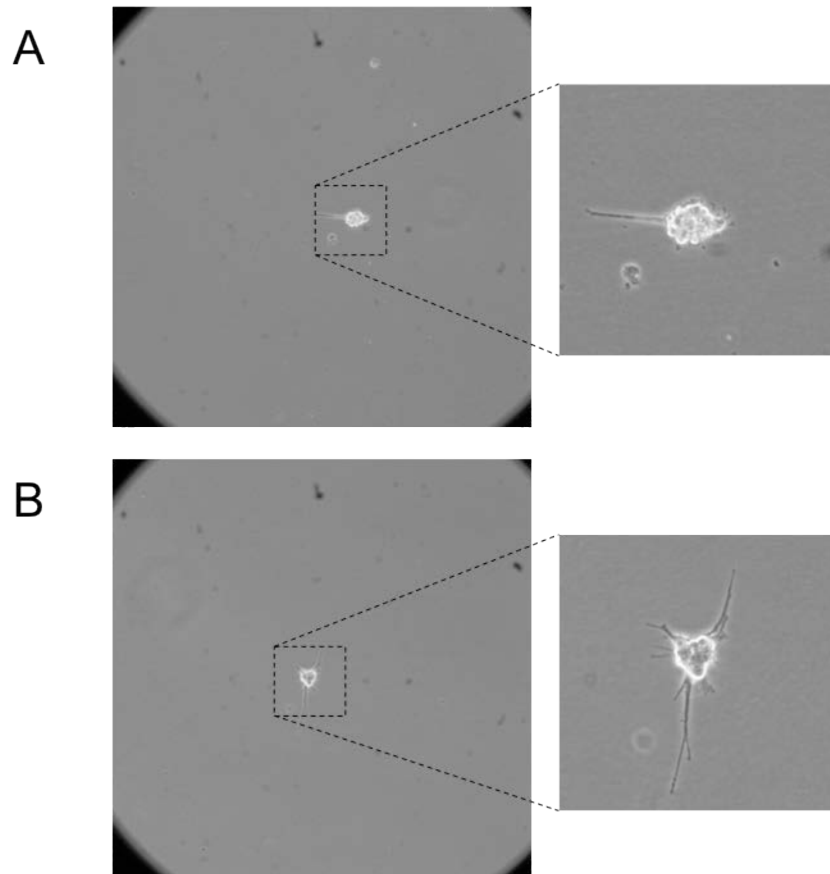


Figure 6.4 Subculture of grade III oligodendroglioma (TB09-0655) cells from SC to SD (2% FCS) media.
Representative images of cells after [A] 7 days and [B] 14 days.

6.4 Discussion

6.4.1 Morphology and genetics of primary cell cultures

The type of cell media used had significant effects on the rate of cell growth, morphology and genetics of the cultured cells. Primary tumour cells cultured in the chemically defined SC media were slow growing, more likely to maintain *IDH1* and/or *TP53* mutations observed in the primary tumour sample but more likely to senesce after only a few passages. Cells that were cultured in SD or ZO media were more likely to multiply rapidly and display fibroblast-like morphology. In all but one case, cells cultured in SD or ZO media did not maintain the *IDH1* or *TP53* mutations observed in the primary tumour.

The characteristics observed in the SC cell lines, such as neurosphere formation and the short time to senescence, are those shared by cultures of neural stem cells, suggesting the primary cell cultures may contain glioma stem cells.

The loss of genetic characteristics of the primary tumour in cells cultured in SD and ZO media (both 10% FCS) could be due to exposure to fetal calf serum. In the primary tumour, the *IDH1* and *TP53* mutations are present in the majority of cells. The minority of cells without mutations, including stromal cells, may have a growth advantage in SD and ZO culture conditions, causing them to differentiate and multiply rapidly. *IDH1* mutations (and in some cases *TP53* mutations) are associated with improved patient survival (van den Bent et al., 2010; Yan et al., 2009; Ino et al., 2001), which may indicate that cells bearing these mutations have a slow growth rate, making them unsuitable for traditional cell culture.

The loss of *IDH1* mutations in primary cell culture is discussed by Piaskowski et al, who note that *IDH1* mutations are present in early passage (2 to 3, up to 96 hours after initial culture) cell lines derived from grade II and III astrocytomas, but are not present in later passages (8 to 10) of these same cell lines (Piaskowski et al., 2011). In the current study, DNA was extracted after cells had been cultured for a minimum of two weeks. Hence it is possible that cells from *IDH1* mutated tumours cultured in SD and ZO media may have retained the *IDH1* mutation for several

days, before losing the marker due to differentiation or preferential growth of un-mutated cells.

LOH status was not maintained in any of the cell lines cultured from primary tumours with LOH of 1p, 19q or both. This result was not surprising, as no available oligodendroglioma cell line has LOH 1p/19q and until 2010 such a cell line had not been described in the literature. While undertaking experiments for this chapter, a study was published describing two oligodendrogliomas that were cultured under stringent conditions to form primary cell lines that maintained LOH 1p/19q, as identified by derivative chromosome t(1;19)(q10;p10) (Kelly et al., 2010). One of these cell lines had a mutation at R132 in the *IDH1* gene and both cell lines had extremely slow growth rates. This was the first report of any sustainable oligodendroglioma cell line that harboured either LOH 1p/19q or an IDH mutation. These two cell lines will be extremely valuable in the investigation of oligodendroglioma biology with respect to LOH 1p/19q.

Glioblastoma cell lines A172 and U87MG have been reported to each carry losses on 1p and 19q (Law et al., 2005). While the cell lines are derived from glioblastoma, plasticity of cell lines in culture and over the passage of time may result in such chromosomal losses. The losses on 1p and 19q in each of these cell lines were not total arm deletions, as most often observed in pure oligodendrogliomas.

6.4.2 Known difficulties in culture of oligodendroglioma cell lines

In this study, almost all SC cell lines and many SD and ZO cell lines either died or senesced at a relatively early passage number. That so few oligodendroglioma cell lines are commercially available is evidence of the difficulty met when attempting to propagate this type of tumour in cell culture.

The cell microenvironment is essential for a cell to display correct markers for its tissue of origin. The differentiation state of oligodendroglioma cells is highly plastic as demonstrated by Tenenbaum et al. who showed that cultured oligodendroglioma cells can express both oligodendrocytic markers (e.g. O1) and astrocytic markers (e.g. GFAP, A2B5), depending on the composition of the culture

media (Tenenbaum et al., 1996). The tumour microenvironment must be mimicked closely in order to study cell behaviour and biology of tumour cells cultured *in vitro*. It is evident that tumour microenvironment conditions were not replicated well enough by standard cell culture techniques as described in this chapter.

6.4.3 Alternative techniques for primary cell culture

The growing surface in flasks used in this chapter was a hydrophilic, negatively charged surface for optimal cell attachment (Corning), which presents a fundamental change from the 3-dimensional tumour microenvironment, and forces cells to attach differently. This causes changed gene expression especially for genes involved in cell polarity, adhesion, attachment, invasion and cell-to-cell interaction. Gene expression in traditionally cultured (2-dimensional) cell lines differs from their tissue of origin by approximately 30% (Sandberg and Ernberg, 2005). The provision of a 3-dimensional substrate for cell growth is likely to induce less of a change, as the artificial extracellular matrix permits cells to attach normally and maintain polarity (Birgersdotter et al., 2005). The use of a 3-dimensional substrate should be considered in future attempts to culture primary glioma specimens.

Alternatively, an ultra-low attachment surface could be utilised to prevent attachment-mediated differentiation and promote growth of cells as neurospheres (Corning Incorporated, 2011).

A better approach to propagate oligodendroglioma cells maintaining LOH 1p/19q and/or *IDH1* mutations may be to implant the tumour cells into the head or flank of a mouse as a xenograft. The *in vivo* environment is a closer match to the tumour microenvironment than traditional cell culture conditions and may better permit growth of tumour cells without inducing differentiation. In the field of stem cell research, the *in vivo* culture of primary cells is preferred over *in vitro* methods, as the cells better retain characteristics of the primary cell (O'Brien et al., 2010).

Together, the findings detailed in this chapter and the recent literature suggest the standard practices of basic 2-dimensional cell culture are not sufficient to culture

primary oligodendroglioma specimens in order for the resulting cell lines to retain the genetic and morphological characteristics of their tissue of origin.

6.4.4 Conclusion

I was successful in culturing a number of oligodendroglioma and astrocytoma cell lines that maintained *IDH1* and/or *TP53* mutations, yet unsuccessful in culturing an oligodendroglioma cell line that maintained LOH 1p/19q. All oligodendroglioma cell lines with the *IDH1* mutation senesced early and would therefore be unsuitable for experiments such as those described in chapter five. The results presented here suggest that optimisation of *in vitro* culture conditions is imperative to the success of primary cell culture and, on review of the literature, that *in vivo* culture may offer better conditions for cells to maintain genetic elements of the primary tumour.

7 Discussion

This thesis pursued an investigation into the occurrence of LOH 1p/19q, its implications for gene expression and survival in oligodendroglioma patients and identified differentially expressed candidate genes that may influence the biology of oligodendroglioma. I first conducted a clinical review of oligodendroglioma cases at a single hospital site (RNSH) to determine the frequency of LOH 1p/19q in grade II and III tumours and the association of LOH 1p/19q with survival and other factors. Seventeen of these oligodendroglioma specimens were analysed by exon microarrays to identify genes that were differentially expressed between tumour groups separated by LOH 1p/19q status. The expression of six differentially expressed and two methylated candidate genes were further validated by qPCR and immunohistochemistry. Investigation of MIG-6 function in glioma cell lines confirmed its potential as a tumour suppressor gene in glioma. Efforts to culture primary low grade glioma specimens in tissue culture were not successful in developing an oligodendroglioma cell line that maintained LOH 1p/19q. In this chapter I will discuss the findings of this thesis in the context of the literature, implications on clinical decision-making, limitations of this research and future directions for the study of LOH 1p/19q in oligodendroglioma.

7.1 Key findings

The clinical review in chapter three of this thesis confirmed that the presence of LOH 1p/19q is a positive prognostic indicator in grade III oligodendroglioma patients in the RNSH cohort. Grade III oligodendroglioma patients without LOH 1p/19q had a median survival time of just 1.2 years (14.4 months). This was significantly shorter than was reported in similar patients in two large clinical trial patient cohorts, which demonstrated median survival between 21.4 and 33.6 months (Cairncross et al., 2006; van den Bent et al., 2006). Survival among our

cohort of grade III oligodendroglioma patients without LOH 1p/19q was actually closer to that observed in glioblastoma patients (12 to 15 months) (Wen and Kesari, 2008). This finding may indicate that our sample group was not typical of the oligodendroglioma population, but it should also influence local treatment decisions.

The proportion of oligodendroglioma patients with LOH 1p/19q was 52.2% and 44.1% in this current cohort for grade II and III patients respectively, each within the range reported by other studies (Cairncross et al., 2006; van den Bent et al., 2006; Kim et al., 2010). Among grade III oligodendrogliomas, the average age of patients with LOH 1p/19q was younger than those without LOH 1p/19q.

