CHAPTER 5

MAINTAINED AND ACQUIRED GENOMIC ALTERATIONS IN PATIENT-MATCHED SETS OF PRIMARY AND RECURRENT PAEDIATRIC EPENDYMOMA

5.1 Introduction

Tumour recurrence is a common feature of paediatric ependymomas. Despite complete surgical excision, recurrence can develop in up to 50 % of patients, even following adjuvant radiotherapy (Hamilton and Pollack 1997; Tabori, Ma et al. 2006). The site of relapse is typically local to that of the primary tumour, although distant CNS recurrences account for approximately 20 – 25 % of cases (Pollack, Gerszten et al. 1995; Needle, Goldwein et al. 1997; Robertson, Zeltzer et al. 1998; Messahel, Ashley et al. 2009). Recurrence often occurs within two years of the original diagnosis (Agaoglu, Ayan et al. 2005) although late relapses up to 15 years after the discontinuation of therapy are not uncommon (Paulino, Wen et al. 2002). The subsequent outcome for relapsed children is extremely poor, with over 70 % of patients dying within five years, primarily due to the ineffective salvage therapies currently available (Kulkarni 2004; Messahel, Ashley et al. 2009). The biology of ependymoma recurrence in childhood therefore warrants consideration.

To date, only three genomic studies have exclusively addressed copy number abnormalities in recurrent paediatric ependymoma cohorts, limited in size by the availability of suitable tumour tissue. Each study analysed the tumour genome at a lower resolution relative to the coverage provided by a SNP array. One such CGH analysis, comparing 11 paediatric recurrent ependymomas with 42 primary tumours, identified chromosome 1q gain as the most frequent genomic imbalance at relapse (Dyer, Prebble et al. 2002). An array CGH analysis of 26 recurrent tumours within a cohort of 59 paediatric intracranial ependymomas also found gain of chromosome 1q and loss of 6q to be acquired at relapse, although gains of 9q33 and 9q34 were the most prevalent imbalances observed. Indeed, subsequent hierarchical clustering of the entire ependymoma cohort revealed gain of 9qter was associated with recurrence (Puget, Grill et al. 2009). The third paediatric study also used array CGH to compare 17 primary ependymomas with 27 patient-matched first or subsequent recurrent tumours, demonstrating loss of 6q25.2 as the only significant genomic acquisition from diagnosis to relapse (Peyre, Commo et al. 2010).

Further work arising from the latter two studies also identified particular genes with dysregulated expression at paediatric ependymoma recurrence. A dual colour gene expression microarray analysis, where differentially labelled primary and relapsed tumour samples competitively hybridise to probes on an individual array, was performed on the aforementioned patient-matched cohort of 44 tumours in order to establish a signature gene profile for ependymoma recurrence in childhood (Peyre, Commo et al. 2010). The altered expression of 87 genes was reported specifically at relapse, including metallothionein gene downregulation and the increased expression of kinetochore genes *ASPM* (1q31.3) and *KIF11* (10q23.33), together with candidates implicated in neural development such as *PROM1* (4p15.32) and genes of the Wnt and Notch pathway (Peyre, Commo et al. 2010). As a continuation of their array CGH work, Puget et al. compared 13 matched primary and recurrent childhood ependymomas for the expression of selected genes located within the regions 9q33 and 9q34 (Puget, Grill et al. 2009). In addition to overexpression of the tenascin gene family member *TNC* (9q33) (discussed further in Chapter 6), the expression of genes involved in the Notch pathway was again found to be altered at tumour recurrence, such as the upregulation of *NOTCH1* (9q34.3), *HES1* (3q29), *HEY2* (6q22.1) and *C-MYC* (8q24.1) and downregulation of the tumour suppressor *FBXW7* (4q31.3) (Puget, Grill et al. 2009). In both studies, the modified expression of selected candidates (*ASPM*, *MT3*, *TNC*, and *HES1*) was confirmed using immunohistochemistry (Puget, Grill et al. 2009; Peyre, Commo et al. 2010).

Other work has identified the dysregulated expression of additional genes at paediatric ependymoma recurrence, such as over-expression of the PTEN homologue *LOC374491* (13q12.12) and under-expression of *NF-KB2* (10q24.32) and *PLEK* (2p14) (Sowar, Straessle et al. 2006). The importance of telomeric maintenance and elongation in ependymoma progression, facilitated by human telomerase protein expression, has also been suggested (Ridley, Rahman et al. 2008). However, these studies were again performed on small paediatric cohorts, comprised of only seven recurrent tumours. Nevertheless, immunohistochemical and expression analysis of 115 paediatric ependymomas also found increased expression of the telomerase subunit hTERT correlated with ependymoma progression and, in conjunction with telomere dysfunction, was associated with a worse recurrence free (and overall) patient survival (Tabori, Wong et al. 2008). Although this may suggest a putative role for hTERT in

paediatric ependymoma relapse, controversy remains regarding the immunohistochemical detection of this protein. A re-evaluation of the hTERT antibody (NCL-hTERT) used by Tabori et al. verified the actual detected target to be Nucleolin, a phosphoprotein which acts as a nuclear chaperone (Wu, Dudognon et al. 2006). This issue is discussed in Chapter 6 which examines putative biological prognostic markers in childhood ependymoma.

While certain genes or proteins have been proposed from these analyses, consistently reported candidates remain elusive. In addition, the majority of previous work has focussed on examining only aberrations acquired at relapse, without considering anomalies that may be present in the primary tumour and sustained into subsequent recurrences. The Affymetrix® 500K SNP array analysis presented in this thesis has been performed on a representative cohort of 63 paediatric ependymomas, including 21 recurrent tumours from 11 children (Chapter 3, section 3.2.1). To date, this represents the largest cohort of recurrent childhood ependymomas analysed on such a high resolution genomic platform. In the preceding chapters, independent analyses of primary and intracranial first recurrent ependymomas were performed. However, incorporated within the SNP array tumour cohort were eight sets of patient-matched primary and recurrent ependymomas. It was hoped that the identification of genomic aberrations that were either maintained or acquired across the primary and recurrent ependymomas of these sets may provide a more refined insight into the potential implication of genomic imbalance in aspects of tumour recurrence.

The following hypotheses were explored:

- The high resolution genomic analysis of paired primary and recurrent paediatric ependymomas will identify shared regions or genes with imbalances which are maintained from primary to recurrent ependymomas, thereby potentially being involved in sustaining tumourigenesis.
- The high resolution genomic analysis of paired primary and recurrent paediatric ependymomas will identify shared regions or genes with imbalances acquired only at tumour recurrence which could potentially be involved in disease progression and therapeutic resistance.

5.2 Materials and methods

5.2.1 The sample cohort

Tumour and blood DNA extraction is outlined in Chapter 2, sections 2.1.3 – 2.1.4. An overview of the 500K SNP array protocol and data processing procedures followed are described in Chapter 2, sections 2.2.1 – 2.2.5. Clinical data for the entire SNP array cohort is summarised in Chapter 3, Figure 3.1 and the comprehensive data set is detailed in Chapter 3, Table 3.1. The eight sets of primary and recurrent tumours comprised 23 tumours, including samples 9P – 9R5 (set A), 16P – 16R1 (set B), 17P – 17R2 (set C), 18P – 18R1 (set D), 20P – 20R1 (set E), 26P – 26R3 (set F), 35P – 35R2 (set G) and 40P – 40R1 (set H). Four sets (A, B, F, and G) consisted of a primary posterior fossa ependymoma and a varying number of local recurrences. Three sets (C, D and E) were comprised of a primary supratentorial tumour and corresponding local recurrences. Set H, by contrast, included a primary posterior fossa ependymoma and its spinal recurrence.

5.2.2 Genomic imbalance data analysis

Global genomic imbalance data analysis was performed as detailed in Chapter 3, section 3.2.1.2, adopting an 80 % chromosome arm and cytoband imbalance threshold whilst excluding probes on chromosomes 21p and X as discussed previously. High resolution analysis was also performed for all tumour samples, generating gene lists from annotated copy number data as described previously in Chapter 4, section 4.2.1.2. Focal regions of maintained or acquired imbalance were thereby identified across individual sets and subsequently the entire cohort to find commonly shared loci (Appendices 10J – M). The formulaic method adopted for this process is provided in Appendix 7. A regional imbalance was defined as maintained if it was present in the primary tumour and all subsequent recurrences for that ependymoma set. A regional imbalance was defined as acquired if it was either gained or lost in all the recurrent tumours of a set, yet had a normal copy number or contrasting imbalance in the corresponding primary tumour.

As before (Chapter 4, section 4.2.1.2), only lists of SNP-assigned genes were prepared, except for focal regions of gain encompassing genes on the long arm of chromosome 9. All genes within these regions were identified using the web-based genomic database Ensembl (http://www.ensembl.org) and included in the lists presented, in order to verify whether candidates identified from the work of Puget et al. (Puget, Grill et al. 2009) were also detected in this study. The Spotfire Decision Site® and SNPview programmes were used to visualise the resulting copy number imbalance data as described in Chapter 2, section 2.2.5. Analysis of the eight primary and recurrent ependymoma sets in this way enabled a comparison of genetic anomalies identified at both presentation and relapse, at varying degrees of genomic resolution.

5.3 Results

5.3.1 Chromosome arm and cytoband imbalance identified in eight primary and recurrent ependymoma sets

A Spotfire Decision Site® generated heatmap enabled a genome wide visualisation of copy number aberrations present in all primary and recurrent ependymomas comprising each of the eight tumour sets (Figure 5.1). The chromosome arm and cytoband imbalances present in the members of each tumour set are also summarised in Table 5.1. In two sets (A and B), the tumour genome was balanced at diagnosis (defined as $\geq$ 95 % of all analysed SNP probes in the primary ependymoma demonstrating a diploid copy number).

Chromosomal regions of genomic imbalance, maintained from the primary tumour to corresponding recurrences, were present in 3/8 ependymomas sets (sets D, F and H). The gain of 9p11.1 - 9p12 was common to two of these sets, while all other maintained aberrations detected were present in a single set (Table 5.1). The exclusive acquisition of genomic anomalies by recurrent tumours was a more frequent phenomenon, occurring in the majority of ependymoma sets (5/8 sets; A, B, D, E and F). Again, whilst most of the acquired anomalies were confined to the recurrent ependymomas within a single set, the gain of smaller regions within chromosome 1q (1q21.1 - 21.2, 1q22, 1q24.1, 1q25.2 - 25.3, 1q32.3 and 1q42.11) was identified as an acquired

alteration in two tumour sets (B and E). Broad imbalances identified as both maintained and acquired at recurrence included gain of chromosome 1q and chromosome 9, gain of regions within chromosome 8 and deletion involving chromosome 22q.

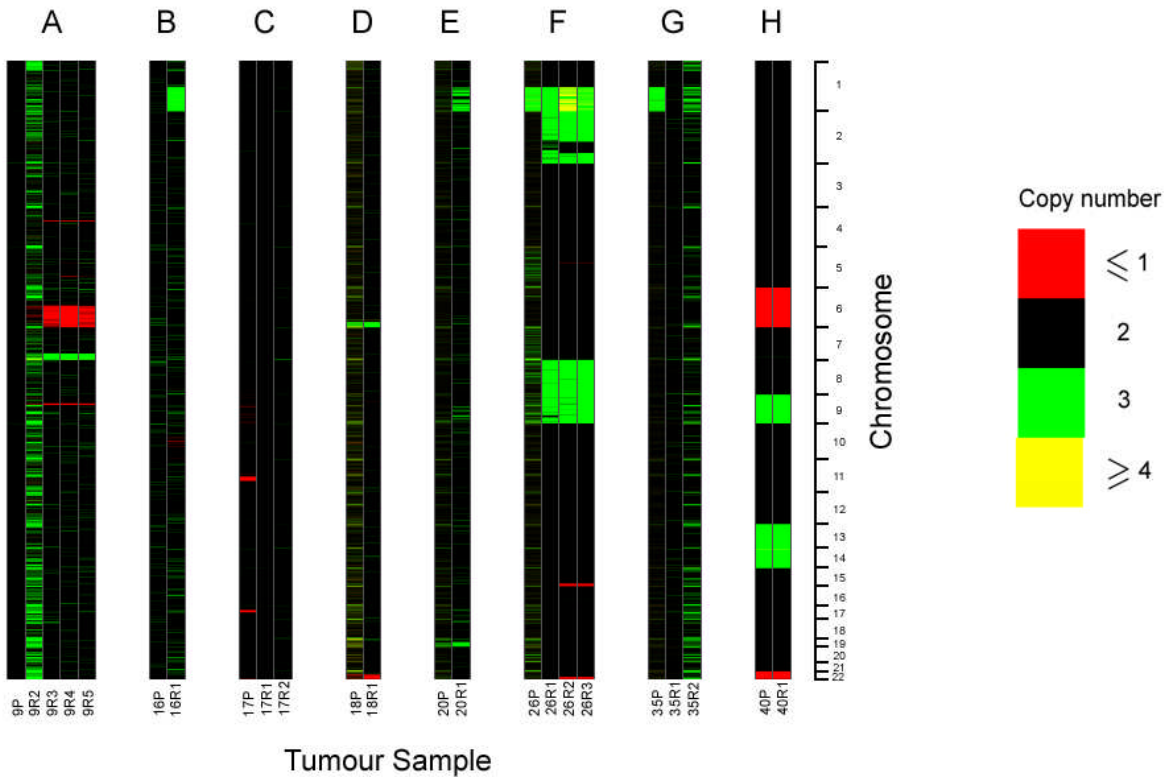

Figure 5.1: Spotfire Decision Site® copy number heatmap demonstrating Affymetrix® 500K SNP array results across the genome for eight patient-matched sets of primary and subsequent recurrent ependymomas. Diploid genomic regions are coloured black. Regions exhibiting genomic loss are coloured red, while regions demonstrating gain are coloured green or yellow, depending on whether the gain represents a copy number of three or greater respectively.