Strong TP53 staining in grade III oligodendrogliomas without LOH 1p/19q demonstrated a non-significant trend towards longer survival. This finding was supported by the literature, as among grade III oligodendroglioma patients without LOH 1p/19q, the presence of a *TP53* mutation was associated with substantially longer overall survival (Ino et al., 2001).

Gene expression was profiled in 17 oligodendroglioma, 4 normal brain and 8 glioblastoma specimens by exon microarrays. 408 genes were differentially expressed between oligodendrogliomas separated by LOH 1p/19q status. Among the genes that were expressed more highly in oligodendrogliomas with LOH 1p/19q were many genes related to biogenesis. Genes under-expressed in oligodendrogliomas with LOH 1p/19q were likely to be involved in cell motility, wound healing and inflammatory response.

A similarity in gene expression profiles between grade III oligodendrogliomas without LOH 1p/19q and glioblastoma was identified. Analysis of gene lists generated by the microarray study revealed that the proneural glioblastoma phenotype was enriched in oligodendrogliomas with LOH 1p/19q. This phenotype is related to longer survival in glioblastoma patients, a phenomenon that is mirrored within the two groups of oligodendroglioma.

Analysis of CpG island methylation in seven oligodendroglioma and four normal brain specimens by the 385K promoter tiling array identified 13 genes on chromosome 1p and 7 genes on chromosome 19q that were methylated in

oligodendrogliomas with LOH 1p/19q but not in normal brain. *PADI2* but not *ALX3* was confirmed by bisulfite sequencing as a methylated gene of interest in oligodendrogliomas with LOH 1p/19q. Additionally, no gene identified by previous studies as methylated in oligodendrogliomas was confirmed by the 385K array. The low rate of target validation indicates that the methylation array used here has potential to be better developed for the identification and confirmation of methylation of candidate genes.

The list of 408 differentially expressed genes was narrowed down to six candidate genes that had been reported as genes of interest in other cancers. Validation of candidate genes *CHI3L1*, *IGF2*, *IQGAP1*, *MIG-6*, *PDPN* and *PLAG1* by qPCR confirmed their differential expression in a cohort of 46 oligodendrogliomas separated by LOH 1p/19q status.

Validation of the candidate genes by immunohistochemistry had limited success. No suitable antibody was found to examine PLAG1 protein expression so it could not be analysed by immunohistochemistry. CHI3L1, IGF2, IQGAP1, MIG-6 and PDPN were examined by immunohistochemistry in a cohort of 24 to 29 grade III oligodendroglioma specimens. Positive staining for CHI3L1 and IQGAP1 protein expression was more frequent in oligodendrogliomas without LOH 1p/19q, but no candidate gene proved to be a robust immunohistochemical marker of overall survival

Functional analysis of *MIG-6* was conducted in H423 glioblastoma cell lines and showed that by restoring *MIG-6* expression, cell migration but not proliferation was inhibited. A 2010 study of *MIG-6* function in glioblastoma reported similar findings for cell migration but showed that reconstitution of *MIG-6* expression reduced cell proliferation in LN319 and LN464 glioblastoma cell lines. Together these findings support the assertion that MIG-6 functions as a tumour suppressor gene in gliomas. It follows then that MIG-6 has the potential to be a factor in tumourigenesis of oligodendroglioma following LOH 1p/19q.

The study of MIG-6 function in oligodendroglioma was impeded, as no oligodendroglioma cell line with LOH 1p/19q is available for research. Such a

model cell line would be required to accurately study the impact of specific genetic elements on the biology of oligodendroglioma.

Primary culture of low grade gliomas in chapter six demonstrated the difficulty inherent in the development of an *in vitro* model of oligodendroglioma with LOH 1p/19q. Slow growth rate and loss of defining genetic characteristics were the two main obstacles faced throughout my attempts to develop such a cell line. A literature search revealed just one study that has successfully propagated two oligodendroglioma specimens into cell lines that maintained a genuine LOH 1p/19q rearrangement (Kelly et al., 2010).

Interestingly, mutations of the *IDH1* gene, which are prevalent in low grade gliomas, were maintained in nine primary cell lines, while LOH 1p/19q was not maintained in any cell lines. This finding suggests that these two events are likely to be regulated by different mechanisms and may have different roles in the biology of oligodendroglioma despite the association of both events with longer patient survival.

7.2 Recommendations from this thesis

A fairly low frequency of LOH 1p/19q in the grade III oligodendroglioma cohort was identified in chapter three. This may be due to the preference of neuropathologists at RNSH to identify a glioma based on histopathological appearance alone, rather than a combination of histopathological and genetic factors, resulting in relatively high rates of inclusion for oligodendroglioma at this centre. At some centres, including Royal Prince Alfred Hospital in Sydney, diagnosis of a tumour as an oligodendroglioma is delayed until the LOH 1p/19q status is confirmed as this test informs the diagnosis. Disagreements on tumour grade and diagnosis have long been an issue within the discipline of neuropathology, as illustrated by low concordance between and within institutions (Coons et al., 1997; Smith et al., 1999). Subjective criteria in all four editions of the WHO Classification of Tumours of the Central Nervous System has permitted significant interobserver variability in the diagnosis of oligodendroglioma (Louis et al., 2007). These criteria should be reviewed and the inclusion of LOH 1p/19q

for the classification of an oligodendroglioma should be considered for future editions.

A trend toward longer survival was observed in grade III oligodendrogliomas without LOH 1p/19q that had strong TP53 staining. It has long been assumed that strong TP53 staining was due to mutations of the *TP53* gene, as such mutations are associated with increased TP53 half life (Hinds et al., 1990). Despite this common assumption, TP53 immunoreactivity is not necessarily correlated with a mutation in the *TP53* gene, as illustrated by one study in which 17/39 oligodendroglioma patients displayed high TP53 immunoreactivity, but only 6/39 patients had a *TP53* mutation confirmed by DNA sequencing (Cairncross et al., 1998). Additionally, *TP53* mutations are primarily associated with astrocytic tumours and are extremely rare in oligodendrogliomas with LOH 1p/19q (Kim et al., 2010; Hartmann et al., 2011; Ohgaki and Kleihues, 2011).One study of mixed oligoastrocytomas found that LOH 1p/19q and *TP53* mutations were confined to the portions of the tumour that appeared oligodendroglioma-like or astrocytoma-like respectively (Qu et al., 2007). Taken together, there is a case to reclassify any grade III glioma that bears a *TP53* mutation as an astrocytic tumour.

This thesis highlighted numerous similarities between grade III oligodendrogliomas without LOH 1p/19q and glioblastomas, observed in patient survival time, similar gene expression profiles and the over-representation of mesenchymal and proliferative gene sets. These findings should influence management of grade III oligodendroglioma patients that are found to have intact 1p and 19q. For such patients, treatment should be administered more aggressively than the standard protocol for grade III oligodendroglioma in order to aggressively target the tumour and prolong survival and quality of life.

The prevalence of *IDH1/2* mutations and CIMP among grade II and III oligodendrogliomas was discovered during the preparation of this thesis (Parsons et al., 2008; Yan et al., 2009; Noushmehr et al., 2010; van den Bent et al., 2011). These two phenomena occur most frequently together with *MGMT* promoter methylation and LOH 1p/19q in oligodendrogliomas (Riemenschneider et al., 2010; Tuononen et al., 2012; Hartmann et al., 2011). Individually, each of these genetic alterations is associated with longer patient survival in both astrocytic and

oligodendroglial tumours, however when all four alterations occur in concert the association with improved survival is more robust (van den Bent et al., 2011). These are recent discoveries and how or why they all occur together it has yet to be understood. One theory for the co-occurrence of these genetic events is that each event is a product of a methylation instability phenotype, which is hypothesised to be the event that confers longer survival. The difficulty with examining such a theory is that *IDH1* mutations, *MGMT* methylation, LOH 1p/19q and CIMP can be tested, whereas methylation instability is not measurable.

The advent of the TCGA has promoted collaboration among scientists and clinicians and led to the rapid discovery of new molecular targets, starting with glioblastoma and ovarian cancer, resulting in 46 publications over the past four years. The TCGA glioblastoma dataset includes clinical information on 599 specimens (582 downloadable), large subsets of which have been analysed by microarray for SNPs, DNA methylation, gene expression and copy number (http://tcga-data.nci.nih.gov/tcga/). Glioblastoma specimens are annotated with mutation data for 45 genes, allowing researchers to group or exclude specimens based on specific genetic alterations. For this thesis, the TCGA database was accessed to validate the copy number of MIG-6 in a set of 267 glioblastoma specimens. Among the TCGA projects that are underway is a Low Grade Glioma project, which at present contains data on a total of 91 tumours including grade II and III astrocytomas, oligodendrogliomas and oligoastrocytomas. The existing Low Grade Glioma dataset has the functionality to be annotated with *IDH* mutation data, however molecular information including LOH 1p/19q and *TP53* mutations are not accounted for.

Following the availability of array data on large glioblastoma datasets, many smaller research centres may now shift their focus to functional analysis of their genes of interest as expensive genomic analyses can be simply downloaded. While data from the TCGA portal is free to be downloaded by any person, significant skill and patience is required to organise the data, conduct analyses and obtain meaningful information. A number of web based and downloadable interfaces are available to query the data, which enables wider use of TCGA data by clinicians and researchers (http://tcga-data.nci.nih.gov/tcga/tcgaAnalytical Tools.jsp). One such

interface, the Cancer Molecular Analysis Portal (https://cma.nci.nih.gov/cma-tcga/), permits anyone to perform limited analyses of glioblastoma TCGA data.

The availability of high quality, standardised microarray data from the TCGA means that smaller research teams may no longer need to carry out expensive microarray experiments, but can instead focus on functional analysis of their genes of interest. There is a growing need for bioinformaticians and training in biostatistics so that research groups can conduct independent analyses on publically available data to suit their own experimental aims.

7.3 Limitations of this study

A number of factors limited the scope and statistical power of this study. Ideally, a microarray study would contain a large number of tumour samples to increase the statistical power of the results. The sample number for this study was limited to 17 tumour and 4 normal specimens by the high cost of microarrays, around $900 per array at the time of the experiment. A second limitation, which also affected the number of samples for validation by qPCR, was the relatively low number of oligodendroglioma specimens from which good quality RNA could be extracted. A larger sample size with greater statistical power would likely have permitted the identification of more robust candidate genes. In the future, statistical power could be improved by implementing tumour banking procedures to ensure that the size of each specimen banked is adequate to extract DNA, RNA and protein for research. Ideally, a worldwide set of guidelines should be drafted to standardise tumour specimen banking, which would enable collaboration and international exchange of banked tissue for the benefit of medical research.

As the sample number for the microarray study in chapter four was small, a public database of microarray data for glioma specimens was accessed to examine gene expression of candidate genes in a larger set of tumours. The National Cancer Institute Repository for Molecular Brain Neoplasia Data (Rembrandt) is a bioinformatics tool that provides access to a large repository of copy number, gene expression and survival data collected on over 550 brain tumour specimens (Madhavan et al., 2009; National Cancer Institute, 2005)

(https://caintegrator.nci.nih.gov/rembrandt/). Specimens in the Rembrandt database are stratified by histopathology into six categories labelled astrocytoma, glioblastoma, mixed, non-tumour, oligodendroglioma and unknown. A major limitation of the Rembrandt database is that it does not provide tumour grade for any specimen, nor does it specify LOH 1p/19q status for oligodendroglioma samples. Because of this limitation, candidate genes identified in this thesis could not be analysed in the Rembrandt database by grade or LOH 1p/19q status. Rembrandt does not provide any other classifying molecular data for glioma specimens such as *IDH1*, *IDH2* or *TP53* mutations, which further limits the scope of its application for most studies. Publicly funded databases such as Rembrandt have potential to be highly valuable resources for researchers and clinicians. As greater emphasis is placed on molecular data in tumour classification and clinical decision making, it should be added to all such databases.

The sample size for immunohistochemistry analysis was relatively small (24 to 29 specimens) and no candidate was significantly correlated with patient survival. The limited numbers of specimens meant that the statistical power was lower than ideal for this analysis. Protein staining in a larger cohort of oligodendrogliomas may yet identify one or more of these candidate genes as a more robust candidate marker for survival.

As no model of oligodendroglioma with LOH 1p/19q (either cell line or animal model) was available, functional analysis of *MIG-6* was limited to two glioblastoma cell lines and two oligodendroglioma cell lines that did not have LOH 1p/19q. The primary cell culture described in chapter six was carried out with the aim of developing an oligodendroglioma cell line with LOH 1p/19q to overcome this limitation. Despite culturing 46 glioma specimens, no oligodendroglioma cell line bearing LOH 1p/19q was successfully cultured.

7.4 Candidate genes in oligodendroglioma

Six differentially expressed candidates and one methylated candidate gene were validated in this thesis (chapter five). While all differentially expressed candidates have previously been implicated in cancer biology, each gene is likely to influence

tumourigenesis or tumour aggression through different pathways and mechanisms.

Under the hypothesis that LOH 1p/19q is an initiating event in oligodendrogliomas, *MIG-6* or *PADI2* could be the elusive tumour suppressor gene lost in the earliest stages of tumourigenesis. *MIG-6* is a known tumour suppressor gene, the loss of which contributes to deregulation of the EGFR pathway and increased cell growth and invasion (Jin et al., 2007; Ferby et al., 2006; Zhang et al., 2007a; Anastasi et al., 2003; Fiorentino et al., 2000; Zhang et al., 2007b). While the role of *PADI2* in degenerative disorders has been examined, no published study has investigated its' potential role in cancer initiation. *PADI2* may also have tumour suppressor qualities, as suggested by preliminary data provided by collaborator Michael Buckland, in which the restoration of *PADI2* expression inhibited the growth trajectory of glioma cells *in vitro* (Buckland et al., manuscript in preparation, 2012).

High expression of each of the five remaining candidate genes is associated with increased tumour aggression. *CHI3L1* is known to promote angiogenesis and may contribute to microvascular proliferation, which is a hallmark of grade III and IV gliomas (Louis et al., 2007; Shao et al., 2009; Francescone et al., 2011). *PLAG1* is a transcriptional regulator of numerous growth factors and their receptors, including *IGF2* (Van Dyck et al., 2007; Hensen et al., 2002; Voz et al., 2004). Overexpression of *IGF2* can lead to uncontrolled cell proliferation and both *IGF2* and *PLAG1* may be involved in the aggressive nature of oligodendrogliomas without LOH 1p/19q.

Recent evidence suggests that *IQGAP1* is highly expressed and is specifically involved in cell migration and invasion in glioblastoma, thyroid and hepatocellular cancers (Fowler et al., 2011; White et al., 2010; Liu et al., 2010). *PDPN* can influence tumour progression by promoting cell migration and invasion in both the presence and absence of an Epithelial-Mesenchymal transition (Martín-Villar et al., 2006; Wicki et al., 2006). It is likely that high *IQGAP1* and *PDPN* expression in oligodendrogliomas without LOH 1p/19q promotes cell migration and invasion, resulting in a more aggressive tumour compared to oligodendrogliomas with LOH 1p/19q.

7.5 Future directions for research

Despite a dramatic increase in the understanding of genetic changes in oligodendroglioma due to LOH 1p/19q over the past decade, numerous questions remain unanswered and are worthy of further research.

Why does LOH 1p/19q confer a better prognosis for oligodendroglioma patients? No single gene on 1p or 19q has proven to be solely responsible for the improved prognosis. As the region of loss in LOH 1p/19q is extremely large, it is likely that the combined loss of multiple genes contributes to this phenomenon. This thesis identified seven candidate genes, five of which (*CHI3L1*, *IGF2*, *IQGAP1*, *PLAG1*, *PDPN*) are likely to be responsible for the difference in prognosis. Additional functional analysis should be conducted to determine the effect of each gene in tumours with LOH 1p/19q.

Why do LOH 1p/19q, *MGMT* methylation, *IDH1/2* mutations and CIMP$^+$ occur most frequently together? The recently discovered genetic characteristics of low grade gliomas (CIMP$^+$, *IDH1/2* mutations) have changed the perspective on LOH 1p/19q and possibly reduced its impact. LOH 1p/19q, *MGMT* methylation *IDH1/2* mutations and CIMP$^+$ together describe a favourable oligodendroglioma genotype. The next interesting question in oligodendroglioma is to understand how these genetic events occur and why they most often occur together. Research into tumourigenesis and development of oligodendroglioma is needed to pinpoint when LOH 1p/19q and other significant genetic events occur and how they influence the phenotype of the resulting tumours.

Why is it so difficult to develop a model of 1p/19q in which to study oligodendroglioma biology? The need to develop a model of oligodendroglioma with LOH 1p/19q is paramount. One possibility is a cell line model, but until now such models have been difficult to culture due to loss of genetic characteristics and an extremely slow growth rate. Changes to the standard procedure for cell culture, such as optimising carbon dioxide and oxygen conditions, alternative cell adhesion substrates, the use of chemically defined or conditioned media, may help in future attempts to create a cell line model of LOH 1p/19q. Xenografts of oligodendroglioma in mice may provide a valuable resource, as this technique

allows cells to retain more genetic characteristics of the primary tumour than compared to *in vitro* cell cultures.

What is the impact on oligodendroglioma biology of each candidate gene identified in this thesis? Each of the seven candidate genes examined in this thesis is worthy of further research to elucidate its role in LOH 1p/19q in oligodendroglioma. Here I had the time to study just one tumour suppressor gene, MIG-6, which is lost in LOH 1p/19q and contributes to tumourigenesis in the early stages of oligodendroglioma development. While the functions of *CHI3L1*, *IGF2*, *IQGAP1*, *MIG-6* and *PDPN* have been studied in glioblastoma or other cancer models, the impact of the loss of these genes in oligodendroglioma with LOH 1p/19q is not yet known and may only be fully elucidated following study in a cell culture or animal model of oligodendroglioma that has LOH 1p/19q. One or more of these candidate genes are likely to be novel molecular targets for therapy.

7.6 Conclusions

This thesis supports the continued use of molecular markers including LOH 1p/19q, *MGMT* methylation, *TP53* and *IDH1/2* mutations to aid diagnosis and management of glioma patients. Oligodendrogliomas that lack positive prognostic markers (LOH 1p/19q, *MGMT* methylation, *IDH1/2* mutations) should be treated more aggressively, especially in the case of a grade III lesion. The findings of this thesis and current opinions in the literature acknowledge that LOH 1p/19q may not be the only defining event in the early development of oligodendroglioma, which may be due to other factors including methylation instability or an *IDH1/2* mutation.

CHI3L1, *IGF2*, *IQGAP1*, *MIG-6*, *PDPN*, *PLAG1* and *PADI2* were identified as candidate genes for tumourigenesis and tumour aggression in oligodendroglioma with and without LOH 1p/19q. *MIG-6* was confirmed as a tumour suppressor gene and is likely to play a role in oligodendroglioma tumourigenesis. Further functional analysis of these candidate genes will help to pinpoint their involvement in the development of oligodendrogliomas with and without LOH 1p/19q and may identify one or more candidate genes as novel molecular targets for therapy.

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Appendices

Appendix 1: List of Reagents and Materials

Material	Supplier	Catalog number	Application
40% Acryl/Bis solution 37.5:1	Bio-Rad	1610148	Polyacrylamide gel
Acidified Phenol	Sigma	P4682	RNA purification
Agarose	Bio-Rad	1613101	Agarose gel
Ammonium Acetate	Sigma	A1542	DNA extraction
Ammonium Persulfate	Sigma	A3678	Polyacrylamide gel
AmpliTaq Gold DNA polymerase	Applied Biosystems	N8080247	PCR
B-27 w/o vitamin A supplement	Invitrogen	12587-01	Primary cell culture
Beta-Fibroblast Growth Factor (b-FGF)	Invitrogen	PHG0023	Primary cell culture
Bovine Serum Albumin Delipidized	BD Biosciences	354331	Cell migration assay
BSA set Standards	Bio-Rad	23208	Protein quantification
Ciproflaxin	MediaTech	61-277-RF	Primary cell culture
Collagen I Bovine Natural	BD Biosciences	354231	Cell migration assay
Collagen I Bovine Natural	BD Biosciences	32623	Trans-well migration assay
Competent cells, DH5@, subcloning efficiency	Invitrogen/Gibco	18265-017	Plasmid amplification
Crystal violet	Sigma	C3886	Cell culture assays
Delipidised Bovine Serum Albumin	BD Biosciences	43295	Trans-well migration assay
DMEM	Gibco	11885-084	Cell culture
DMEM-HG, with Na pyruvate, glutamine	Invitrogen/Gibco	11995-065	Cell culture
DNA 1 Kb ladder	Promega	G5711	Agarose gel
DNA puc19/HpaII ladder	Bresatec/ Geneworks	DMW-P1	Polyacrylamide gel
DNase Set (RNase free)	Qiagen	79254	RNA purification
Dulbecco's PBS, w/o Ca, Mg	Invitrogen/Gibco	14190-144	Cell Culture, Western blot
ECL™ Anti-mouse IgG, HRP linked whole antibody	GE Healthcare	NA931	Western blot
ECL™ Anti-rabbit IgG, HRP linkes whole antibody	GE Healthcare	NA934V	Western blot
Epitect Bisulfite Kit	Qiagen	59104	Methylation analysis
Epithelial Growth Factor (EGF)	Chemicon	GF001	Primary cell culture
ERRFI1 antibody, Rabbit polyclonal	Proteintech Group, Inc	11630-1-AP	Western blot
Fetal Bovine Serum, Heat-inact.	Hyclone	SH30071.03HI	Cell culture
Fetal Calf Serum	Gibco	16000-044	Cell culture
GAPDH antibody, Rabbit polyclonal	Santa Cruz	sc-25778	Western blot
GeneChip™ Human Exon 1.0 ST array	Affymetrix	900651	Exon gene expression microarray
Gentamicin	Invitrogen	15750-060	Primary cell culture