Within set A, which consisted of four recurrences, certain chromosome arm imbalances were only present in individual recurrent ependymomas and were thereby not classified as maintained or acquired aberrations as they were neither present in the primary tumour (sample 9P), nor sustained in all other recurrences of the set. These included the gain of chromosomes 19 and 22q in sample 9R2, and the loss of chromosome 6q in sample 9R4. Furthermore, in two tumour sets (C and G), no broad genomic imbalances were designated as maintained or acquired. Gain of chromosome 1q was evident in the primary sample (35P) of set G, although this was not maintained in either of the two recurrent samples, 35R1 and 35R2, while chromosome arm or cytoband copy number aberrations were completely absent in any of the tumours of ependymoma set C. Nevertheless, this did not preclude the potential of subsequent higher resolution analysis to detect maintained or acquired copy number aberration within more focal genomic regions of these, or any of the eight ependymoma sets.

Table 5.1: Broad genomic regions of maintained and acquired copy number imbalance (whole chromosome, chromosome arm or cytoband) in the eight primary and recurrent paediatric ependymoma sets.

Ependymoma tumour set	Regions of maintained genomic gain (chromosome, arm or cytoband)	Regions of maintained genomic loss (chromosome, arm or cytoband)	Regions of acquired genomic gain (chromosome, arm or cytoband)	Regions of acquired genomic loss (chromosome, arm or cytoband)
A (samples 9P - 9R5)			7q32.2 - 7q36.3	
B (samples 16P - 16R1)			1q	
C (samples 17P - 17R2)				
D (samples18P - 18R1)	6q25.2 - 6q27			22q12.3 - 22q13.33
E (samples 20P - 20R1)			1q21.1 - 21.2, 1q22, 1q24.1, 1q25.2 - 25.3, 1q32.3, 1q42.11 19q13.11 - 19q13.31, 19q13.42	
F (samples 26P - 26R3)	1q 8p23.3, 8q12.2 9p11.1 - 9p12		2, 8, 9	
G (samples 35P - 35R2)				
H (samples 40P - 40R1)	9, 13, 14	6, 22q		

Note: Regional copy number imbalances (chromosome arm or cytoband) were defined using an 80 % imbalance threshold (Chapter 3, section 3.2.1.2). P = primary, R1 – R5 = 1st – 5th recurrence.

5.3.2 Common focal regions of maintained copy number alterations

The in-house SNPview program was used to produce a genome wide visualisation of the copy number alterations maintained from primary to recurrent tumours within the eight paediatric ependymoma sets A – H (Figure 5.2). Focal regions of maintained genomic gain and loss shared across the eight tumour sets were identified through the generation of gene lists assembled from individual sets (section 5.2.2, Appendices 10J and 10K), then ordered according to frequency of occurrence (Tables 5.2 and 5.3).

Maintained regions of genomic gain were observed more frequently than loss. The most common focal maintained gains involved chromosomes 1q and 9, specifically within the regions 1q41 – 1q44, 9p13 and 9q21.33 – 9q34.11 (affecting 3/8 sets, 38 %). Some of the focal loci identified within these regions were found to encompass genes already identified as putative ependymoma oncogenes (*COL27A1* (9q32)) (Modena, Lualdi et al. 2006), or reported to regulate processes such as mitosis (*CENPF* (1q41)), cell invasiveness (*CDC42BPA/CDC42MRCK* (1q42.13)) and neurite formation (*DNM1* (9q34.11)) (Torre, McNiven et al. 1994; Liao, Winkfein et al. 1995; Wilkinson, Paterson et al. 2005).

The most common focal regions of maintained genomic loss were exclusively on chromosome 22, specifically within the regions 22q12.2 – 12.3 and 22q13.31 – 13.33 (2/8 sets, 25 %), encompassing candidate tumour suppressor genes identified from other studies, including *LARGE* (22q12.3) and the metalloproteinase inhibitor *TIMP3* (22q12.3) (Darnton, Hardie et al. 2005; de Bernabe, Inamori et al. 2009).

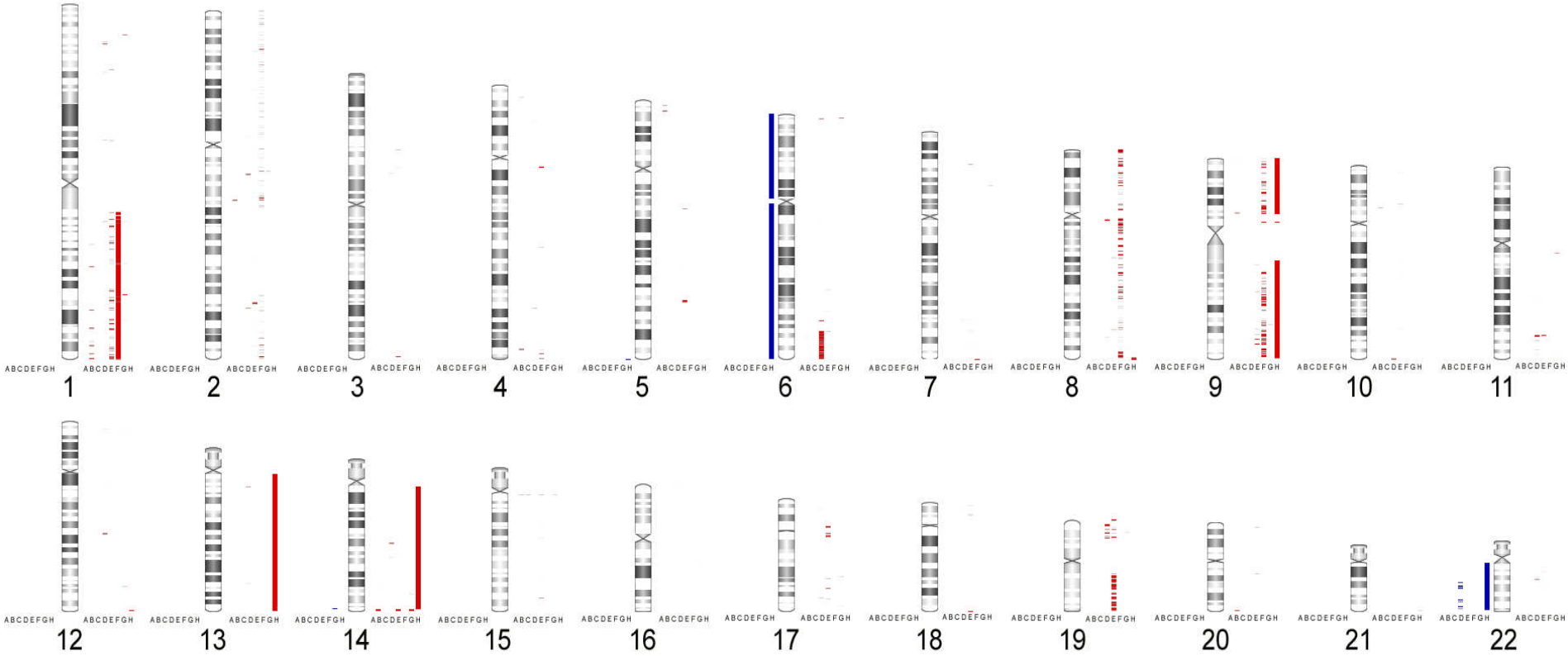

Figure 5.2**:** SNPview chromosome ideogram of maintained copy number imbalances in the eight patient-matched primary and recurrent ependymoma sets analysed using Affymetrix® 500K SNP arrays. The imbalances correspond to a particular ependymoma set (identified by the labels A – H). Maintained genomic gains are shown to the right of each chromosome (coloured red), while maintained genomic losses are to the right (coloured blue).

Table 5.2: The most common focal regions of maintained copy number gain in the eight primary and recurrent ependymoma sets.

Cytoband	Start (bp)	End (bp)	Gene symbol	Additional genes in this region	Frequency
1q41	212729463	212921197	*PTPN14* (i) – *KCNK2* (u)	*CENPF*	3/8
1q42.13	225524609	225736961	*CDC42BPA* (i) - *ENST00000385421* (u)	*ENST00000358560*	3/8
1q43	234907042	234923247	*ACTN2* (u - i)		3/8
1q44	247069146	247135059	*OR5BU1* (u) – *ZNF692* (u)	*SH3BP5L, ZNF672*	3/8
9p13.1	38364977	38616309	*ALDH1B1* (u) – *ENST00000377679* (d)	*ALDH1B1, IGFBPL1, ANKRD18A, ENST00000357927, ENST00000377679*	3/8
9q22.31	93153985	93232625	*AUH* (i) – *ROR2* (u)	*NFIL3*	3/8
9q32	115948274	115961754	*KIF12* (d) – *COL27A1* (i)		3/8
9q33.1	119539087	119657773	*TLR4* (d)		3/8
9q33.2	123248672	123301053	*ENST00000373793* (i)		3/8
9q33.3	126575813	127263851	*OLFML2A* (u) – *MAPKAP1* (i)	*OLFML2A, ENST00000373579, WDR38, RPL35, ARPC5L, GOLGA1, C9orf126, PPP6C, RABEPK, GAPVD1, ENST00000336505*	3/8
9q34.11	129895082	130612724	*SLC25A25* (i) – *C9orf114* (i)	*C9orf16, DNM1, GOLGA2, TRUB2, COQ4, URM1, ODF2, GLE1, SPTAN1, SET, ZER1, ZDHHC12, PKN3, PDGES2, TBC1D13, WDR34, CERCAM, SLC27A4, C9orf119, CIZ1, LCN2,*	3/8

Note: The gene symbols representing the start and end of each specified genomic region are shown, together with additional encompassed genes identified from the Affymetrix® annotation file (Netaffx file build 07.12.07). bp = base pair, i = intronic, u = upstream of annotated gene, d = downstream of annotated gene.

Table 5.3: The most common focal regions of maintained copy number loss in the eight primary and recurrent ependymoma sets.

Cytoband	Start (bp)	End (bp)	Gene symbol	Additional genes in this region	Frequency
22q12.2	30384415	30661594	*ENST00000336566* (i) –*ENST00000248984* (i)	*C22orf30, ENST00000327423, DEPDC5, ENST00000365711*	2/8
22q12.3	31559830	31773903	*SYN3* (i) – *TIMP3* (d)	*TIMP3*	2/8
22q12.3	31998879	33103088	*LARGE* (i) – *ISX* (u)		2/8
22q12.3	33794973	34067214	*ISX* (i) – *TOM1* (i)	*HMG2L1*	2/8
22q12.3	34136238	34394033	*MCM5* (i) – *APOL6* (3'UTR)	*RASD2, MB*	2/8
22q12.3	34930787	34978498	*APOL4* (5'UTR) – *APOL1* (u)	*APOL2*	2/8
22q13.2	41986266	42527300	*SCUBE1* (i) – *EFCAB6* (i)	*MPPED1*	2/8
22q13.31	43112879	43311741	*LDOC1L* (u-d)		2/8
22q13.31	46003628	46109538	*TBC1D22A* (d)		2/8
22q13.32	47717016	47832385	*FLJ44385* (u)		2/8
22q13.33	48885887	48939672	*MOV10L1* (i)		2/8

Note: The gene symbols representing the start and end of each specified genomic region are shown, together with additional encompassed genes identified from the Affymetrix® annotation file (Netaffx file build 07.12.07). bp = base pair, i = intronic, u = upstream of annotated gene, d = downstream of annotated gene, 3'UTR = 3' untranslated region, 5'UTR = 5'untranslated region.

5.3.3 Common focal regions of acquired copy number alterations

SNPview was again used to provide an overview across the tumour genome of the copy number alterations acquired at recurrence within the eight paediatric ependymoma sets (Figure 5.3). As for maintained aberrations, focal regions of acquired genomic gain and loss shared across the eight tumour sets were identified through the generation of gene lists assembled from individual sets (section 5.2.2, Appendices 10L and 10M), then ordered according to frequency of occurrence (Tables 5.4 and 5.5).

Regions of genomic gain were more frequently acquired at tumour recurrence than loss. The most common focal regions of acquired copy number gain were observed within various regions across chromosomes 1q, 2q, 7q, 8q and 9q, in addition to regions confined within 2p23.1, 6q25.3 – 6q26 and 17p13.2 (affecting 3/8 sets in each case, 38 %). Several of these focal regions were found to encompass genes reported to be involved with neurite growth and guidance, cell adhesion and cell migration, such as *LAMC1* (1q25.3), *CNTN2/TAX1* (1q32.1), *ALK* (2p23.1) and *MKLN1* (7q32.3) (Adams, Seed et al. 1998; Rickman, Tyagi et al. 2001; Wiksten, Liebkind et al. 2003; Motegi, Fujimoto et al. 2004). Genes reported to have a role in mediating mitosis or inhibiting apoptosis were also identified within certain loci, including *HAX1* (1q21.3), *KIF12* (9q32) and *PAXIP1/PTIP* (7q36.2) (Cho, Prindle et al. 2003; Lakshmikanth, Warrick et al. 2004; Vafiadaki, Sanoudou et al. 2007).

Focal regions of acquired genomic loss were not shared between the ependymoma groups, occurring only in individual sets, particularly A, B and D (1/8 sets, 13 %). The majority of these focal loci mapped to regions spanning the chromosome arms of 6q, 10q and 22q, together with smaller regions within 1q23.3, 2q24.3, 9p21.1 – 21.3 and 21q22.3. Certain focal regions of loss were found to harbour tumour suppressor genes previously identified in ependymoma, such as *CDKN2A*, *CDKN2B* (9p21.3) and *PTEN* (10q23.2)(Taylor, Poppleton et al. 2005; Johnson, Wright et al. 2010).

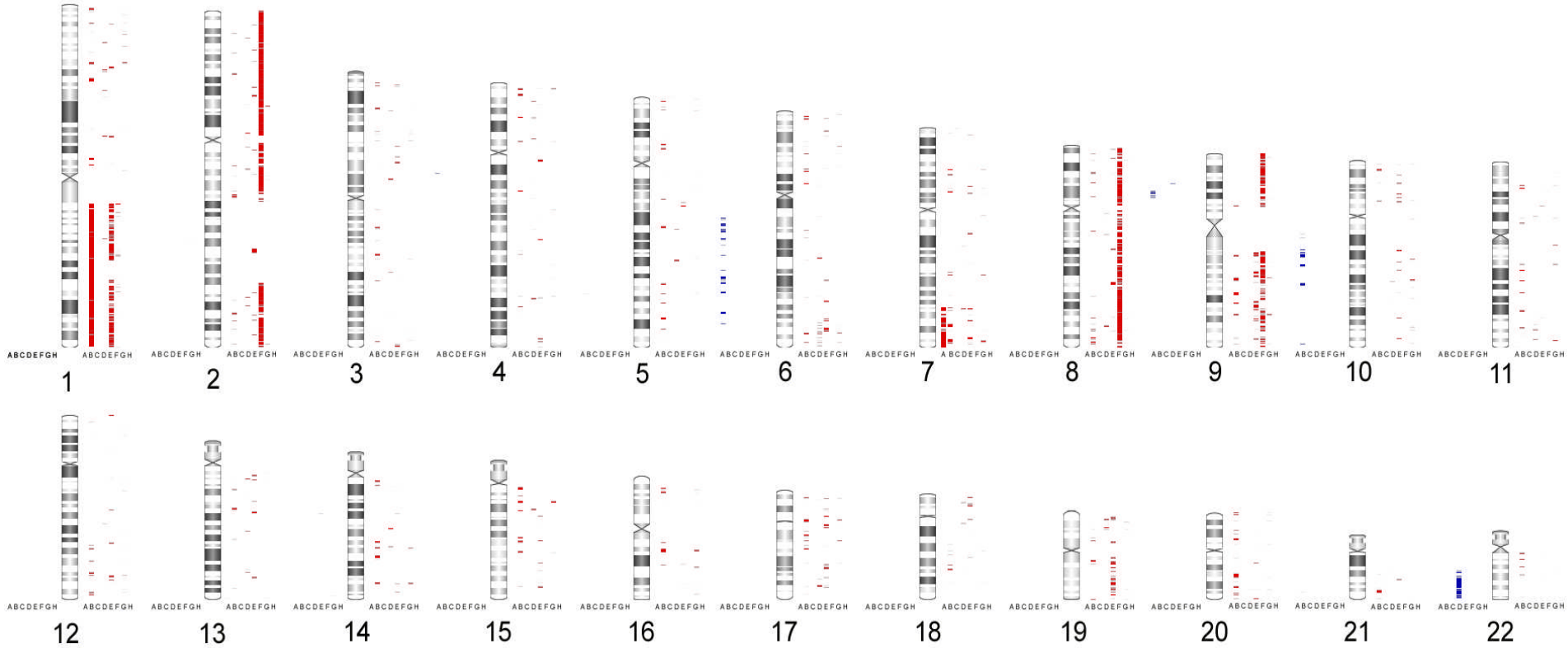

Figure 5.3**:** SNPview chromosome ideogram of acquired copy number imbalances in the eight primary and recurrent ependymoma sets analysed using Affymetrix® 500K SNP arrays. The imbalances correspond to a particular ependymoma set (identified by the labels A – H). Maintained genomic gains are shown to the right of each chromosome (coloured red), while maintained genomic losses are to the right (coloured blue).

Table 5.4: The most common focal regions of acquired copy number gain in the eight primary and recurrent ependymoma sets.

Cytoband	Start (bp)	End (bp)	Gene symbol	Additional genes in this region	Frequency
1q32.2	206609806	206620808	*PLXNA2* (d)		4/8
1q21.1	142756696	149763764	*ENST00000360154* (i) – *CGN* (i)	*PDE4DIP, NOTCH2NL, HFE2*	3/8
1q21.3	152491968	152513878	*UBAP2L* (i) – *HAX1 (i)*		3/8
1q25.2	177104622	177190048	*RALGPS2* (i) – *ANGPTL1* (d)	*ANGPTL1*	3/8
1q25.3	179663847	179693533	*ENST00000367573* (i)		3/8
1q25.3	180322120	180530619	*ZNF648* (u)		3/8
1q25.3	181280422	181383596	*LAMC1* (i) – *LAMC2* (u)		3/8
1q32.1	203287311	203431777	*CNTN 2*(i) – *RIPK5* (i)		3/8
1q42.11	222387408	222417646	*FBXO28* (i-d)		3/8
2p23.1	29807920	29973277	*ALK* (i)		3/8
2p23.1	30011689	30145982	*YPEL5* (u)		3/8
2q13	112179426	112184559	*ANAPC1* (d)		3/8
2q14.2	119269235	119294068	*INSIG2* (d)		3/8
2q36.1	223539272	223702372	*ACSL3* (u) – *KCNE4* (d)	*KCNE4*	3/8
2q37.3	240142568	240199105	*HDAC4* (d) – *NDUFA10* (u)	*FLJ45964*	3/8
6q25.3- 6q26	160711970	161096246	*SL22A3* (i) – *PLG* (d)	*LPA, LPA2, PLG*	3/8
7q32.2	129805023	129814869	*CPA1* (u-i)		3/8
7q32.3	130410338	130881572	*MKLN1* (u) – *PODXL* (i)	*MKLN1*	3/8
7q36.2	154134150	154409671	*DPP6* (i) – *PAXIP1* (i)		3/8
7q36.2	154471567	154695638	*HTR5A* (u) - *ENST00000389257* (i)	*HTR5A*	3/8
7q36.3	154730440	155073465	*INSIG1* (i-d)	*ENST00000389257, ENST00000321736, EN2*	3/8
8q24.22	133471013	133485000	*KCNQ3* (i)		3/8
8q24.3	139181340	140766331	*FAM135B* (d) – *KCNK9* (i)		3/8
9q21.13	73784219	73955600	*C9orf85*(i) – *GDA(i)*	*C9orf57*	3/8
9q21.33	89358871	89516915	*DAPK1*(i)		3/8
9q22.2	92751273	92795018	*SYK* (d)		3/8
9q22.23	100251588	100291497	*GABBR2* (i)		3/8
9q32	115811870	115939079	*ZNF61 8*(i) – *COL27A1* (d)	*AMBP, KIF12*	3/8

Note: The gene symbols representing the start and end of each specified genomic region are shown, together with additional encompassed genes identified from the Affymetrix® annotation file (Netaffx file build 07.12.07). bp = base pair, i = intronic, u = upstream of annotated gene, d = downstream of annotated gene, 3'UTR = 3' untranslated region, 5'UTR = 5'untranslated region.

Table 5.5: The most common focal regions of acquired copy number loss in the eight primary and recurrent ependymoma sets.

Cytoband	Start (bp)	End (bp)	Gene symbol	Additional genes in this region	Frequency
1q23.3	162509350	162537740	*ENST00000362979* (u)		1/8
4q13.1	65457872	65599025	*EPHA5* (d)		1/8
6q14.1	78195443	78854198	*HTR1B* (u) – *IRAK1BP1* (u)	*HTR1B*	1/8
6q14.1	81282268	81811390	*BCKDHB* (d)		1/8
6q14.1	82325403	82887157	*ENST00000237194* (d) – *IBTK* (u)	*FAM46A*	1/8
6q14.3	85111610	85301899	*KIAA1009* (u)		1/8
6q14.3	85564031	85707419	*ENST00000230608* (d) – *ENST00000330469* (u)		1/8
6q14.3	86786670	87557058	*ENST00000365233* (d) – *HTR1E* (u)		1/8
6q16.1	91958814	92462813	*MAP3K7* (u)		1/8
6q16.1	92735946	92969850	*ENST00000363622* (d) – *ENST00000386471* (u)		1/8
6q16.1	96830505	97115399	*FUT9* (d) – *FHL5* (u)	*KIAA0776*	1/8
6q16.3	104113500	104180806	*GRIK2* (d) – *HACE1* (u)		1/8
6q22.31	119664378	121037192	*MAN1A1* (i) - *ENST00000384130* (u)		1/8
6q22.31	121334610	121425761	*C6orf170* (d)		1/8
6q22.31	122657393	122731099	*HSF2* (u)		1/8
6q22.31	123633123	124813067	*TRDN* (i) – *TBCA1* (i)	*ENST00000334268*	1/8
6q22.33	130137425	130298039	*C6orf191* (u) – *L3MBTL3* (u)	*C6orf191*	1/8
6q23.1	130562636	131203958	*SAMD3* (i) – *EPB41L2* (i)	*ENST00000368134, KIAA1913*	1/8
6q24.2	145020197	145939315	*UTRN* (i) – *EPM2A* (u)		1/8
6q25.2	153799127	154049312	*ENST00000364238* (d) – *OPRM1* (u)		1/8
9p21.3	21899000	22166961	*ENST00000380190* (i) – *CDKN2A* (d)	*CDKN2A, CDKN2B*	1/8
9p21.2	27715305	28084657	*C9orf72* (d) – *LINGO2* (i)		1/8
9p21.1	28807957	29075968	*LINGO2* (d)		1/8
9p21.1	30799413	31139228	*ENST00000360120* (u)		1/8
10q21.1	53347641	53839390	*PRKG1* (i) – *MBL2* (d)		1/8
10q21.1	55658985	56081922	*PCDH15* (i)		1/8
10q21.2	64320624	65105984	*EGR2* (d) – *REEP3* (d)	*ENST00000362576, NRBF2, JMJD1C, REEP3*	1/8
10q21.3	68107070	76813473	*CTNNA3 (i) – ZNF503 (d)*	*SIRT1, HERC4, DNAJC12, MYPN, PBLD, ENST00000358410, SLC25A16, AP3M1, ADK, MYST4, DUPD1, DUSP13, SAMD8, VDAC2*	1/8

Cytoband	Start (bp)	End (bp)	Gene symbol	Additional genes in this region	Frequency
10q23.2	88987279	89858910	*ENST00000330762* (i) – *C10orf59* (u)	*MINPP1, PAPSS2, ATAD1, PTEN*	1/8
10q23.31	90242704	90490501	*C10orf59* (i) – *ENST00000371932* (i)	*LIPJ, LIPF*	1/8
10q26.3	132490668	132630798	*TCERG1L* (d)		1/8
21q22.3	42049134	42159421	*RIPK4* (i) – *PRDM15* (i)		1/8
22q12.2	29431646	30373630	*OSPB2* (i) – *ENST00000336566* (i)	*MORC2, PIB5PA, PLA2G3, RNF185, LIMK2, PIK3IP1, PATZ1, ENST00000331488, EIF4, ENIF1, FSI1, PISD*	1/8
22q12.3	Inclusive				1/8
22q13.1	Inclusive				1/8
22q13.2	Inclusive				1/8
22q13.31	Inclusive				1/8
22q13.32	Inclusive				1/8
22q13.33	Inclusive				1/8

Note: The gene symbols representing the start and end of each specified genomic region are shown, together with additional encompassed genes identified from the Affymetrix® annotation file (Netaffx file build 07.12.07). All genes within the region of 22q12.3 – 22q13.33 are included in this table. The term 'inclusive' signifies this to prevent the table from being exhaustive. bp = base pair, i = intronic, u = upstream of annotated gene, d = downstream of annotated gene, 3'UTR = 3' untranslated region, 5'UTR = 5'untranslated region.