Material	Supplier	Catalog number	Application
Gentra PureGene Blood Kit	Gentra/Qiagen	158467	DNA extraction
Gluta-max I	Invitrogen	A12860-01	Primary cell culture
Heparin	Sigma	H3149-100KU	Primary cell culture
Human DNA Methylation 3x720K CpG Island Plus RefSeq Promoter Array	NimbleGen	05924529001	Methylation array
Human Serum Albumin	Albumin Bioscience	9803	Primary cell culture
HyBlot CL™ Autoradiography film	Denville Scientific	E3018	Western blot
IGF2 antibody Clone S1F2	Upstate Cell Signalling Solutions	05-166	Immunohistochemsitry
Improved MEM Zinc Option Media	Invitrogen/Gibco	86-0194DJ	Cell culture
KAPA2G Fast HotStart DNA Polymerase	KAPA Biosystems	KK5500	PCR
Laemmli Sample Buffer	Bio-Rad	1610737	Western blot
Leukaemia Inhibitory Factor (LIF)	Chemicon	LIF1010	Primary cell culture
Liberase	Roche	05401160001	Primary cell culture
Lipofectamine 2000	Invitrogen	11668-019	Transfection
Lithium Chloride 7.5 M	Life Technologies (Ambion)	AM9480	RNA purification
MagicMark™ XP Western Standard	Invitrogen	LC5602	Western blot
MEM/F12 low osmolality w/o L-glutamine	Gibco	12660-012	Primary cell culture
MTT reagent: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (Thiazolyl blue tetrazolium bromide)	Research Organics	2165D	MTT assay
Non-essential Amino Acids	Gibco	11140-050	Primary cell culture
Nonidet P-40 (IGEPAL® CA-630)	Sigma	56741-50ML-F	Protein extraction
NuPAGE 4-12% BisTris gel	Invitrogen	NP0332BOX	Western blot
NuPAGE MES SDS Running Buffer	Invitrogen	NP0002	Western blot
NuPAGE transfer buffer	Invitrogen	NP0006-1	Western blot
Opti-MEM	Gibco	31985-062	Transfection
pGEM-T Easy Vector System II	Promega	A1380	Methylation analysis
Platinum Taq DNA Polymerase	Invitrogen	10966-026	PCR, LOH assay
Protein Assay Dye Reagent Concentrate	Bio-Rad	500-0006EDU	Western blot
Proteinase K	NEB	P8102S	DNA extraction
QIAamp DNA Mini Kit	Qiagen	51306	DNA extraction
QIAquick Gel Extraction Kit	Qiagen	28704	Methylation analysis
QIAzol Lysis Reagent	Qiagen	79306	RNA extraction

Material	Supplier	Catalog number	Application
QuickLyse Miniprep Kit	Qiagen	27405	Methylation analysis
RNA 6000 Nano Assay	Agilent	5067-1511	Agilent bioanalyser
RNase OUT	Invitrogen	10777-019	Reverse transcription
RNaseZap	Ambion	AM9780	RNA extraction
RNeasy Mini Kit	Qiagen	74104	RNA extraction
SeeBlue® pre-stained standard	Invitrogen	LC5625	Western blot
Sodium Acetate 3 M	Sigma	71196	RNA purification
Sodium Chloride	Sigma	S3014	Protein extraction
Sodium Fluoride	Sigma	34247	Protein extraction
Superfrost Ultra Slides	Thermo Scientific	1014356145	Immunohistochemistry
Superscript® III First Strand Synthesis System	Invitrogen	18080-051	Reverse transcription
SuperSignal® West Femto Maximum Sensitivity Substrate	Thermo	34096	Western blot
SYBR green dye	Sigma	S9430	Agarose gel
Taqman universal PCR master mix,no AmpE	Applied Biosystems	4324018	qPCR
TaqMan® Gene Expression assay	Applied Biosystems	various - see table 2.6	qPCR
TEMED	Sigma	T7024	Polyacrylamide gel
TP53 antibody DO7 clone	Novacastra	NCL-L-TP53-DO7	Immunohistochemistry
Transwell inserts, 24-well format, 8 μm pore size, polycarbonate membrane	Costar	3422	Cell migration assay
Tris-Acetic Acid-EDTA (TAE) Buffer (50X concentrate)	Bio-Rad	161-0773	Agarose gel electrophoresis
Trizma® Tris base	Sigma	T1503	General reagent
Tungsten Carbide Beads, 3 mm	Qiagen	69997	RNA extraction
TWEEN-20	Sigma	P2287	Western blot
Vysis LSI 1p36 / LSI 1q25 and LSI 19q13/19p13 Dual-Color Probe	Abbot Molecular	07J73-001	FISH LOH detection
Water, Ultra, DNAse/RNAse free	Invitrogen/Gibco	10977-015	PCR, qPCR

Appendix 2: Neuroendocrine Tumour Bank Patient Consent Form and Patient Information Sheet

Kolling Institute of Medical Research Cancer Genetics Unit & University of Sydney Endocrine Surgical Unit

Consent to Participate in a Neuroendocrine Tumour & Tissue Bank

I, ____ __________________
(name of participant) (date of birth)

of ____
(address)

____ ____________
(phone number)

have been invited to have tissue from my operation kept in a tumour & tissue bank. In relation to this project I have read the attached Patient Information Sheet and have been informed of the following points:

1. Approval has been given by the Human Research Ethics Committee (HREC) of Northern Sydney Health for the collection of tumour and tissue samples

2. Specific approval will also be given by the Human Research Ethics Committee for any specific research project on the samples which may include research projects interstate and overseas as well as locally

3. The aim of the project is to collect and store tissue and tumour samples for molecular genetics research specifically, and only, in the field of endocrine and neuroendocrine tumours. They will be stored for as long as the tissue is useable.

4. The results obtained from any studies may or may not be of direct benefit to my medical management.

5. The procedure will involve collecting and storing excess samples of tissue or tumour and a blood specimen, all removed at operation. The specimen will be identified by a code kept separate from my personal details, and will be stored in the Kolling Institute laboratories.

6. There are some possible adverse effects or risks related to this project which include the possibility of slight bruising at the site of the blood test.

7. Should I develop a problem which I suspect may have resulted from my involvement in this project, I am aware that I may contact Professor Bruce Robinson on 9926 7267 or Professor Leigh Delbridge on 9926 7014

8. Should I have any problems or queries about the way in which the study was conducted, and I do not feel comfortable contacting the research staff, I am aware that I may contact the Patient Representative who is an independent person within the Hospital on 9926 7612.

Date: ______________________________ Witness: _______________________
(Please print name)

Signature: __________________________ Signature: _______________________
(of participant) (of witness)

Investigator's Signature: ____

Kolling Institute of Medical Research Cancer Genetics Unit & University of Sydney Endocrine Surgical Unit

Consent to Participate in Neuro Endocrine Tumour & Tissue Bank

9. I can refuse to take part in this project or withdraw at any time without affecting my medical care.

10. Participation in this project will not result in any extra medical and hospital costs to me.

11. I will not receive any financial benefit from the project.

12. If the results of my tests or information regarding my medical history is published, my identity will not be revealed

13. If findings are made that have implications for my family or myself, I would like myself/my family doctor to be contacted. (If you choose not to be informed please delete and initial)

14. My privacy will be protected and all information will be kept absolutely confidential on a secure database separate from the site of tumour storage.

15. I agree/disagree to the researchers being given relevant clinical information from my GP, clinician and/or access to hospital medical records that would assist with the study. I understand that this would only be done by a researcher who is fully aware of their responsibility with regard to confidentiality.

16. I agree/disagree to researchers accessing tissue (paraffin blocks) stored in Anatomical Pathology following routine histopathological examination by the pathologist at the time of my surgery.

17. My tissue can be withdrawn from the tumour bank by contacting either Professor Robinson (ph. 9926 7267) or Professor Delbridge (ph. 9926 7014). The samples will then be destroyed.

After considering all these points I accept the invitation to participate in this project
I also state that ***I have/have not*** participated in any other research project in the past 3 months. If I have, the details are as follows:

____

Date: ____________________________ Witness: ______________________
(Please print name)

Signature: _________________________ Signature: ______________________
(of participant) (of witness)

Investigator's Signature: ____

____

Kolling Institute of Medical Research Cancer Genetics Unit & University of Sydney Endocrine Surgical Unit

Patient Information Sheet for Neuro and Endocrine Tumour & Tissue Bank

What type of research will be done with my tissue?
Your operation will result in the removal of a tumour, abnormal tissue, and surrounding normal tissue. These either go to pathology for testing or are transplanted back into your body. By participating in the Neuro Endocrine Tumour & Tissue bank, a small part of that specimen will be stored frozen for future research into genetic abnormalities associated with tumour development in endocrine glands. There may be no direct benefit to you from participating in this research. Results from this work, however, are likely to help people who have cancer and other diseases in the future.

How is my tissue collected and stored?
There are two methods of collection of tumour or tissue. After removal of most tumours, and before they are sent to pathology for routine examination, the surgeon will take a very small piece of the tissue for storage. This process will not affect the way in which your tumour is analysed by the pathologists nor the amount of parathyroid tissue that would have been transplanted. This tumour or tissue is placed into liquid nitrogen and sent to a secured storage facility in the Kolling Institute. All specimens are coded prior to storage with all relevant clinical details kept in a separate secure database in the University of Sydney Endocrine Surgical Unit. In addition, researchers may require access to tissue (paraffin blocks) stored in Anatomical Pathology following routine histopathological examination by the pathologist at the time of your surgery.

Blood collection and testing. Two small blood samples (10 mls = about 2 teaspoons) will also be collected and this will be done while you are under the anaesthetic. One of the blood samples will be centrifuged at high speed to separate the plasma and serum. The serum will be removed and stored at -80deg C. The other whole blood sample will be directly stored at -80 deg C. Both the serum and the total blood sample will help researchers find a test for different cancers or diseases, and may help to determine if a person is carrying an altered gene that predisposes them to tumour development. Such an altered gene is known as a germline mutation and the analysis performed on genes purified from the blood cells.

Are there any adverse effects?
There could be slight bruising at the site of the blood test.

Will I find out the results of the research using my tissue?
You will receive the pathology results of your biopsy or surgery from your surgeon independently of your involvement in our research projects. You would not normally receive any specific results of ongoing research studies. However, if some research findings become apparent that have clinical relevance to you and your family, we may contact your doctor in the future with these findings. If required you will have access to a genetic counsellor. For example if a genetic abnormality in a family member was found, the relative risk of other family members may be able to be predicted. This would have implications for you and your family and we would be ethically obliged to contact you through your doctor if that is what you requested on the consent form. However you may choose not to be contacted or you can change your mind at any time.

The National Statement on Ethical Conduct in Research Involving Humans strongly recommends respecting privacy and confidentiality rights of individuals. "When information relevant to the future health of participants and their offspring may be generated the research protocol should provide for consent procedures, counselling, test quality and test result confidentiality."

How do researchers get to use my tissue?
Samples collected during your surgery are stored in a secure facility at the Kolling Institute of Medical Research. All research projects must be approved by the Human Research Ethics Committee (HREC) of Northern Sydney Health and in some cases also by the University of Sydney HREC. Only if these committees consider it ethical and approve the research project can the researcher have access to the samples. They will have in place a process for clinical staff to discuss with patients any changes that may be found. It is possible that tissue will be sent to collaborating institutions interstate or overseas for molecular genetics studies relating to endocrine and neuroendocrine tumours. Specific approval would be obtained from the HREC for such studies and any such specimens would be returned where possible.