5.4 Discussion

This SNP array analysis of copy number alteration across eight sets of patient-matched primary and recurrent ependymomas enabled the identification of genomic aberrations that were either maintained throughout diagnosis and relapse, or only acquired with recurrence, thereby providing a more precise assessment of genomic imbalance in paediatric ependymoma initiation, maintenance and progression than previous studies.
In general, genomic gains were observed more frequently than losses, both as maintained and acquired copy number aberrations. This suggests that oncogenic activation may be a more common phenomenon than the deletion of tumour suppressor genes, potentially driven at recurrence by genomic instability secondary to adjuvant therapy (Goldberg 2003). The majority of ependymoma sets (A, B, D, E, F and H) demonstrated broad genomic imbalances that were either maintained or acquired. Of the remaining two sets, set G revealed numerous acquired aberrations at high resolution analysis (Appendices 10L and 10M), highlighting the value of the 500K SNP array to discover copy number alterations which would have been undetectable using preceding platforms such as conventional and array CGH. Set C did not contain significant copy number alteration in either the primary tumour (17P) or the recurrences (17R1 and 17R2). This indicates that, at least in a subset of paediatric ependymomas, alternative mechanisms to genomic imbalance are responsible for disease initiation and progression. These potentially include the dysregulation of ependymoma gene expression profiles by epigenetic phenomena such as gene promoter methylation or histone deacetylation. Indeed, a methylation profile analysis of ependymomas from these patient-matched primary and recurrent tumour sets will be performed in the future (Chapter 7, section 7.2).

The high genomic resolution of this SNP array analysis also enabled identification of the most common focal regions of maintained and acquired copy number alteration across the panel of ependymoma sets. Although no focal aberration was universal to all eight tumour groups, one acquired genomic gain on chromosome 1q was present in half of the sets, while the majority of the other most frequent changes were evident in over one-third of the cohort. This suggests that these alterations could have important roles in

aspects of tumour recurrence for a significant subpopulation of paediatric ependymomas.

5.4.1 Candidate regions of maintained and acquired copy number gain

This comparison of copy number imbalance across the eight paediatric ependymoma sets revealed aberrant genomic regions implicated in recurrence that have been observed in other analyses, while also identifying novel potential candidate genes warranting further assessment, some of which are now discussed.

Focal regions of genomic gain within chromosome 1q were amongst the most common maintained and acquired alterations identified across the tumour panel (Tables 5.2 and 5.4). This supports the observation from the CGH work of Dyer et al. that 1q gain was the most frequent imbalance seen at ependymoma relapse in children (Dyer, Prebble et al. 2002). Indeed, the meta-analysis of all CGH studies performed on paediatric ependymomas (Chapter 1, section 1.5.2) (Kilday, Rahman et al. 2009) also revealed chromosome 1q gain as the most frequent aberration in both 187 primary and 50 recurrent paediatric intracranial tumours, suggesting genes within this chromosome arm may have a role in tumourigenesis, maintenance and progression.

In this study, the most common focal regions of maintained genomic gain on chromosome 1q were confined within loci 1q41 – 1q44, containing genes such as *CENPF* (1q41) and *CDC42BPA* (1q42.13) (Table 5.2). *CENPF* has been implicated in mitotic regulation by encoding a component of the kinetochore (Liao, Winkfein et al. 1995) and was one of the 87 genes recently reported to contribute to the expression signature of paediatric ependymoma recurrence through its increased expression (Peyre, Commo et al. 2010). *CDC42BPA* is located within a novel region of amplification for ependymomas (Johnson, Wright et al. 2010). This gene encodes a member of the serine/threonine protein kinase family that is thought to be a downstream activator of the GTPase CDC42 (Govek, Newey et al. 2005). *CDC42BPA* may mediate CDC42 induced peripheral actin formation, thereby contributing to the contractility required for cell invasiveness (Wilkinson, Paterson et al. 2005). In addition, CDC42 is required for apical neuroepithelial progenitor cells to retain their capacity for self renewal (Cappello,

Attardo et al. 2006), suggesting that the genomic gain of *CDC42BPA* could consequently contribute to both ependymoma initiation and subsequent recurrence.

In contrast, the most common focal regions of acquired 1q gain were evident across the chromosome arm, encompassing the 1q21.1-32.1 region associated with recurrence from an array CGH analysis of 68 ependymomas from a mixed age cohort (Mendrzyk, Korshunov et al. 2006) (Table 5.4). Genes identified included the anti-apoptotic gene *HAX1* (1q21.3) which has been shown *in vitro* to promote cell migration and invasion in head and neck tumours (Ramsay, Keppler et al. 2007) and *CNTN2/TAX1* (1q32.1), which encodes a neuronal membrane protein thought to be involved in axon connection formation in the developing nervous system (Karagogeos, Pourquie et al. 1997). While immunohistochemical analysis has revealed CNTN2 overexpression in glioblastoma multiforme, *in vitro* studies have suggested CNTN2 may be implicated in the invasiveness and migration of high grade glioma cells (Rickman, Tyagi et al. 2001). Moreover, *CNTN2/TAX1* was another of the overexpressed signature genes reported by Peyre et al. to be specifically associated with childhood ependymoma recurrence (Peyre, Commo et al. 2010). These genes therefore warrant functional investigation for a role in ependymoma progression, as discussed in the final chapter.

Focal gains within chromosome 9q were also identified to be among the most common maintained and acquired genomic alterations across the eight ependymoma sets of this study. Common focal regions of maintained, although not acquired gain were revealed within the subtelomeric regions of chromosome 9 (Table 5.2), lending some credence to the observation by Puget et al. of an association between chromosome 9q33-34 gain and paediatric ependymoma recurrence from array CGH work (Puget, Grill et al. 2009). However, the genes within the focal regions of 9q33 – 34 identified from this analysis were different to the candidates postulated by Puget, possibly reflecting the discordant genomic coverage and precision of the two array platforms. One such example was the maintained gain of *DNM1*, detected in 3/8 ependymoma sets (38 %). The gene is located within 9q34.11, a region already associated with anaplastic intracranial ependymomas from preceding aspects of this work (Chapter 3, Table 3.9). *DNM1* is thought to play a role in synaptogenesis and neural plasticity and has been shown to have a dysregulated expression profile in particular subgroups of ependymoma (Torre, McNiven et al. 1994; Johnson, Wright et al. 2010). In addition, this study identified

numerous other focal loci spanning the chromosome 9q arm which may be implicated in ependymoma maintenance or progression (Tables 5.2 and 5.4). For instance, the gain of the *COL27A1* within 9q32 was identified as both a maintained and acquired alteration in 3/8 sets (38 %). *COL27A1* encodes a member of the extracellular matrix collagen family (Pace, Corrado et al. 2003). Array CGH and expression work has already shown *COL27A1* to be amplified and overexpressed in primary ependymomas from infants, suggesting copy number driven dysregulation (Modena, Lualdi et al. 2006). This study now suggests that, in addition to tumourigenesis, the gene may also have a role in paediatric ependymoma recurrence.

In Chapter 3 of this work, a comparison of SNP derived chromosome arm imbalances between paediatric ependymoma subgroups revealed gain of chromosome 8 was associated with intracranial recurrent tumours (Chapter 3, section 3.3.4). Indeed, gain of chromosome 8 or 8q have been proposed as markers of tumour recurrence in other solid cancers including Ewing's sarcoma (Tarkkanen, Kiuru-Kuhlefelt et al. 1999) and high grade prostate cancer (Visakorpi, Kallioniemi et al. 1995). In this refined analysis of ependymoma recurrence, gain of chromosome 8 was observed as an acquired alteration in only one of the ependymoma sets (set F) (Table 5.1). Nevertheless, high resolution analysis identified focal regions of acquired gain within 8q24.22 and 8q24.3 in 3/8 sets (38 %). These focal regions were found to encompass genes encoding potassium channels responsible for neuronal excitation such as *KCNQ3* (8q24.22) and *KCNK9* (8q24.3), a candidate oncogene for recurrence known to be amplified and overexpressed in ependymoma and several other cancers such as breast, lung, colon cancer (Mu, Chen et al. 2003; Kim, Cho et al. 2004; Johnson, Wright et al. 2010).

Focal gains within other chromosomes were identified in this study, particularly as acquired alterations (Table 5.4). These included gain of *PAXIP1/PTIP* (7q36.2), a member of the paired box gene family (3/8 sets, 38 %). *PAXIP1/PTIP* encodes a protein involved in maintaining genomic stability and cellular proliferation which has been shown *in vitro* to promote cellular resistance to ionising radiation, a principal adjuvant therapy in paediatric ependymoma (Jowsey, Doherty et al. 2004). In addition, a review of the most common focal regions of acquired gain revealed an enrichment for genes regulating neurite growth and guidance, cell adhesion and cell migration including *ALK* (2p23.1), *MKLN1* (7q32.2) and genes on chromosome 1q such as *LAMC1* (1q25.3) and

the aforementioned *CNTN2/TAX1* (1q32.1) (Adams, Seed et al. 1998; Rickman, Tyagi et al. 2001; Wiksten, Liebkind et al. 2003; Motegi, Fujimoto et al. 2004). Indeed, the most frequent region of acquired gain was found downstream of *PLXNA2* (1q32.2), a gene encoding a member of the plexin A-family of semaphorin co-receptors implicated in axon guidance, invasive cell growth and migration (reviewed by (Trusolino and Comoglio 2002; Negishi, Oinuma et al. 2005)). Some of the most frequently maintained focal regions of gain in this analysis were also found to encompass genes involved in neurite growth. Further functional assessment of the mechanisms by which these genes appear implicated in paediatric ependymoma recurrence should be considered as activation may promote cell invasiveness into surrounding structures and tumour spread. Consequently, this could facilitate local relapse by reducing the possibility of achieving complete surgical resection, while increased cell migration may encourage the development of more distant tumour recurrences. It may also help elucidate whether future therapy should aim to target a particular mechanism or pathway promoting disease progression, rather than individual genes. Again, such analysis is discussed in the last chapter.

5.4.2 Candidate regions of maintained and acquired copy number loss

Although less frequent than gain, focal regions of genomic loss within chromosome 22q were evident from this analysis as both maintained and acquired alterations, making chromosome 22q the most common site for genomic deletions implicated in ependymoma recurrence. This is consistent with results from the meta-analysis of all reported paediatric CGH studies performed on recurrent intracranial ependymomas, which also found chromosome 22q deletion to be the most frequent genomic loss (Chapter 1, section 1.5.2) (Kilday, Rahman et al. 2009).

The most common focal regions of maintained genomic loss were located within 22q12.2 – 12.3 and 22q13.31 – 13.33 (2/8 sets, 25 %), encompassing genes such as *LARGE* and *TIMP3* (22q12.3) (Table 5.3). These regions were also the site of acquired copy number loss in a further ependymoma set (set D) (Table 5.5, Appendix 10M). *LARGE* encodes a glycosyltransferase which participates in the glycosylation of the epithelial cell basement membrane receptor dystroglycan (Grewal, McLaughlan et al.

2005). *In vitro* studies have shown repression of the LARGE protein is responsible for reduced cell adhesion and increased migration in lung, colon and breast cancer cells, implicating defective dystroglycan glycosylation as a factor in tumourigenesis and cancer progression (de Bernabe, Inamori et al. 2009). *TIMP3* encodes a metalloproteinase inhibitor which can induce cell death and suppress tumour cell growth and invasion *in vitro* (Darnton, Hardie et al. 2005). Reduced TIMP3 protein expression has been associated with increased invasiveness and recurrence in breast cancer (Mylona, Magkou et al. 2006) and is an adverse prognostic marker in other cancers such as oesophageal and gastric cancer (Darnton, Hardie et al. 2005; Gu, Xing et al. 2008). Moreover, methylation of *TIMP3* has been shown frequently in ependymoma (Chapter 1, section 1.5.4). Since deletions of these genes were observed as both maintained and acquired abnormalities in this analysis, they are candidates potentially implicated in ependymoma initiation, preservation and advancement.