Will there be any cost to me?
There will be no extra cost to you for your participation in this research. It is possible that the results of our research using many patient samples may lead to intellectual property which may later be commercialised or lead to diagnostic testing. However as no information can be generated from an individual sample there is no financial benefit to individual patients through participation in this type of research. If a diagnostic testing program becomes available for a gene abnormality that might be relevant to your tumour type, upon discussion with your doctor, you may choose to be tested. This testing is separate to your participation in research and it is possible that it may incur a fee.

Does the decision to participate affect my care in any way?
The choice to let us keep tumour or tissue for research is entirely up to you. Your care will not be affected in any way by your decision.

What if I wish to withdraw my tissue?
My tissue can be withdrawn by contacting Professor Robinson (ph. 9926 7267) or Professor Delbridge (ph. 9926 7014). Your samples will then be destroyed.

Why do you need information from my health records?
In order to interpret results researchers may need to know things such as age, sex, race, family history of disease, treatment etc. This may help to determine when tumours may develop and what may influence them, which may in turn improve the diagnosis and treatment of this disease. All individual clinical information is kept separate from the Tumour & Tissue Bank. The researchers may need to access relevant clinical information from your GP, clinician or from your hospital medical records. This would be done by a researcher approved by the Department Head as being suitably aware of confidentiality issues.

How is my privacy protected?
All your information is confidential and is stored in a secure database. Your identity will not be revealed in any publications in scientific journals that may arise.

What if I change my mind later?
If you decide now that your tissue can be kept for research, you can change your mind at any time. Just contact us and let us know that you do not want us to use your tissue and it will be appropriately destroyed.
If you require any more information or have any other queries please feel free to contact either Professor Bruce Robinson at the Cancer Genetics Unit on 9926 7267 or Professor Leigh Delbridge at the University of Sydney Endocrine Surgical Unit on 9926 7014

Appendix 3A: Brain tumour specimens used in this thesis for exon array, quantitative PCR, immunohistochemistry and CpG array

Sample number	Sex	Age	Type	1p Status	19q Status	IDH1 Status	exon array	qPCR	IHC	CpG array
3265	F	32	OII	LOH	LOH	R132H	✔	✔	-	✔
3610	M	39	OII	LOH	LOH	R132H	✔	✔	-	-
3583	M	32	OII	LOH	LOH	wt	✔	✔	-	-
3622	F	46	OII	LOH	LOH	wt	✔	✔	-	✔
3837	M	39	OII	LOH	LOH	R132H	✔	✔	-	✔
3037	M	37	OII	No Loss	No Loss	R132H	✔	✔	-	-
3506	M	36	OII	No Loss	No Loss	wt	✔	✔	-	-
3034	M	31	OIII	LOH	LOH	R132H	✔	✔	✔	✔
3202	M	44	OIII	LOH	LOH	R132H	✔	✔	✔	-
3591	M	50	OIII	LOH	LOH	wt	✔	✔	✔	-
3122	F	31	OIII	LOH	LOH	R132H	✔	✔	-	✔
3283	M	34	OIII	LOH	LOH	R132H	✔	✔	-	-
3042	F	40	OIII	No Loss	No Loss	wt	✔	✔	✔	-
3590	F	73	OIII	No Loss	No Loss	wt	✔	✔	✔	-
3124	M	77	OIII	No Loss	No Loss	wt	✔	✔	-	-
3173	M	63	OIII	No Loss	No Loss	wt	✔	✔	-	-
3571	M	75	OIII	No Loss	No Loss	wt	✔	✔	-	-
3371	M	54	OIII	Gain	Gain	-	✔	✔	-	-
1379	-	51	GBM	-	-	-	✔	-	-	-
3146	-	34	GBM	-	-	-	✔	-	-	-
3166	-	48	GBM	-	-	-	✔	-	-	-
3195	-	58	GBM	-	-	-	✔	-	-	-
3239	-	35	GBM	-	-	-	✔	-	-	-
3667	-	46	GBM	-	-	-	✔	-	-	-
3743	-	54	GBM	-	-	-	✔	-	-	-
R143	-	51	GBM	-	-	-	✔	-	-	-
3019	F	50	OII	LOH	LOH	R132H	-	✔	-	✔
3594	F	51	OII	LOH	LOH	R132H	-	✔	-	-
3612	M	45	OII	LOH	LOH	R132H	-	✔	-	-
3757	M	32	OII	LOH	LOH	R132H	-	✔	-	-
3721	M	41	OII	LOH	LOH	R132H	-	✔	-	-
30009	F	-	OII	LOH	LOH	R132H	-	✔	-	-
3117	M	58	OII	LOH	Partial Loss	R132H	-	✔	-	-
3108	?	21	OII	No Loss	No Loss	wt	-	✔	-	-
3561	F	38	OII	No Loss	No Loss	R132H	-	✔	-	-
3587	M	72	OII/OIII	LOH	LOH	R132H	-	✔	-	-
3028	F	73	OIII	LOH	LOH	wt	-	✔	✔	-
3062	F	55	OIII	LOH	LOH	R132H	-	✔	✔	-
3276	M	35	OIII	LOH	LOH	wt	-	✔	✔	-
3679	F	56	OIII	LOH	LOH	R132H	-	✔	✔	-
3843	F	34	OIII	LOH	LOH	R132H	-	✔	✔	-
3253	M	45	OIII	LOH	LOH	R132H	-	✔	-	-
3392	M	45	OIII	LOH	LOH	R132H	-	✔	-	✔
3966	M	44	OIII	LOH	LOH	R132H	-	✔	-	-
3983	F	56	OIII	LOH	LOH	R132H	-	✔	-	-
3426	F	53	OIII	LOH	Partial Loss	R132H	-	✔	-	-
3378	M	62	OIII	No Loss	No Loss	wt	-	✔	✔	-
3540	F	65	OIII	No Loss	No Loss	wt	-	✔	✔	-
3578	F	72	OIII	No Loss	No Loss	wt	-	✔	✔	-
3681	M	43	OIII	No Loss	No Loss	wt	-	✔	✔	-
3070	F	53	OIII	No Loss	No Loss	wt	-	✔	-	-
3585	M	66	OIII	No Loss	No Loss	wt	-	✔	-	-
3615	M	79	OIII	No Loss	No Loss	wt	-	✔	-	-
30006	F	59	OIII	No Loss	No Loss	wt	-	✔	-	-
3566	F	73	OIII	Partial Loss	LOH	R132H	-	✔	-	-
3682	M	49	OIII	LOH	LOH	-	-	-	✔	-

Sample number	Sex	Age	Type	1p Status	19q Status	IDH1 Status	exon array	qPCR	IHC	CpG array
3954	F	67	OIII	LOH	LOH	-	-	-	✔	-
3973	F	63	OIII	LOH	LOH	-	-	-	✔	-
4048	M	71	OIII	LOH	LOH	-	-	-	✔	-
4097	F	41	OIII	LOH	LOH	-	-	-	✔	-
4647	F	33	OIII	LOH	LOH	-	-	-	✔	-
4650	M	69	OIII	LOH	LOH	-	-	-	✔	-
4658	M	48	OIII	LOH	LOH	-	-	-	✔	-
4663	M	51	OIII	LOH	LOH	-	-	-	✔	-
4667	M	63	OIII	LOH	LOH	-	-	-	✔	-
117801B1	M	34	OIII	LOH	LOH	-	-	-	✔	-
3397	F	71	OIII	LOH	NI	-	-	-	✔	-
3002	F	45	OIII	LOH	No Loss	-	-	-	✔	-
4665	M	30	OIII	LOH	No Loss	-	-	-	✔	-
3930	M	31	OIII	No Loss	LOH	-	-	-	✔	-
3633	M	64	OIII	No Loss	No Loss	-	-	-	✔	-
3971	F	48	OIII	No Loss	No Loss	-	-	-	✔	-
4158	M	67	OIII	No Loss	No Loss	-	-	-	✔	-
4644	M	36	OIII	No Loss	No Loss	-	-	-	✔	-
4645	F	58	OIII	No Loss	No Loss	-	-	-	✔	-
4651	M	64	OIII	No Loss	No Loss	-	-	-	✔	-
4653	F	55	OIII	No Loss	No Loss	-	-	-	✔	-
4655	M	62	OIII	No Loss	No Loss	-	-	-	✔	-
4657	F	56	OIII	No Loss	No Loss	-	-	-	✔	-
4661	F	46	OIII	No Loss	No Loss	-	-	-	✔	-
4666	M	32	OIII	No Loss	No Loss	-	-	-	✔	-

GBM – glioblastoma; IHC – immunohistochemistry; OII – grade II oligodendroglioma; OII/OIII – grade II oligodendroglioma with grade III features; OIII grade III oligodendroglioma.

Appendix 3B: Normal Brain purchased specimens (RNA): Donors of Normal Brain RNA

RNA	Donor age	Sex	Race	Ambion Catalog#
Whole Brain	68	M	Caucasian	AM6050
	59	F	Caucasian	
	63	M	Caucasian	
	73	F	Caucasian	
	59	F	Caucasian	
	23	M	Caucasian	
	81	M	Caucasian	
	84	F	Caucasian	
	54	M	Caucasian	
	79	M	Caucasian	
	61	M	Unknown	
	86	M	Caucasian	
	85	F	Caucasian	
	78	F	Caucasian	
	81	M	Caucasian	
	70	M	Caucasian	
	55	M	Caucasian	
	74	F	Caucasian	
	60	M	Caucasian	
	59	F	Caucasian	
	54	M	Caucasian	
	86	F	Caucasian	
	80	F	Caucasian	
Orbital frontal Cortex	-	-	-	AM6786
Parietal Cortex Superior	-	-	-	AM6790
Parietal Cortex Posterior	-	-	-	AM6788

Appendix 4: 408 genes differentially expressed in 17 oligodendrogliomas separated by LOH status

#	Gene Symbol	Chromosome	p-value (LOH 1p19q vs. No LOH)	Fold-Change(LOH 1p19q vs. No LOH)	T(LOH 1p19q vs. No LOH)
1	DHRS3	1p36.1	4.73E-06	-2.2068	-6.43028
2	ZNF436	1p36	5.97E-06	-2.45398	-6.31344
3	SRPX2	Xq21.33-q23	9.21E-06	-3.84336	-6.09908
4	ITGAX	16p11.2	1.93E-05	-2.15932	-5.7405
5	C4B	6p21.3	4.29E-05	-4.20568	-5.36034
6	LAMA2	6q22-q23	5.91E-05	-2.91105	-5.20994
7	LDHA	11p15.4	7.93E-05	-2.31538	-5.07271
8	SH3BGRL3	1p35-p34.3	9.64E-05	-2.13086	-4.98253
9	PLAG1	8q12	0.000116383	-2.07078	-4.89582
10	IGF2	11p15.5	0.000127639	-4.82353	-4.85339
11	SLC2A5	1p36.2	0.000130664	-2.49922	-4.84264
12	MAN1C1	1p35	0.000157314	-2.66541	-4.7576
13	TYROBP	19q13.1	0.000184175	-2.34001	-4.68559
14	ADORA3	1p13.2	0.000202116	-2.31534	-4.64322
15	AVIL	12q14.1	0.000211902	-2.12376	-4.62169
16	SLA	8q22.3-qter|8q24	0.000241231	-2.72735	-4.56275
17	TPPP3	16q22.1	0.000245443	-2.91872	-4.55488
18	UCP2	11q13	0.000265409	-2.00086	-4.51938
19	KLHL4	Xq21.3	0.000289099	-2.98827	-4.48062
20	LHFPL2	5q14.1	0.000298161	-2.34497	-4.46664
21	FLNA	Xq28	0.000298367	-2.60407	-4.46632
22	TFPI	2q32	0.000332729	-2.46994	-4.41699
23	STK40	1p34.3	0.000333719	-2.09808	-4.41564
24	CD68	17p13	0.000336642	-2.32076	-4.4117
25	SERPINB8	18q21.3	0.000414129	-2.18895	-4.31812
26	LILRB4	19q13.4	0.000417363	-2.09415	-4.31461
27	GPNMB	7p15	0.000421541	-3.99039	-4.31011
28	SFRP4	7p14.1	0.000453215	-2.90485	-4.27744
29	EMP1	12p12.3	0.000470954	-5.65205	-4.26013
30	C5AR1	19q13.3-q13.4	0.000508898	-2.47392	-4.22522
31	FOSL2	2p23.3	0.000521473	-2.08058	-4.21423
32	CRABP1	15q24	0.000522839	-2.04562	-4.21305
33	SLC13A3	20q12-q13.1	0.000545352	-2.2315	-4.19407
34	ANXA1	9q12-q21.2|9q12-q21.2	0.0005494	-6.35492	-4.19074
35	SLC1A5	19q13.3	0.000557136	-2.04026	-4.18445
36	CSF1R	5q33-q35	0.000654902	-2.25342	-4.11173
37	CD53	1p13	0.000722936	-2.88877	-4.06732
38	OLFML3	1p13.2	0.000734364	-2.32189	-4.06027
39	SLC2A3	12p13.3	0.000749301	-3.30793	-4.05122
40	LAPTM5	1p34	0.000765595	-2.93905	-4.04156
41	NDRG1	8q24.3	0.000791326	-4.44737	-4.02671
42	SERPINE1	7q21.3-q22	0.000812079	-6.40574	-4.01509
43	ITGB2	21q22.3	0.000847189	-2.01712	-3.99608
44	SLC11A1	2q35	0.000866448	-2.349	-3.98599
45	LCP1	13q14.3	0.000868314	-2.58668	-3.98502
46	MMP19	12q14	0.000881204	-2.09836	-3.97841
47	CD300A	17q25.1	0.000916933	-2.19758	-3.96057
48	C1QC	1p36.11	0.000955772	-2.21565	-3.94195
49	PSRC1	1p13.3	0.000972848	-2.64056	-3.934
50	VSIG4	Xq12-q13.3	0.00099427	-2.69188	-3.92422

#	Gene Symbol	Chromosome	p-value (LOH 1p19q vs. No LOH)	Fold-Change(LOH 1p19q vs. No LOH)	T(LOH 1p19q vs. No LOH)
51	NCKAP1L	12q13.1	0.0010174	-2.28794	-3.9139
52	ERRFI1	1p36	0.001028	-2.27427	-3.90926
53	FLNC	7q32-q35	0.00103248	-3.35042	-3.9073
54	TNFAIP3	6q23	0.00104755	-2.35138	-3.9008
55	ELK3	12q23	0.00111823	-2.12828	-3.8715
56	BLID	11q24.1	0.00113326	-2.28797	-3.86551
57	PTAFR	1p35-p34.3	0.00114391	-2.3265	-3.86131
58	PPP1R15A	19q13.2	0.00115159	-2.19019	-3.85831
59	LAIR1	19q13.4	0.00116901	-2.51074	-3.85157
60	JUN	1p32-p31	0.00120768	-2.19658	-3.83697
61	ADAM28	8p21.2	0.00121647	-2.19192	-3.83371
62	CLIC2	Xq28	0.00126881	-2.00516	-3.81481
63	SLC16A12	10q23.31	0.00129874	-2.20459	-3.80435
64	PDPN	1p36.21	0.00130124	-3.5588	-3.80349
65	FCGR2A	1q23	0.00132093	-2.79856	-3.79675
66	ALOX5AP	13q12	0.00136174	-3.7285	-3.78309
67	SULF1	8q13.2-q13.3	0.00136504	-3.9502	-3.78201
68	GEM	8q13-q21	0.00139772	-2.61663	-3.77139
69	CD274	9p24	0.00143846	-2.13085	-3.7585
70	GADD45A	1p31.2-p31.1	0.00143964	-2.18973	-3.75813
71	SLN	11q22-q23	0.00145874	-2.71118	-3.75221
72	HK2	2p13	0.0015442	-2.51465	-3.72666
73	ACTN1	14q24.1-q24.2	0.00158574	-2.68001	-3.71474
74	AQP1	7p14	0.00162128	-4.09444	-3.70479
75	FTL	19q13.33	0.00163882	-2.44998	-3.69996
76	C17orf87	17p13.2	0.00175936	-2.48654	-3.66808
77	NCF2	1q25	0.001764	-2.07469	-3.6669
78	GPR37	7q31	0.00184262	-3.43516	-3.64731
79	ITIH2	10p15	0.00192829	-3.36565	-3.62689
80	BGN	Xq28	0.00205491	-2.78078	-3.59831
81	NCRNA00152	2p11.2	0.00211366	-2.79664	-3.58564
82	CD84	1q24	0.00216725	-2.07491	-3.57438
83	FYB	5p13.1	0.0022377	-2.16309	-3.55999
84	LOX	5q23.2	0.00227909	-5.376	-3.55174
85	COLEC12	18pter-p11.3	0.00229768	-2.57851	-3.54809
86	GABRE	Xq28	0.00234827	-2.05368	-3.53829
87	GAS2L3	12q23.1	0.00245103	-2.11178	-3.51901
88	SEC24D	4q26	0.00251575	-2.43907	-3.50728
89	FPR1	19q13.4	0.00256141	-2.09869	-3.49918
90	PLIN2	9p22.1	0.00260347	-3.0073	-3.49184
91	LAMB1	7q22	0.00268662	-2.61852	-3.47768
92	FBXO32	8q24.13	0.00275116	-2.51628	-3.46698
93	HLA-DMB	6p21.3	0.00277421	-2.2024	-3.46322
94	CITED1	Xq13.1	0.00294254	-2.63393	-3.43665
95	ALOX5	10q11.2	0.00307495	-2.01027	-3.4168
96	SLC6A6	3p25-p24	0.00309435	-2.12995	-3.41396
97	ISLR	15q23-q24	0.00309896	-3.78055	-3.41328
98	DMD	Xp21.2	0.00318221	-2.39401	-3.40132
99	DOCK8	9p24.3	0.00318369	-2.02889	-3.40111
100	BCAT1	12p12.1	0.00320885	-3.2285	-3.39755
101	ESM1	5q11.2	0.00327014	-2.80841	-3.38901
102	HMOX1	22q12|22q13.1	0.0032724	-3.53699	-3.3887
103	ITGB3	17q21.32	0.00329211	-2.97055	-3.38598
104	TAGLN	11q23.2	0.00331317	-2.55481	-3.3831
105	PDK1	2q31.1	0.00336308	-2.02241	-3.37635

#	Gene Symbol	Chromosome	p-value (LOH 1p19q vs. No LOH)	Fold-Change(LOH 1p19q vs. No LOH)	T(LOH 1p19q vs. No LOH)
106	COL11A1	1p21	0.00341024	-3.03723	-3.37005
107	PLEK	2p14-p13.3	0.00343808	-2.30352	-3.36638
108	TMSB10	2p11.2	0.00345339	-2.09804	-3.36437
109	CLDN11	3q26.2-q26.3	0.00346272	-3.47086	-3.36315
110	C3AR1	12p13.31	0.00355486	-2.36513	-3.35128
111	IGFBP3	7p13-p12	0.00363909	-2.84559	-3.34069
112	DNAJB1	19p13.2	0.00364799	-2.19899	-3.33958
113	HIST1H1A	6p21.3	0.00378327	-2.6366	-3.3231
114	C1orf162	1p13.2	0.00379587	-2.19723	-3.3216
115	OLR1	12p13.2-p12.3	0.00380257	-2.4668	-3.3208
116	ANKRD22	10q23.31	0.0038615	-2.44357	-3.31384
117	FAP	2q23	0.00386748	-2.95189	-3.31314
118	EFNB2	13q33	0.00388188	-2.3158	-3.31145
119	LCP2	5q33.1-qter	0.00393026	-2.00112	-3.30585
120	HS3ST1	4p16	0.00393354	-2.01541	-3.30547
121	TTYH1	19q13.4	0.00400413	-2.13899	-3.29741
122	PLAUR	19q13	0.00405292	-2.1521	-3.29192
123	MSR1	8p22	0.00407406	-2.97809	-3.28957
124	IFI30	19p13.1	0.00407844	-2.70427	-3.28908
125	SRGN	10q22.1	0.00409601	-2.05847	-3.28713
126	CYP1B1	2p21	0.00418954	-3.58218	-3.27689
127	TM4SF1	3q21-q25	0.00422379	-3.03575	-3.2732
128	SPP1	4q21-q25	0.00446487	-4.88398	-3.24802
129	PSMB9	6p21.3	0.00449209	-2.16535	-3.24526
130	MSN	Xq11.2-q12	0.00455192	-2.92367	-3.23925
131	MET	7q31	0.00473208	-2.75979	-3.22162
132	CHRNA9	4p14	0.00479605	-2.06982	-3.21551
133	PLOD2	3q23-q24	0.0047962	-2.02875	-3.2155
134	LOXL2	8p21.3-p21.2	0.00482939	-2.01584	-3.21236
135	FCER1G	1q23	0.00486167	-2.37113	-3.20934
136	AR	Xq11.2-q12	0.0048734	-2.12144	-3.20824
137	CXCR4	2q21	0.00488909	-2.29026	-3.20678
138	DOCK7	1p31.3	0.00495753	-2.37134	-3.20045
139	RGS1	1q31	0.00500559	-3.81107	-3.19607
140	IQGAP1	15q26.1	0.00514717	-2.177	-3.18337
141	FAM129A	1q25	0.00522664	-2.07443	-3.1764
142	FCGR3A	1q23	0.00537389	-2.6604	-3.16374
143	GNG12	1p31.3	0.0054407	-3.16104	-3.15811
144	HLA-DPA1	6p21.3	0.00554993	-2.38435	-3.14905
145	OLFML2B	1q23.3	0.00555477	-2.0522	-3.14865
146	TLR1	4p14	0.00559626	-2.10064	-3.14525
147	SLC6A13	12p13.3	0.00581752	-2.25685	-3.12756
148	SQRDL	15q15	0.00584059	-2.09493	-3.12576
149	GJC1	17q21.31	0.00589612	-2.66888	-3.12143
150	C10orf10	10q11.21	0.00608966	-2.03314	-3.10668
151	FMOD	1q32	0.00614666	-3.13614	-3.10242
152	SERPINA3	14q32.1	0.00636002	-5.73502	-3.08682
153	TLR2	4q32	0.00638358	-2.56467	-3.08513
154	LPAR1	9q31.3	0.00651893	-2.86107	-3.07552
155	VIM	10p13	0.00656743	-2.60022	-3.07213
156	FKBP5	6p21.3-p21.2	0.00657585	-2.8425	-3.07154
157	HLA-DPB1	6p21.3	0.00665242	-2.22053	-3.06624
158	MFAP4	17p11.2	0.00674992	-2.11803	-3.05958
159	EDNRA	4q31.22	0.00680746	-2.23018	-3.05569
160	CCDC109B	4q25	0.00692813	-2.74832	-3.04763