Focal regions of acquired genomic loss were only evident in individual sets (A, B and D) (Table 5.5, Appendix 10M). Nevertheless, the predominant locations for these focal losses were within the three most common regions of deletion identified in paediatric intracranial recurrent ependymomas from the CGH meta-analysis; chromosomes 6q, 10q, and 22q (Chapter 1, section 1.5.2) (Kilday, Rahman et al. 2009). A number of the focal regions were found to encompass putative tumour suppressor genes identified from other studies, such as the serotonin receptor gene *HTR1B* (6q14.1) (Jin, Oksenberg et al. 1992) and the urotrophin encoding *UTRN* (6q24.2) (Tinsley, Blake et al. 1992). Deletion of *HTR1B* has previously been reported in glioblastomas (Korshunov, Sycheva et al. 2006), while *in vitro* studies have demonstrated inactivating mutations of *UTRN* to occur in neuroblastoma and breast cancer (Li, Huang et al. 2007). Recognised tumour suppressor genes previously implicated in ependymoma pathogenesis were also identified within other loci, including the cell cycle regulators *CDKN2A*, *CDKN2B* (9p21.3) and the phosphatase encoding *PTEN* (10q23.2) (Rousseau, Ruchoux et al. 2003; Taylor, Poppleton et al. 2005). Besides tumourigenesis, *CDKN2A* deletion is now also considered to be a critical event in cancer progression (reviewed by (Rocco and Sidransky 2001)), while loss of *PTEN* has been associated with an increased incidence of tumour relapse in several malignancies including breast, hepatocellular and prostate cancers (Halvorsen, Haukaas et al. 2003; Hu, Huang et al. 2003). This study suggests that genomic deletion of these established genes may have a role in paediatric

ependymoma recurrence which, since infrequent, may be restricted to a particular subpopulation of tumours. The deletion of *CDKN2A* and *CDKN2B* has been discussed in Chapter 4.

5.5 Summary

The outcome for children who develop ependymoma recurrence remains very poor. Since current clinical factors cannot reliably predict the likelihood of relapse, the biology of paediatric ependymoma recurrence merits further assessment. This 500K SNP array study examined genetic copy number alterations across eight sets of patient-matched primary and relapsed ependymomas, allowing the role of genomic imbalance in paediatric ependymoma recurrence to be assessed at a higher resolution than previous reports.

Many of the aberrations detected were located on chromosomes 1q, 9q and 22q, reflecting the findings of current literature. Shared regions of focal copy number imbalance sustained from primary to recurrent tumours were identified across this ependymoma panel, encompassing genes potentially important for tumourigenesis and tumour maintenance. These included gain of *CENPF* (1q41), *CDC42BPA* (1q42.13) *DNM1* (9q34.11) and *COL27A1* (9q32), together with loss of *LARGE* and *TIMP3* (22q12.3). Shared focal aberrations acquired only at ependymoma recurrence were also identified, encompassing genes conceivably contributing to ependymoma progression and therapeutic resistance. Such imbalances included *COL27A1* gain once again, together with gain of *HAX1* (1q21.3), *CNTN2/TAXI* (1q32.1) *PAXIP1/PTIP* (7q36.2) and *KCNK9* (8q24.3). In addition, a review of the copy number alterations identified at ependymoma recurrence discovered an enrichment for genes involved in neurite growth, guidance and cell migration. It is hypothesised that activation of such genes or mechanistic pathways may promote neoplastic cell invasiveness and spread, facilitating local relapse by influencing tumour resectability whilst encouraging more distant CNS recurrences to arise.

The genomic alterations of these candidate genes require validation using the techniques of qPCR and FISH (as described in Chapter 2, sections 2.4 and 2.5). Differentiation of

genomic imbalances that drive corresponding gene and protein expression, from those that do not, would subsequently be essential. This is particularly pertinent for acquired anomalies detected in this study as they may have arisen as a consequence of therapeutic induced genomic instability and are, per se, mere ‘passenger’ aberrations. Candidates established from expression studies could then be assessed functionally for a role in ependymoma maintenance and progression, using cell lines derived in-house from primary and recurrent paediatric ependymomas. Such *in vitro* analysis is discussed further in Chapter 7.

The most common focal copy number alterations identified in this analysis were detected across a proportion of the eight ependymoma sets analysed. Incorporating the data from this study into larger SNP array analyses of patient matched primary and recurrent ependymomas would provide a more accurate evaluation of how common these imbalances are. This may become possible through improved tumour banking procedures and international collaboration. Whilst not appearing universal, these alterations could nevertheless be complemented with epigenetic data to provide an insight into different mechanisms of tumour recurrence for particular biological subpopulations of paediatric ependymoma that are becoming apparent from literature.

CHAPTER 6

PROSPECTIVE ANALYSIS OF PUTATIVE BIOLOGICAL PROGNOSTIC MARKERS IN PAEDIATRIC INTRACRANIAL EPENDYMOMA

6.1 Introduction

Several putative biological prognostic markers for ependymoma have been reported, using either genomic analysis or immunohistochemistry (Chapter 1, Table 1.8). In spite of this, very few candidates have been exclusively analysed in large, childhood ependymoma cohorts to allow an informative evaluation of their impact on survival in this age group (Chapter 1, section 1.5.7). Of the paediatric markers assessed, the contradictory results for ERBB2/ERBB4 and Ki-67 expression (Bennetto, Foreman et al. 1998; Gilbertson, Bentley et al. 2002; Ridley, Rahman et al. 2008), controversial immunohistochemical detection of hTERT (Wu, Dudognon et al. 2006; Ridley, Rahman et al. 2008) and lack of reproducible findings for Nucleolin expression (Ridley, Rahman et al. 2008) have meant a definitive and consistent biological correlate of outcome in childhood ependymoma remains elusive. In addition, no marker has ever been assessed against a clinical trial cohort. All preceding analyses to date have been performed on retrospective tumour groups, where patients have already received a heterogeneous range of therapy which in itself may influence patient survival.

To address these issues, a novel prognostic marker tissue microarray analysis was performed using FISH and immunohistochemistry on age-defined prospective cohorts of paediatric ependymomas, accrued from children being treated uniformly in accordance with two independent clinical trials (UK CCLG 1992 04 and SIOP 1999 04). The UK CCLG 1992 04 trial was intended for paediatric ependymoma patients aged under three years (Grundy, Wilne et al. 2007). It was a post-surgical chemotherapy-based therapeutic regimen designed to avoid or delay radiotherapy in order to minimise long-term toxicity to the immature central nervous system. In contrast, the SIOP 1999 04 trial was for children aged above three years diagnosed with intracranial ependymomas (Massimino, Gandola et al. 2004), where surgery and radiotherapy were the principal treatment modalities. An overview of the therapeutic strategies adopted by each trial is included in sections 6.2.1.1 and 6.2.1.2 of this chapter.

A panel of six proposed biological prognostic markers were assessed. This included chromosome 1q gain (specifically 1q25, as determined using the LSI 1p36/LSI 1q25 FISH probe, Vysis, USA) and the expression of PRUNE, NAV1, Tenascin-C, Ki-67 and

Nucleolin. All had been reported to be of prognostic significance for paediatric ependymoma or were associated with ependymoma relapse from preceding retrospective analyses, including the 500K SNP array analysis of this work.

Copy number gain of chromosome 1q has been discussed previously (Chapter 3, section 3.4.3.1). Using aCGH detection and 1q25 FISH validation, 1q gain has been identified as an independent marker of recurrence and unfavourable outcome in ependymoma from independent retrospective mixed age cohorts (Mendrzyk, Korshunov et al. 2006; Korshunov, Witt et al. 2010). Less evidence exists for a prognostic role exclusive to the paediatric setting (Dyer, Prebble et al. 2002), although the preceding SNP array work of this thesis has shown 1q gain to be associated with reduced overall patient survival on multivariate analysis, in addition to a frequent imbalance in intracranial recurrent ependymomas (Chapter 3, section 3.3.4).

Higher resolution cytoband analysis of chromosome 1q from the 500K SNP array data, presented in previous chapters, identified 1q21.2 – 21.3 to be the most frequently imbalanced region in a group of ependymomas characterised by an adverse patient outcome ('group two') and was also the most frequently gained chromosome 1q locus across the primary and recurrent cohorts (Chapter 3, section 3.3.5). Within this 1q21.2 – 21.3 locus, gain of a focal region incorporating *PRUNE* (1q21.3) was associated with a particularly poor event-free survival on univariate analysis and was an independent predictor of reduced overall patient survival when assessed across the entire primary ependymoma cohort (Chapter 4, section 4.3.2). In addition, genomic gain encompassing *NAV1* (1q32.1) was among the most frequent imbalances of the intracranial recurrent tumours analysed in the SNP array study (Chapter 4, Table 4.3) and was associated with an adverse event-free survival when evaluated by multivariate analysis across the primary ependymoma group (Chapter 4, section 4.3.2). This immunohistochemical analysis aimed to initially verify whether the genomic gains identified in these two candidate genes translated into increased expression of the corresponding encoded proteins, before subsequently investigating the prognostic role of NAV1 and PRUNE in childhood ependymoma.

Increased expression of the extracellular matrix glycoprotein Tenascin-C (TNC) has been reported in paediatric recurrent intracranial ependymoma when compared to

primary tumours, along with a corresponding upregulation of the encoding gene *TNC* (Puget, Grill et al. 2009). This has led to the hypothesis that Tenascin-C may be involved in the progression of childhood ependymomas and is potentially associated with adverse outcome when overexpressed. However, this theory had never been tested on a large sample cohort for which patient survival data was readily available, as was the case with this analysis.

As discussed (Chapter 1, section 1.5.7), previous immunohistochemical work had identified hTERT protein expression as an independent marker of survival in paediatric ependymoma (Tabori, Ma et al. 2006). However, re-evaluation of the antibody used to detect hTERT verified the target to be Nucleolin, a nuclear phosphoprotein chaperone for hTERT, or a Nucleolin-like protein (Wu, Dudognon et al. 2006; Ridley, Rahman et al. 2008). While low Nucleolin expression has since been shown to be a predictor of beneficial outcome in paediatric intracranial ependymoma (Ridley, Rahman et al. 2008), no other study had replicated this finding on an independent, prospective tumour cohort.

Certain large studies have proposed an adverse prognostic role for increased expression of the cell proliferation marker Ki-67 in paediatric ependymoma (Bennetto, Foreman et al. 1998; Gilbertson, Bentley et al. 2002). Other work has failed to establish this association in children but has identified a correlation between Ki-67 and ependymoma histological grade (Ridley, Rahman et al. 2008), thereby supporting the view that increased proliferation appears a feature of anaplasia (Rushing, Brown et al. 1998; Suzuki, Oka et al. 2001; Suri, Tatke et al. 2004). Therefore, this analysis aimed to clarify if Ki-67 was a marker of ependymoma histology and/or patient outcome.

By analysing ependymoma tissue microarrays from the two clinical trial cohorts, the following hypotheses were explored:

- Copy number gain of chromosome 1q25 by FISH has prognostic significance for children with intracranial ependymomas
- PRUNE overexpression is an adverse prognostic marker for children with intracranial ependymomas
- NAV1 overexpression is an adverse prognostic marker for children with intracranial ependymomas

- Tenascin-C overexpression is an adverse prognostic marker for children with intracranial ependymomas
- Low Nucleolin expression is a beneficial prognostic marker for children with intracranial ependymomas
- Ki-67 expression is a marker of cell proliferation and not a prognostic marker for children with intracranial ependymomas

6.2 Materials and methods

6.2.1 The sample cohorts

6.2.1.1 UK CCLG 1992 04 clinical trial cohort

Paediatric patients were eligible for treatment according to this clinical trial if they had been diagnosed with an intracranial tumour which had been histologically confirmed as an ependymoma, were aged three years or younger at diagnosis, and had not received previous adjuvant therapy (Grundy, Wilne et al. 2007).

Table 6.1: Chemotherapy schedule for the UK CCLG 1992 04 paediatric ependymoma clinical trial.

Course 1 (day 0)
Vincristine (IV bolus) Carboplatin (IV infusion)
Course 2 (day 14)
Vincristine (IV bolus) High Dose Methotrexate (IV infusion) Folinic acid (IV infusion)
Course 3 (day 28)
Vincristine (IV bolus) Cyclophosphamide (IV infusion) Mesna (IV infusion)
Course 4 (day 42)
Cisplatin (IV infusion for 48 hours)

One cycle of therapy is shown in the table (Grundy, Wilne et al. 2007). Each cycle lasted for 56 days. Seven cycles were administered to each enrolled patient. IV = intravenous.

Treatment comprised of maximal surgical resection, followed by four courses of alternating myelosuppressive and non-myelosuppressive chemotherapy given at 14 day intervals, which was repeated for seven cycles (Table 6.1). Radiotherapy was only given if progressive disease had been identified on surveillance neuro-imaging. This was either directed at the site of local relapse, or to the entire neuroaxis in cases of metastatic recurrence.

In total, 89 intracranial ependymomas (60 primary and 29 recurrent) from 71 patients registered on the UK CCLG 1992 04 trial were examined. The clinical details of these children are summarised in Figure 6.1, while the comprehensive data set is detailed in Table 6.2.

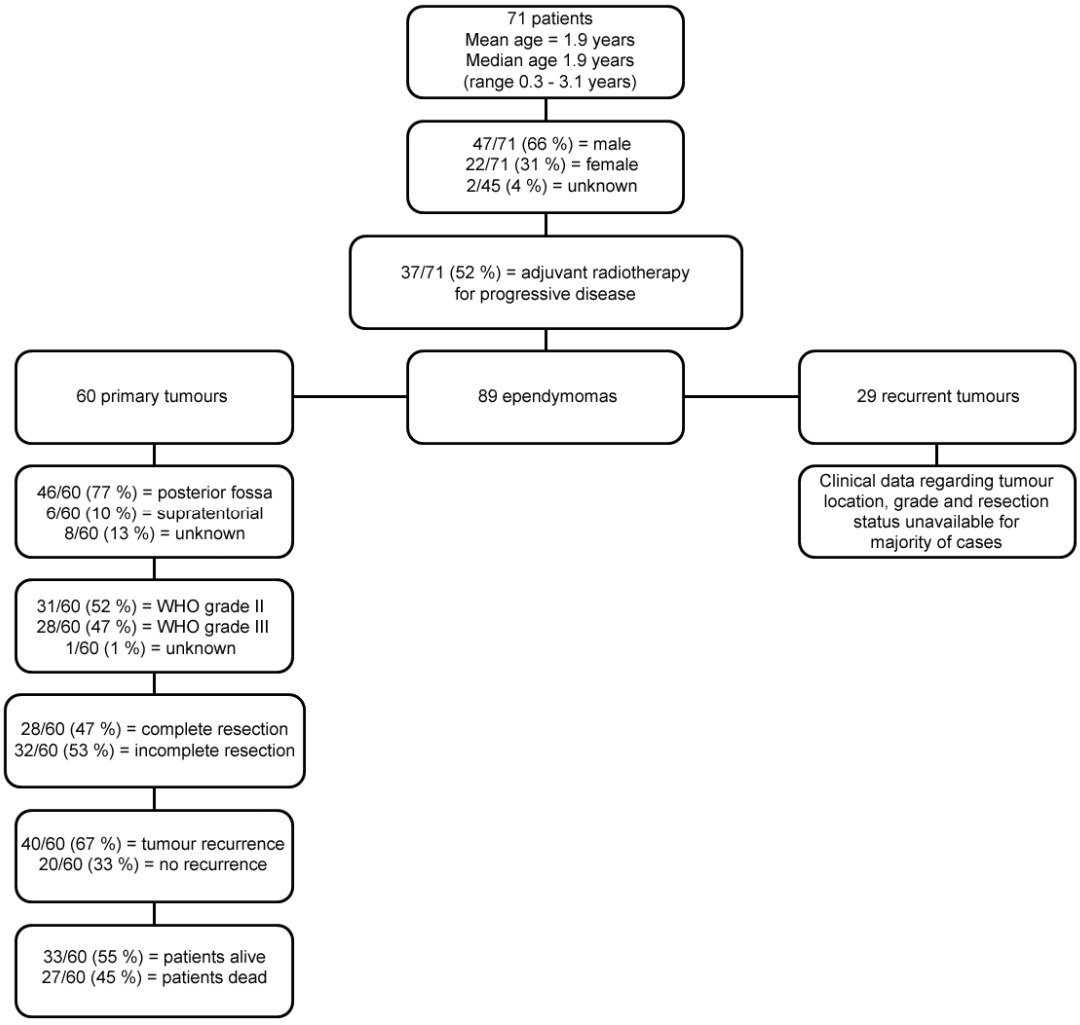

Figure 6.1: Clinical data summary for the 71 patients that constitute the UK CCLG 1992 04 clinical trial cohort. Of the 89 intracranial ependymomas analysed, 77 were from 60 patients who had contributed either a primary tumour alone, or primary and subsequent recurrent tumours. The cohort also contained 12 ependymomas from 11 patients contributing only recurrent tumours.

Table 6.2: Clinical parameters of the UK CCLG 1992 04 clinical trial cohort.

Sample ID	Trial Number	Age at Diagnosis (yrs)	Sex	Tumour Location	WHO Grade	Primary Tumour Resection Status	Received RT	Status	EFS (yrs) (> if censored)	OS (yrs) (> if censored)
9204 – 1P	170	0.3	F	ST	II	Incomplete	No	A(r)	9.5	12.8
9204 – 2R1	182	0.7	M	/	/	/	Yes	D	0.6	3.4
9204 – 3P	89	0.8	F	PF	II	Complete	No	D	0.7	0.9
9204 – 3R1	89	as above	as above	/	/	/	/	as above	as above	as above
9204 – 4R1	159	0.8	M	/	/	/	No	A(r)	1.8	8.9
9204 – 5P	158	0.9	F	ST	III	Complete	No	ADF	9.3	9.3
9204 – 6P	128	1.0	M	PF	III	Complete	Yes	D	1.5	3.2
9204 – 7P	133	1.0	M	PF	/	Complete	Yes	D	1.2	2.8
9204 – 7R1	133	as above	as above	/	II	/		as above	as above	as above
9204 – 8P	210	1.0	M	PF	II	Complete	Yes	D	1.4	2.1
9204 – 9R2	96	1.2	F	/	/	/	No	A(r)	3.8	13.1
9204 – 9R3	96	as above	as above	/	/	/	/	as above	as above	as above
9204 – 10P	15	1.3	F	PF	II	Complete	Yes	D	1.4	3.5
9204 – 11P	21	1.3	F	PF	III	Complete	Yes	D	2.5	3.8
9204 – 12P	70	1.3	M	PF	III	Complete	Yes	D	2.1	4.1
9204 – 13P	97	1.3	M	PF	III	Incomplete	Yes	D	3.4	8.9
9204 – 13R1	97	as above	as above	/	/	/	/	as above	as above	as above
9204 – 14R1	117	1.3	M	/	/	/	No	D	1.5	1.7
9204 – 15P	118	1.3	F	PF	III	Complete	No	ADF	11.2	11.2
9204 – 16P	199	1.3	F	PF	III	Incomplete	No	D	1.5	1.6
9204 – 17P	203	1.3	M	/	II	Complete	Yes	D	1.4	2.9
9204 – 17R1	203	as above	as above	/	/	/	/	as above	as above	as above
9204 – 18P	213	1.3	/	/	III	Incomplete	Yes	A(r)	0.4	1.8
9204 – 19P	10	1.4	M	PF	II	Incomplete	No	ADF	10.8	10.8
9204 – 20P	30	1.4	F	ST	III	Incomplete	No	ADF	12.3	12.3
9204 – 21P	137	1.4	M	PF	/	Incomplete	Yes	D	2.2	2.7
9204 – 22P	168	1.4	M	ST	III	Incomplete	No	ADF	6.8	6.8
9204 – 23P	189	1.4	M	PF	III	Complete	No	D	0.4	0.6
9204 – 24P	201	1.5	F	/	III	Complete	No	ADF	2.5	2.5
9204 – 25P	207	1.5	M	/	III	Complete	No	ADF	1.8	1.8
9204 – 26R1	14	1.6	M	/	/	/	Yes	A(r)	10.2	11.3
9204 – 27P	32	1.7	M	PF	II	Incomplete	Yes	D	2.0	2.9
9204 – 28P	101	1.7	M	PF	II	Complete	No	ADF	10.4	10.4

Sample ID	Trial Number	Age at Diagnosis (yrs)	Sex	Tumour Location	WHO Grade	Primary Tumour Resection Status	Received RT	Status	EFS (yrs) (> if censored)	OS (yrs) (> if censored)
9204 – 29P	138	1.7	F	PF	II	Incomplete	Yes	A(r)	1.2	8.1
9204 – 29R1	138	as above	as above	/	II	/		as above	as above	as above
9204 – 30P	13	1.8	M	PF	II	Incomplete	Yes	D	2.8	6.5
9204 – 30R1	13	as above	as above	/	/	/	as above	as above	as above	as above
9204 – 31P	56	1.8	M	PF	III	Incomplete	Yes	D	1.1	3.7
9204 – 31R1	56	as above	as above	/	/	/	/	as above	as above	as above
9204 – 31R2	56	as above	as above	/	/	/	/	as above	as above	as above
9204 – 31R3	56	as above	as above	/	/	/	/	as above	as above	as above
9204 – 32R1	103	1.8	M	/	/	/	Yes	A(r)	1.5	2.1
9204 – 33R1	180	1.8	F	/	/	/	No	A(r)	2.2	6.8
9204 – 34P	164	1.8	M	PF	II	Complete	Yes	A(r)	2.7	8.3
9204 – 35P	204	1.8	M	/	III	Incomplete	No	ADF	2.4	2.4
9204 – 36P	83	1.9	M	PF	III	Incomplete	Yes	A(r)	2.5	14.2
9204 – 36R1	83	as above	as above	/	/	/		as above	as above	as above
9204 – 37R1	122	1.9	M	/	/	/	Yes	A(r)	1.4	9.7
9204 – 38P	197	1.9	M	PF	III	Incomplete	No	ADF	0.0	0.0
9204 – 39P	18	2.0	F	PF	III	Complete	No	ADF	15.2	15.2
9204 – 40R1	72	2.0	M	/	/	/	No	D	1.4	3.8
9204 – 41R1	190	2.0	M	/	/	/	Yes	D	0.8	2.3
9204 – 42P	200	2.0	F	/	III	Complete	Yes	A(r)	2.3	2.6
9204 – 42R1	200	as above	as above	/	/	/	/	as above	as above	as above
9204 – 43P	81	2.1	M	PF	III	Incomplete	Yes	D	0.3	1.7
9204 – 44P	129	2.1	M	PF	II	Complete	No	ADF	9.9	9.9
9204 – 45P	139	2.2	F	PF	II	Incomplete	No	A(r)	4.8	8.6
9204 – 46P	145	2.2	F	PF	II	Complete	Yes	A(r)	5.8	10.6
9204 – 47P	58	2.3	M	PF	III	Complete	Yes	A(r)	2.5	12.9
9204 – 48P	112	2.3	M	PF	II	Incomplete	Yes	D	1.6	3.3
9204 – 49P	161	2.3	M	PF	II	Complete	Yes	D	5.6	6.4
9204 – 49R1	161	as above	as above	/	/	/	/	as above	as above	as above
9204 – 50P	162	2.3	M	PF	II	Incomplete	Yes	D	2.3	3.8
9204 – 51P	176	2.3	F	ST	II	Incomplete	No	A(r)	0.9	8.5
9204 – 52P	187	2.3	F	PF	II	Incomplete	Yes	D	2.9	5.4
9204 – 53P	208	2.3	M	/	III	Incomplete	No	A(r)	1.3	1.3
9204 – 53R1	208	as above	as above	/	/	/	/	as above	as above	as above

Sample ID	Trial Number	Age at Diagnosis (yrs)	Sex	Tumour Location	WHO Grade	Primary Tumour Resection Status	Received RT	Status	EFS (yrs) (> if censored)	OS (yrs) (> if censored)
9204 – 54P	188	2.4	M	PF	III	Incomplete	No	ADF	7.3	7.3
9204 – 55P	196	2.4	M	PF	III	Complete	No	A(r)	0.8	5.4
9204 – 56P	51	2.5	M	PF	II	Incomplete	Yes	D	1.3	3.7
9204 – 56R1	51	as above	as above	/	II	/	/	as above	as above	as above
9204 – 57P	174	2.5	M	PF	III	Incomplete	Yes	D	2.2	6.9
9204 – 57R3	174	as above	as above	/	III	/	/	as above	as above	as above
9204 – 58P	185	2.5	F	ST	III	Complete	No	ADF	7.3	7.3
9204 – 59P	212	2.5	/	/	II	Incomplete	No	D	1.2	1.3
9204 – 60P	116	2.6	F	PF	III	Incomplete	Yes	D	0.6	3.0
9204 – 60R1	116	as above	as above	/	III	/	/	as above	as above	as above
9204 – 61P	132	2.6	F	PF	II	Complete	Yes	A(r)	0.7	6.9
9204 – 62P	181	2.6	M	PF	II	Incomplete	No	D	1.8	1.9
9204 – 63P	1	2.7	M	PF	II	Incomplete	No	ADF	11.0	11.0
9204 – 64P	131	2.7	M	PF	II	Incomplete	No	ADF	11.3	11.3
9204 – 65P	171	2.8	M	PF	II	Complete	Yes	ADF	12.8	12.8
9204 – 66P	194	2.8	M	PF	II	Incomplete	No	ADF	6.1	6.1
9204 – 67R1	177	2.9	M	/	II	/	Yes	A(r)	0.3	10.1
9204 – 68P	184	2.9	M	PF	III	Complete	Yes	D	2.3	6.8
9204 – 68R1	184	as above	as above	/	II	/	/	as above	as above	as above
9204 – 69P	192	2.9	F	PF	II	Incomplete	Yes	D	1.7	4.1
9204 – 70P	46	3.0	M	PF	II	Complete	No	ADF	13.5	13.5
9204 – 71P	166	3.1	M	PF	II	Complete	No	ADF	8.2	8.2

P = primary, R – R5 = 1^{st} – 5^{th} recurrence, M = male, F = female, PF = posterior fossa/infratentorial, ST = supratentorial, C = chemotherapy, RT = radiotherapy, A(r) = alive but relapsed, ADF = alive and disease free, D = dead of disease, EFS = event-free survival, OS = overall survival.