#	Gene Symbol	Chromosome	p-value (LOH 1p19q vs. No LOH)	Fold-Change(LOH 1p19q vs. No LOH)	T(LOH 1p19q vs. No LOH)
161	APOL4	22q11.2-q13.2	0.00700379	-2.03463	-3.04265
162	HSPA6	1q23	0.00704231	-3.42517	-3.04014
163	DDR2	1q23.3	0.00706702	-2.68314	-3.03853
164	NNMT	11q23.1	0.0071428	-2.34727	-3.03364
165	IFI16	1q22	0.00722415	-2.17282	-3.02844
166	GBP7	1p22.2	0.00724275	-2.23254	-3.02726
167	TNFRSF19	13q12.11-q12.3	0.00727227	-2.08245	-3.02539
168	CCR1	3p21	0.0073353	-2.56734	-3.02143
169	PMP22	17p12-p11.2	0.00746497	-2.32524	-3.01338
170	ADAM12	10q26.3	0.00748128	-3.03665	-3.01237
171	C1R	12p13	0.00749529	-2.84819	-3.01151
172	TCTEX1D1	1p31.3	0.00770267	-2.14797	-2.99897
173	ABCC3	17q22	0.00777883	-2.07278	-2.99444
174	MOXD1	6q23.1-q23.3	0.00781612	-2.14938	-2.99224
175	CYBRD1	2q31.1	0.00789914	-2.53257	-2.98738
176	MAN1A1	6q22	0.00799785	-2.13262	-2.98166
177	HOXD10	2q31.1	0.00820488	-2.35406	-2.96989
178	IL8	4q13-q21	0.00848113	-4.28112	-2.95463
179	METTL7B	12q13.2	0.00870848	-3.01552	-2.94242
180	FN1	2q34	0.00905347	-2.88799	-2.92448
181	DPYD	1p22	0.00922188	-2.03751	-2.91596
182	C7orf68	7q32.1	0.00950587	-2.51026	-2.90192
183	ACSL1	4q34-q35	0.00958815	-2.06388	-2.89793
184	KIAA1199	15q24	0.00973839	-3.04342	-2.89073
185	HRH1	3p25	0.00991843	-2.05514	-2.88224
186	LGALS1	22q13.1	0.00994162	-2.33166	-2.88116
187	TMBIM1	2p24.3-p24.1	0.00994304	-2.86754	-2.88109
188	SLCO1C1	12p12.2	0.00994859	-3.19865	-2.88083
189	SAMD9L	7q21.2	0.0101911	-2.43688	-2.86966
190	ABCA9	17q24.2	0.0102603	-2.28091	-2.86652
191	SYNJ2	6q25.3	0.0103742	-2.15272	-2.86139
192	TMTC2	12q21.31	0.0110839	-2.09179	-2.83063
193	ATP10B	5q34	0.0111036	-2.14186	-2.8298
194	CASP1	11q23	0.011206	-2.16179	-2.82553
195	SDC2	8q22-q23	0.0113671	-2.4007	-2.81888
196	HSPA1B	6p21.3	0.0114869	-2.47232	-2.81399
197	EGR1	5q31.1	0.0118748	-2.23473	-2.7985
198	OR52H1	11p15.4	0.0119119	-2.10641	-2.79704
199	CFI	4q25	0.0123882	-2.9216	-2.77873
200	EMP3	19q13.3	0.0123983	-3.7595	-2.77835
201	TNFRSF12A	16p13.3	0.0124232	-2.05087	-2.77741
202	ECE1	1p36.1	0.0125388	-2.08798	-2.77308
203	CNR1	6q14-q15	0.0125982	-2.40817	-2.77087
204	GBP5	1p22.2	0.0127096	-2.35043	-2.76675
205	CYBB	Xp21.1	0.0127225	-2.42135	-2.76628
206	CD44	11p13	0.013085	-2.93797	-2.75312
207	MYOF	10q24	0.0132601	-2.19369	-2.74689
208	TREM1	6p21.1	0.0134	-2.93299	-2.74197
209	GPR65	14q31-q32.1	0.0134551	-2.05108	-2.74004
210	RPL41	12q13	0.0134949	-2.25258	-2.73865
211	CYR61	1p31-p22	0.0138449	-2.20409	-2.72663
212	CASP4	11q22.2-q22.3	0.0140396	-2.22571	-2.72007
213	CPA4	7q32	0.0140571	-2.13691	-2.71949
214	C13orf18	13q14.13	0.0141147	-2.18558	-2.71757
215	COL6A3	2q37	0.0142791	-2.7358	-2.71212

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216	SOD2	6q25.3	0.0143355	-2.31207	-2.71027
217	OMD	9q22.31	0.0145292	-2.64772	-2.70395
218	THBS1	15q15	0.0147041	-3.02667	-2.69832
219	COL5A2	2q14-q32	0.014912	-2.12488	-2.6917
220	AKAP12	6q24-q25	0.0154672	-2.04005	-2.67446
221	IGF2BP2	3q27.2	0.0156289	-4.12734	-2.66955
222	IGFBP2	2q33-q34	0.0157825	-2.75053	-2.66493
223	EPHA3	3p11.2	0.0159049	-2.26712	-2.66128
224	CAV1	7q31.1	0.0159097	-2.51073	-2.66114
225	ITGA5	12q11-q13	0.0164155	-2.32153	-2.64634
226	PLXDC2	10p12.31	0.0169032	-2.24076	-2.63247
227	MGST1	12p12.3-p12.1	0.0170252	-2.64671	-2.62906
228	C3	19p13.3-p13.2	0.0174969	-2.69463	-2.61609
229	COL1A1	17q21.33	0.0176092	-3.72762	-2.61305
230	HGF	7q21.1	0.0176546	-2.74884	-2.61183
231	SERPING1	11q12-q13.1	0.0178654	-2.54547	-2.60619
232	AQP9	15q22.1-q22.2	0.0179102	-2.28899	-2.605
233	TMEM71	8q24.22	0.0181574	-2.35365	-2.59848
234	IBSP	4q21-q25	0.018406	-2.51827	-2.59201
235	SDC4	20q12	0.0187279	-2.69894	-2.58375
236	PLCE1	10q23	0.018898	-2.30221	-2.57944
237	CFH	1q32	0.0190545	-2.99412	-2.57551
238	PREX1	20q13.13	0.0191012	-2.141	-2.57434
239	SLC22A15	1p13.1	0.0192995	-2.30035	-2.56941
240	ICAM1	19p13.3-p13.2	0.0193223	-2.22615	-2.56885
241	IGFBP5	2q33-q36	0.0193231	-2.28208	-2.56883
242	CDC20	1p34.1	0.0195229	-2.12037	-2.56392
243	TNFRSF11B	8q24	0.0206079	-2.11232	-2.53805
244	COL1A2	7q22.1	0.020727	-2.79558	-2.53529
245	TAGLN2	1q21-q25	0.0209359	-2.41774	-2.53049
246	C1S	12p13	0.0209785	-2.13726	-2.52951
247	GDPD2	Xq13.1	0.0209968	-2.22501	-2.52909
248	SLC6A20	3p21.3	0.0212017	-2.16631	-2.52444
249	MMP9	20q11.2-q13.1	0.0216234	-2.43139	-2.51498
250	CBR1	21q22.13	0.021818	-2.72385	-2.51068
251	NNAT	20q11.2-q12	0.0219369	-2.45009	-2.50807
252	FZD5	2q33-q34	0.0220069	-2.2845	-2.50654
253	GLIPR1	12q21.2	0.0221514	-2.13096	-2.50339
254	CHI3L1	1q32.1	0.0228223	-7.72239	-2.48903
255	PYGL	14q21-q22	0.0228819	-2.21507	-2.48777
256	GBP1	1p22.2	0.0230077	-2.26126	-2.48513
257	OSMR	5p13.1	0.023162	-2.6727	-2.48191
258	MS4A4A	11q12	0.0236253	-2.14075	-2.47235
259	POSTN	13q13.3	0.0242656	-5.68471	-2.45943
260	CD14	5q22-q32|5q31.1	0.0243919	-2.15086	-2.45692
261	TLR4	9q32-q33	0.0244022	-2.10438	-2.45672
262	GPX8	5q11.2	0.0251579	-2.86428	-2.44196
263	DYNLT3	Xp21	0.0251637	-2.53302	-2.44184
264	STC1	8p21-p11.2	0.0254394	-2.16175	-2.43656
265	AHNAK2	14q32.33	0.0256959	-2.24729	-2.43169
266	SERPINF1	17p13.1	0.0257328	-2.06168	-2.431
267	ITGA3	17q21.33	0.0259056	-2.16283	-2.42775
268	CD163	12p13.3	0.0260809	-3.12351	-2.42448
269	DCN	12q21.33	0.026084	-2.13684	-2.42442
270	SNX10	7p15.2	0.0267101	-2.72866	-2.41289