6.2.1.2 SIOP 1999 04 clinical trial cohort

Patients were eligible for treatment according to this clinical trial if they had been diagnosed with a non-metastatic intracranial tumour which had been histologically confirmed as an ependymoma, were aged above three years but below 21 years at diagnosis, and had not received previous adjuvant therapy (Massimino, Gandola et al. 2004). A summary of the therapeutic regimen is shown (Figure 6.2). All patients received involved field radiotherapy, although the timing of this was dependent on the outcome of initial neurosurgery. Following a complete resection, radiotherapy was administered immediately within four weeks of surgery. In cases of incomplete resection, surgery could be re-attempted to achieve complete excision but only if this was deemed feasible. If this surgery was successful, radiotherapy was then administered within the subsequent four weeks.

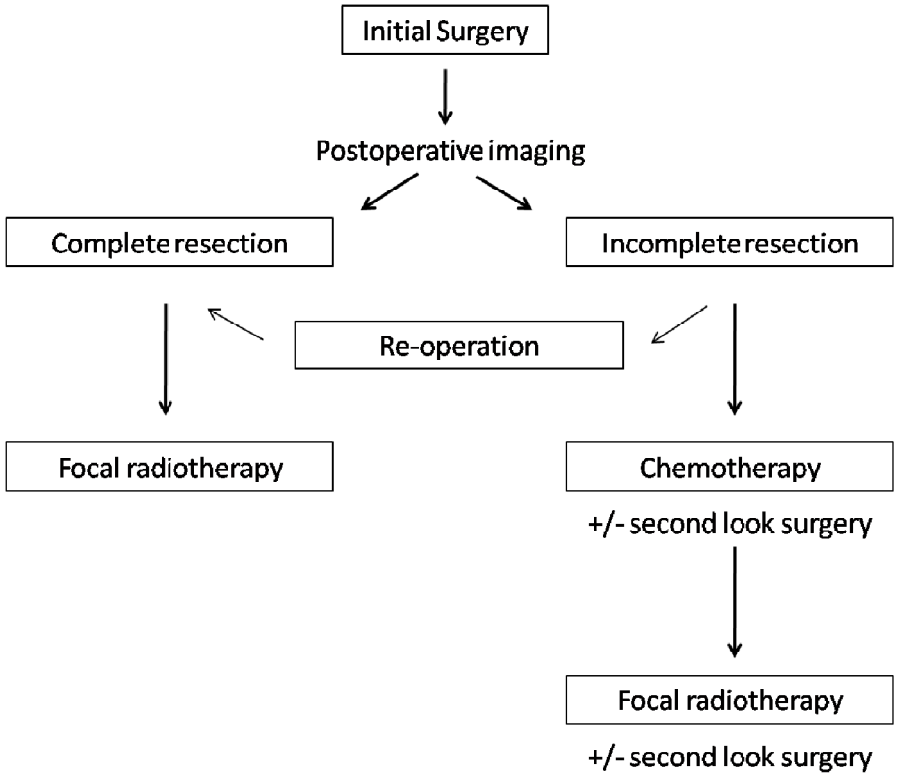

Figure 6.2: Flow diagram overview of the treatment regimen adopted in the SIOP 1999 04 paediatric ependymoma clinical trial (Massimino, Gandola et al. 2004).

All cases of residual disease, after initial or re-attempted surgery, were treated with chemotherapy followed by focal radiotherapy four weeks later. Further surgical resection of tumour residuum was optional after either adjuvant therapy schedule if deemed appropriate by a neurosurgeon. The chemotherapy regimen consisted of intravenous vincristine, etoposide and cyclophosphamide, administered every four weeks for four cycles (Table 6.3).

Table 6.3: Chemotherapy schedule for the SIOP 1999 04 paediatric ependymoma clinical trial patients with residual disease post surgical resection.

Course 1 (day 1)
Vincristine (IV bolus) Etoposide (IV infusion) Cyclophosphamide (IV infusion) Mesna (IV infusion)
Course 1 (day 2)
Etoposide (IV infusion)
Course 1 (day 3)
Etoposide (IV infusion)
Course 1 (day 8)
Vincristine (IV bolus)
Course 1 (day 15)
Vincristine (IV bolus)

One cycle of therapy is shown in the table (Massimino, Gandola et al. 2004). Each cycle lasted for 28 days. Four cycles were administered to each enrolled patient. IV = intravenous.

A total of 69 intracranial ependymomas (47 primary and 22 recurrent) from 47 paediatric patients registered on the SIOP 1999 04 trial were examined. The clinical details for these children are summarised in Figure 6.3, while the comprehensive data set is detailed in Table 6.4.

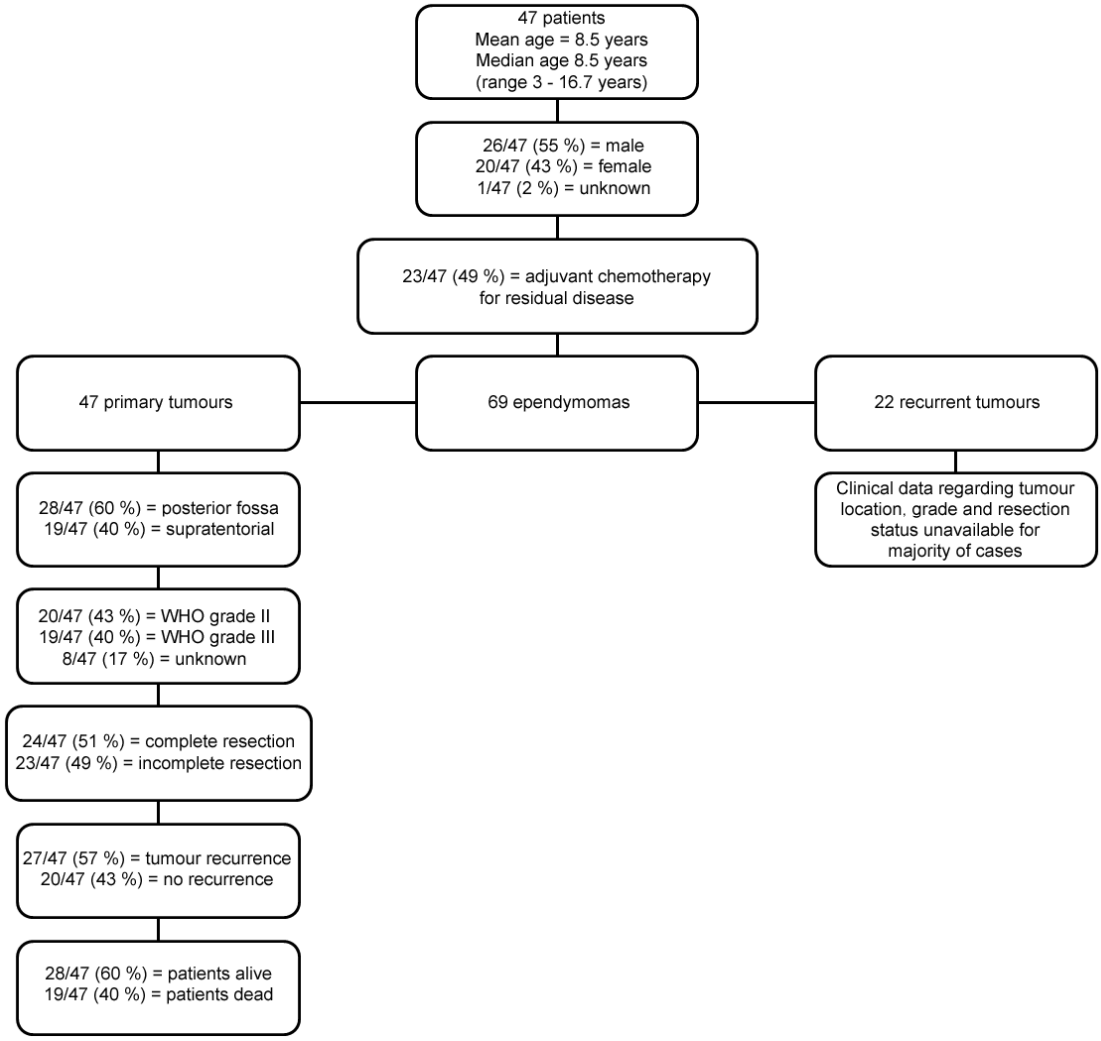

Figure 6.3: Clinical data summary for the 47 patients that constitute the SIOP 1999 04 clinical trial cohort. All of the 69 intracranial ependymomas analysed were obtained from these 47 patients, who had either contributed a primary tumour alone, or primary and subsequent recurrent tumours.

Table 6.4: Clinical parameters of the SIOP 1999 04 clinical trial cohort.

Sample ID	Trial Number	Age at Diagnosis (yrs)	Sex	Tumour Location	WHO Grade	Primary Tumour Resection Status	Status	EFS (yrs) (> if censored)	OS (yrs) (> if censored)
9904 – 1P	75	3.0	M	ST	/	Incomplete	D	1.2	1.8
9904 – 1R1	75	as above	as above	/	/	/	as above	as above	as above
9904 – 2P	79	3.0	M	PF	/	Complete	ADF	3.3	3.3
9904 – 3P	55	3.2	F	PF	II	Incomplete	ADF	3.8	3.8
9904 – 4P	9	3.3	M	PF	II	Incomplete	ADF	8.8	8.8
9904 – 5P	3	3.6	M	PF	/	Incomplete	D	2.3	4.1
9904 – 5R1	3	as above	as above	PF	/	/	as above	as above	as above
9904 – 6P	20	3.7	M	PF	II	Complete	ADF	5.5	5.5
9904 – 7P	26	4.3	M	PF	III	Complete	D	2.3	2.6
9904 – 8P	62	4.3	M	PF	III	Complete	D	1.8	3.5
9904 – 8R1	62	as above	as above	/	/	/	as above	as above	as above
9904 – 9P	37	4.5	M	PF	/	Incomplete	D	1.4	5.8
9904 – 9R1	37	as above	as above	/	/	/	as above	as above	as above
9904 – 10P	41	4.5	F	PF	III	Complete	A(r)	7.9	8.2
9904 – 11P	46	4.9	M	PF	II	Incomplete	D	1.7	3.4
9904 – 11R1	46	as above	as above	/	/	/	as above	as above	as above
9904 – 11R2	46	as above	as above	/	/	/	as above	as above	as above
9904 – 11R3	46	as above	as above	/	/	/	as above	as above	as above
9904 – 12P	54	5.2	F	PF	II	Incomplete	ADF	3.7	3.7
9904 – 13P	56	5.2	F	PF	III	Incomplete	ADF	6.6	6.6
9904 – 14P	24	5.6	M	ST	II	Incomplete	D	0.8	1.0
9904 – 14R1	24	as above	as above	/	II	/	as above	as above	as above
9904 – 15P	6	5.7	M	ST	III	Complete	ADF	9.7	9.7
9904 – 16P	27	6.3	M	ST	II	Incomplete	A(r)	0.3	5.3
9904 – 17P	52	6.3	F	ST	III	Complete	D	1.9	5.1
9904 – 17R1	52	as above	as above	/	/	/	as above	as above	as above
9904 – 17R2	52	as above	as above	/	/	/	as above	as above	as above
9904 – 17R3	52	as above	as above	/	/	/	as above	as above	as above
9904 – 17R4	52	as above	as above	/	/	/	as above	as above	as above
9904 – 17R5	52	as above	as above	/	/	/	as above	as above	as above
9904 – 18P	35	6.5	M	PF	III	Incomplete	D	2.6	4.0