#	Gene Symbol	Chromosome	p-value (LOH 1p19q vs. No LOH)	Fold-Change(LOH 1p19q vs. No LOH)	T(LOH 1p19q vs. No LOH)
271	MS4A6A	11q12.1	0.0268977	-2.01814	-2.40949
272	TIMP1	Xp11.3-p11.23	0.0269998	-4.04722	-2.40764
273	GLDN	15q21.2	0.0272008	-2.25882	-2.40403
274	C7	5p13	0.0279215	-2.81624	-2.39129
275	SLC41A3	3q21.2-q21.3	0.0283251	-2.0986	-2.38428
276	ADAMTS3	4q13.3	0.0284594	-2.02891	-2.38197
277	IFI44L	1p31.1	0.0286015	-2.30596	-2.37954
278	ADM	11p15.4	0.0292983	-2.58778	-2.36777
279	PCOLCE	7q22	0.0294083	-2.07771	-2.36594
280	COL3A1	2q31	0.0294084	-2.88132	-2.36593
281	FXYD1	19q13.1	0.0298696	-2.15088	-2.35831
282	CXCL9	4q21	0.0302591	-2.2702	-2.35196
283	RDH10	8q21.11	0.0307951	-2.39481	-2.34335
284	LGALS3	14q21-q22	0.0312243	-2.03144	-2.33655
285	SEMA3E	7q21.11	0.0315954	-2.16126	-2.33074
286	B3GALT1	2q24.3	0.0335945	-2.09274	-2.3005
287	HIST1H4L	6p22-p21.3	0.0338276	-2.10715	-2.29708
288	FAM114A1	4p14	0.0347185	-2.05035	-2.28422
289	TGFB2	1q41	0.0347258	-2.97267	-2.28411
290	EYA4	6q23	0.0350815	-2.50963	-2.27906
291	SLC13A4	7q33	0.0357346	-2.39856	-2.26991
292	COL4A2	13q34	0.0368349	-2.27553	-2.25484
293	HLA-DRA	6p21.3	0.0376077	-2.06968	-2.24451
294	CLEC5A	7q33	0.0376313	-3.14616	-2.24419
295	LIF	22q12.2	0.0379426	-2.78474	-2.24009
296	PTGDS	9q34.2-q34.3	0.0380234	-2.31832	-2.23903
297	CDK6	7q21-q22	0.0380988	-2.35104	-2.23804
298	MICAL2	11p15.3	0.0389475	-2.27971	-2.22704
299	SEMA3A	7p12.1	0.0390611	-2.96569	-2.22559
300	TMTC1	12p11.22	0.0393602	-2.0112	-2.22177
301	NIPAL3	1p36.12-p35.1	0.0407736	-2.03954	-2.2041
302	UCHL1	4p14	0.0416222	-2.55551	-2.19377
303	RGS6	14q24.3	0.0420182	-2.33455	-2.18901
304	NAMPT	7q22.3	0.043686	-2.05949	-2.16941
305	F3	1p22-p21	0.0439708	-2.67229	-2.16613
306	FBLN5	14q32.1	0.04582	-2.18319	-2.1453
307	SGMS2	4q25	0.0460261	-2.02385	-2.14303
308	CYP4F11	19p13.1	0.0466266	-2.26273	-2.13646
309	HS3ST3B1	17p12-p11.2	0.0482402	-2.69596	-2.11918
310	WWTR1	3q23-q24	0.0484444	-2.00177	-2.11703
311	CPNE8	12q12	0.0487771	-2.08112	-2.11355
312	CLIC1	6p22.1-p21.2	0.0496624	-2.02063	-2.10438
313	NRN1	6p25.1	0.0499256	-3.36802	-2.10168
314	GABRB3	15q11.2-q12	0.0497971	2.37976	2.103
315	T1560	8q12.1	0.0485664	2.99748	2.11575
316	PCDHB2	5q31	0.0481464	2.33852	2.12017
317	KCNIP2	10q24	0.0480039	2.0647	2.12168
318	SHC3	9q22.1	0.0477238	2.08196	2.12465
319	ACCN4	2q35	0.0468838	2.07299	2.13367
320	GPR17	2q21	0.0463048	4.08413	2.13997
321	MCF2	Xq27	0.0417822	2.04172	2.19184
322	LRRTM4	2p12	0.0417507	3.18015	2.19222
323	ACTL6B	7q22	0.0415417	2.01038	2.19474
324	C11orf41	11p13	0.0409925	2.14989	2.20142
325	SLC8A3	14q24.1	0.0364072	2.15022	2.26065

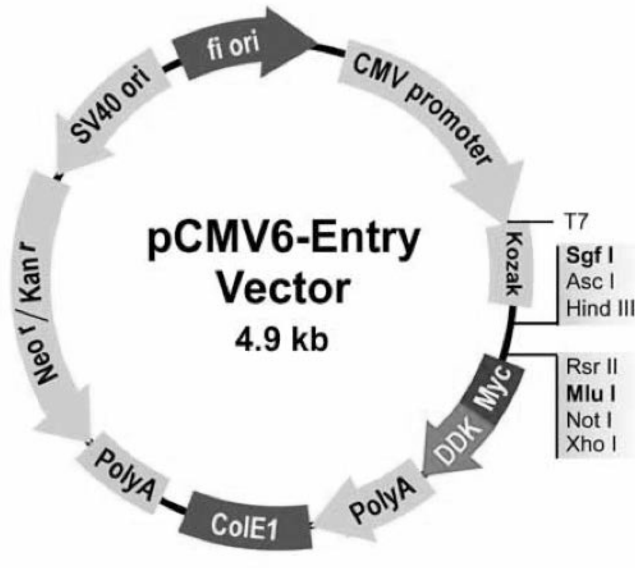
#	Gene Symbol	Chromosome	p-value (LOH 1p19q vs. No LOH)	Fold-Change(LOH 1p19q vs. No LOH)	T(LOH 1p19q vs. No LOH)
326	RIMS1	6q12-q13	0.0361964	2.41814	2.26354
327	ZNF99	19p12	0.0356571	2.11402	2.27099
328	SHD	19p13.3	0.0354395	2.80666	2.27403
329	INA	10q24.33	0.035012	5.18496	2.28005
330	NMNAT2	1q25	0.0346696	2.62547	2.28491
331	ATCAY	19p13.3	0.0339555	3.23823	2.29521
332	DDX25	11q24	0.0338205	2.60833	2.29718
333	RIMS2	8q22.3	0.0323328	4.22773	2.31938
334	FAM181B	11q14.1	0.0316876	2.00423	2.32931
335	TNR	1q24	0.0313903	4.49427	2.33394
336	BMP2	20p12	0.0309554	2.29498	2.3408
337	PHYHIPL	10q11	0.0305321	2.8061	2.34756
338	BEX1	Xq21-q23|Xq22	0.0292053	2.28286	2.36933
339	NRXN1	2p16.3	0.0288117	2.93579	2.37596
340	HES6	2q37.3	0.0276671	2.1782	2.39575
341	ADAMTS20	12q12	0.0261395	2.99964	2.42339
342	GNG4	1q42.3	0.0232528	2.28549	2.48002
343	GPR19	12p12.3	0.0231707	2.21169	2.48173
344	NRSN1	6p22.3	0.0231488	3.22908	2.48218
345	CRTAC1	10q22	0.0229975	2.44709	2.48534
346	RPL17	18q21	0.0222209	2.03969	2.50188
347	ACCN2	12q12	0.0204931	2.28655	2.54073
348	DOK6	18q22.2	0.0198472	2.07533	2.55605
349	GRIN3A	9q31.1	0.0198359	3.54434	2.55632
350	GLRA3	4q33-q34	0.0197504	3.73555	2.55838
351	NXPH1	7p22	0.0172584	2.07066	2.6226
352	SATB1	3p23	0.0172236	2.5682	2.62356
353	CHRNB2	1q21.3	0.0172114	2.27068	2.6239
354	ESRRG	1q41	0.0170132	2.16488	2.62939
355	FAM190A	4q22.1	0.0164763	2.31251	2.64459
356	HTR1A	5q11.2-q13	0.0158651	2.3343	2.66247
357	GLRB	4q31.3	0.0154261	2.30055	2.67572
358	TRIM67	1q42.2	0.0152319	3.18542	2.6817
359	RANBP17	5q34	0.0151116	2.60921	2.68544
360	PID1	2q36.3	0.0146889	2.4298	2.6988
361	ANK3	10q21	0.0143024	2.77495	2.71135
362	CHRDL1	Xq23	0.0135033	3.69115	2.73836
363	NOG	17q21-q22	0.0134978	3.66557	2.73856
364	CHGB	20pter-p12	0.0125295	4.30669	2.77343
365	PDCD4	10q24	0.0116113	2.05976	2.80897
366	GDAP1L1	20q12	0.00961501	2.36986	2.89663
367	ABCC8	11p15.1	0.00936401	2.82914	2.90888
368	ZDHHC22	14q24.3	0.00930296	3.26676	2.91191
369	SEZ6L	22q12.1	0.0087561	4.20069	2.9399
370	SHANK2	11q13.3-q13.4	0.00834598	2.03508	2.96204
371	HNRNPM	19p13.3-p13.2	0.00834134	2.22378	2.96229
372	OR2L2	1q44	0.00831493	3.47495	2.96375
373	HIP1R	12q24	0.00817636	2.5063	2.9715
374	SLC17A8	12q23.1	0.00806219	4.37429	2.97797
375	ANO5	11p14.3	0.00733522	4.58533	3.02143
376	KCNIP3	2q21.1	0.00650536	2.22673	3.07648
377	KCNJ16	17q23.1-q24.2	0.00625283	3.12526	3.09459
378	FAM110B	8q12.1	0.00569817	2.47012	3.13702
379	KCNN2	5q22.3	0.00564319	3.0989	3.14145
380	CCDC26	8q24.21	0.00469088	2.11451	3.22559

#	Gene Symbol	Chromosome	p-value (LOH 1p19q vs. No LOH)	Fold-Change(LOH 1p19q vs. No LOH)	T(LOH 1p19q vs. No LOH)
381	CDHR1	10q22.1-q22.3	0.00447494	2.52069	3.24699
382	CACNG2	22q13.1	0.00442693	5.97935	3.25189
383	H2AFY2	10q22	0.00436	2.20377	3.2588
384	MMD2	7p22.1	0.00435742	3.74579	3.25907
385	CT45A5	Xq26.3	0.00427585	2.44058	3.26765
386	LOC84856	10q11.21	0.00424664	2.48009	3.27076
387	RGMB	5q15	0.00407431	2.50061	3.28954
388	CHORDC1	11q14.3	0.00405144	2.38895	3.29209
389	OR2AK2	1q44	0.0039988	2.20468	3.29801
390	SCG5	15q13-q14	0.00398549	2.06642	3.29952
391	OMG	17q11.2	0.00366846	2.16761	3.33705
392	ZNF681	19p12	0.00347901	2.72127	3.36103
393	SCML2	Xp22	0.00313459	2.00202	3.40812
394	LYRM7	5q23.3	0.00248891	2.10268	3.51211
395	OR2L8	1q44	0.00237902	3.36629	3.53243
396	CMBL	5p15.2	0.0020556	2.34097	3.59816
397	AS3MT	10q24.32	0.00186137	2.68385	3.64277
398	KIAA1409	14q32.12	0.00177547	4.91433	3.66399
399	OR2L3	1q44	0.00157151	3.05924	3.71879
400	SEZ6L2	16p11.2	0.000743419	2.11049	4.05476
401	THEM4	1q21	0.000722548	2.04577	4.06756
402	KIF21B	1pter-q31.3	0.000605118	3.45183	4.14728
403	MLLT3	9p22	0.000457428	2.48106	4.27327
404	LMBR1	7q36	0.000192893	2.01269	4.6645
405	FAM36A	1q44	0.00019078	2.24925	4.66952
406	POLR2F	22q13.1	0.000188417	2.80016	4.67521
407	MOSC1	1q41	0.000159997	2.44887	4.74987
408	SART3	12q24.1	7.40E-05	2.12843	5.1052

Appendix 5: pCMV6-Entry-MIG6 plasmid map

pCMV6-Entry-MIG6 plasmid map (6287 bp)

MIG-6 coding sequence was inserted in frame into pCMV6-Entry at *AscI* and *RsrII* sites.



Sequence features:
Multiple Cloning Site (bold)
AscI site (thick underline)
MIG-6 coding sequence (double underline)
RsrII site (wave underline)

AACAAAATATTAACGCTTACAATTTCCATTCGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGT
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