Sample ID	Trial Number	Age at Diagnosis (yrs)	Sex	Tumour Location	WHO Grade	Primary Tumour Resection Status	Status	EFS (yrs) (> if censored)	OS (yrs) (> if censored)
9904 – 18R1	35	as above	as above	/	/	/	as above	as above	as above
9904 – 18R2	35	as above	as above	/	/	/	as above	as above	as above
9904 – 19P	72	6.5	F	PF	III	Incomplete	D	1.1	3.1
9904 – 20P	78	6.5	F	PF	II	Incomplete	D	0.6	1.4
9904 – 21P	80	6.5	F	PF	II	Complete	ADF	2.4	2.4
9904 – 22P	40	6.7	F	ST	II	Complete	ADF	7.1	7.1
9904 – 23P	42	6.7	M	PF	II	Incomplete	A(r)	0.7	6.8
9904 – 24P	23	6.8	F	ST	II	Complete	ADF	6.8	6.8
9904 – 25P	88	6.9	M	PF	III	Complete	D	0.9	1.8
9904 – 26P	58	7.1	F	ST	III	Complete	D	1.5	5.6
9904 – 26R1	58	as above	as above	/	/	/	as above	as above	as above
9904 – 26R2	58	as above	as above	/	/	/	as above	as above	as above
9904 – 27P	81	8.0	F	PF	/	Complete	ADF	2.9	2.9
9904 – 28P	67	8.2	/	PF	II	Incomplete	A(r)	5.6	5.6
9904 – 29P	73	9.2	M	ST	III	Complete	ADF	4.8	4.8
9904 – 30P	60	9.6	M	ST	III	Complete	D	3.1	5.8
9904 – 30R1	60	as above	as above	/	III	/	as above	as above	as above
9904 – 31P	71	9.9	M	PF	III	Complete	ADF	4.8	4.8
9904 – 32P	44	10.1	M	PF	II	Complete	D	2.5	5.6
9904 – 32R1	44	as above	as above	/	/	/	as above	as above	as above
9904 – 33P	39	10.3	F	ST	II	Complete	D	1.7	2.4
9904 – 33R1	39	as above	as above	/	/	/	as above	as above	as above
9904 – 34P	83	10.9	F	ST	/	Incomplete	ADF	1.8	1.8
9904 – 35P	76	12.0	F	ST	/	Incomplete	D	0.3	3.0
9904 – 36P	57	12.2	F	PF	III	Incomplete	A(r)	2.2	5.8
9904 – 36R1	57	as above	as above	/	III	/	as above	as above	as above
9904 – 37P	8	12.7	M	ST	III	Incomplete	A(r)	0.3	9.0
9904 – 38P	18	12.7	F	PF	II	Incomplete	D	2.3	5.4
9904 – 38R1	18	as above	as above	/	/	/	as above	as above	as above
9904 – 39P	29	12.9	M	PF	III	Complete	ADF	6.7	6.7
9904 – 40P	87	14.6	F	ST	/	Complete	ADF	2.7	2.7
9904 – 41P	17	14.7	M	PF	III	Complete	ADF	7.6	7.6
9904 – 42P	68	14.8	M	ST	II	Complete	A(r)	3.6	4.0

Sample	Trial Number	Age at Diagnosis (yrs)	Sex	Tumour Location	WHO Grade	Primary Tumour Resection Status	Status	EFS (yrs) (> if censored)	OS (yrs) (> if censored)
9904 – 43P	31	14.9	F	ST	II	Incomplete	ADF	8.0	8.0
9904 – 44P	53	14.9	M	PF	II	Complete	ADF	3.3	3.3
9904 – 45P	64	15.5	F	ST	III	Complete	ADF	1.7	1.7
9904 – 46P	89	16.0	M	ST	III	Incomplete	D	1.4	2.2
9904 – 47P	65	16.7	M	PF	II	Incomplete	A(r)	0.6	5.1

P = primary, R1 – R5 = 1^{st} – 5^{th} recurrence, M = male, F = female, PF = posterior fossa/infratentorial, ST = supratentorial, C = chemotherapy, RT = radiotherapy, A(r) = alive but relapsed, ADF = alive and disease free, D = dead of disease, EFS = event-free survival, OS = overall survival, NK = not known.

6.2.2 Fluorescent *in situ* hybridisation

The protocol adhered to, control tissue and method of scoring used are described in Chapter 2, section 2.5.

6.2.3 Immunohistochemistry

The protocol adhered to, control tissues and methods of scoring used are described in Chapter 2, section 2.6.

6.2.4 Statistical analysis

The statistical tests performed in this chapter are described in Chapter 2, section 2.7. Multivariate survival analysis for the UK CCLG 1992 04 cohort included the clinical variables of tumour location, WHO histological grade and resection status, together with any biological marker associated with survival on univariate analysis. Adjuvant radiotherapy was not included as a variable for overall survival as it was selectively administered to patients with a conferred survival disadvantage as a consequence of experiencing tumour relapse. Multivariate survival analysis for the SIOP 1999 04 cohort included the clinical variables of tumour location, WHO histological grade and resection status/adjuvant chemotherapy, again in addition to any biological marker associated with survival on univariate analysis.

6.3 Results

6.3.1 Clinical associations: the UK CCLG 1992 04 trial cohort

Statistical analysis of the primary UK CCLG 1992 04 cohort revealed the only significant association between clinical parameters was concerning posterior fossa ependymomas in male patients and supratentorial tumours in females ($p = 0.015$, two-tailed Fisher's exact test).

The estimated mean overall survival time for patients in the cohort was 9.3 ± 0.8 years (range 0 – 15.2 years). The mean time for follow up was 6.2 ± 0.5 years (range 0 – 15.2 years). Two-thirds of the primary cohort had relapsed (40/60, 67 %), with a mean time to recurrence of 2.1 ± 0.3 years (range 0.3 – 10.2 years). The percentage of the cohort achieving five year event-free survival was 35.7 ± 6.4 %, while five year overall survival was achieved by 61.1 ± 6.7 % (Figure 6.4).

Univariate survival analysis revealed the only clinical factor associated with adverse patient outcome was tumour location. Posterior fossa ependymomas were associated with a worse five year overall survival compared to supratentorial tumours (57.8 % versus 100 %, p = 0.028; all supratentorial cases were censored so mean survival times not calculated) and a trend towards a worse five year event-free survival (31.1 ± 6.9 % versus 83.3 ± 15.2 %, p = 0.087).

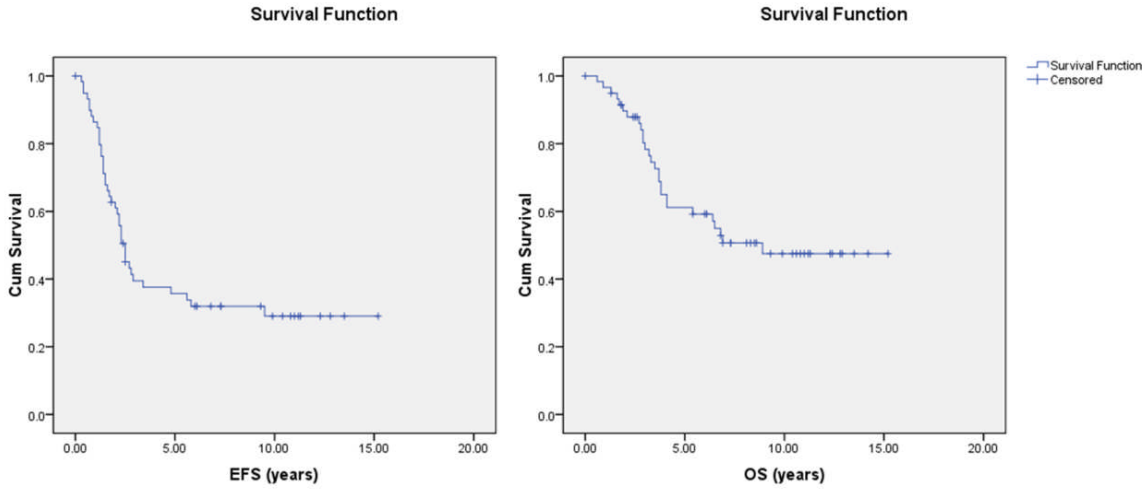

Figure 6.4: Kaplan-Meier EFS and OS curves for the UK CCLG 1992 04 primary tumour cohort of 60 patients. The estimated mean event-free survival for the cohort was 6 ± 0.8 years, while the estimated mean overall survival was 9.3 ± 0.8 years. Censored cases are marked as indicated. Cum survival = cumulative survival.

6.3.2 Clinical associations: the SIOP 1999 04 trial cohort

Statistical analysis of the primary 1999 04 cohort for associations between clinical factors revealed the mean age of patients with posterior fossa ependymomas was lower than those with supratentorial tumours (7.5 ± 0.7 years versus 9.8 ± 0.9 years; p = 0.05, independent t-test). No other associations were identified.

The estimated mean overall survival time for patients in the cohort was 6.6 ± 0.5 years (range 1 – 9.7 years). The mean time for documented follow up was 4.7 years ± 0.3 years (range 1 – 9.7 years). Tumour recurrence occurred in the majority of patients (27/47, 57 %), with a mean time to relapse of 1.9 ± 0.3 years (range 0.3 – 7.9 years). The percentage of the cohort achieving five year event-free survival was 44.2 ± 7.6 %, while five year overall survival was achieved by 69 ± 7.3 % (Figure 6.5).

Univariate survival analysis revealed the only clinical factor associated with adverse patient outcome was tumour resection status. Incomplete resection was associated with a worse estimated mean event-free survival compared to complete resection (3.4 years ± 0.7 years versus 6 years ± 0.8 years, p = 0.016) although this effect was not seen on overall survival (5.9 years ± 0.7 years versus 7 years ± 0.7 years, p = 0.387).

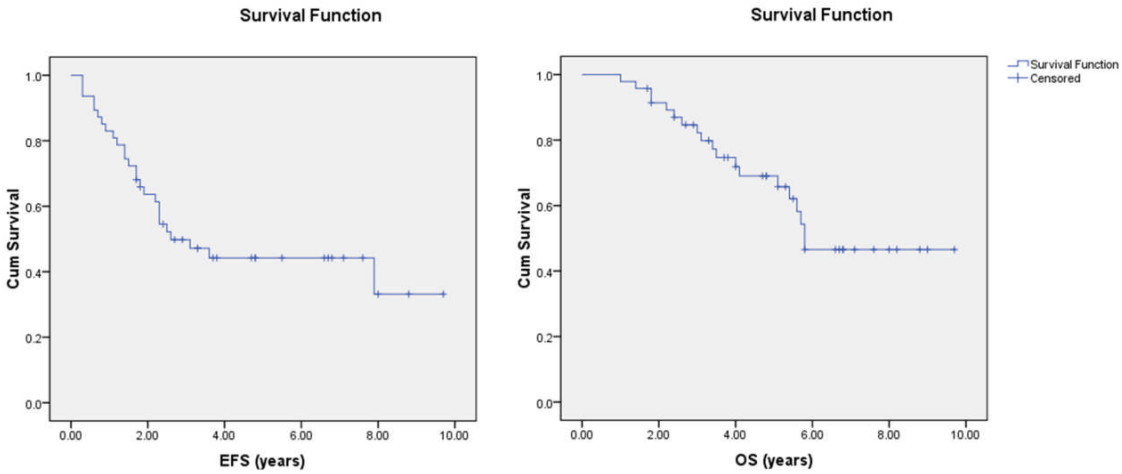

Figure 6.5**:** Kaplan-Meier EFS and OS curves for the SIOP 1999 04 primary tumour cohort of 47 patients. The estimated mean event-free survival for the cohort was 4.9 ± 0.6 years, while the estimated mean overall survival was 6.6 ± 0.5 years. Censored cases are marked as indicated. Cum survival = cumulative survival.

6.3.3 Overview of FISH scores and IHC staining results

All FISH and immunohistochemistry results from the biological marker analysis performed on the UK CCLG 1992 04 and SIOP 1999 04 tissue microarray cohorts are shown in Appendices 8 and 9 respectively. These results are summarised in Table 6.5.

Table 6.5: Summary of results for the prognostic marker analysis on both paediatric ependymoma clinical trial cohorts.

Markers & thresholds		1992 04 clinical trial cohort		1999 04 clinical trial cohort	
		60 primary	29 recurrent	47 primary	22 recurrent
1q25 FISH	**No gain (< 15 %)**	41 (68.3 %)	14 (48.3 %)	30 (63.8 %)	10 (45.5 %)
	Gain (≥15 %)	11 (18.3 %)	2 (6.9 %)	8 (17 %)	5 (22.7 %)
	No data	8 (13.4 %)	13 (44.8 %)	9 (19.2 %)	7 (31.8 %)
PRUNE	**Negative**	3 (5 %)	1 (3.4 %)	4 (8.5 %)	2 (9.1 %)
	Weak	5 (8.3 %)	5 (17.2 %)	8 (17 %)	2 (9.1 %)
	Moderate	20 (33.3 %)	2 (6.9 %)	6 (12.8 %)	2 (9.1 %)
	Strong	29 (48.3 %)	16 (55.2 %)	25 (53.2 %)	14 (63.6 %)
	No data	3 (5 %)	5 (17.2 %)	4 (8.5 %)	2 (9.1 %)
NAV1	**Negative**	5 (8.3 %)	6 (20.7 %)	4 (8.5 %)	2 (9.1 %)
	Weak	12 (20 %)	4 (13.8 %)	8 (17 %)	1 (4.5 %)
	Moderate	10 (16.7 %)	5 (17.2 %)	6 (12.8 %)	5 (22.7 %)
	Strong	31 (51.7 %)	9 (31 %)	25 (53.2 %)	11 (50 %)
	No data	2 (3.3 %)	5 (17.2 %)	4 (8.5 %)	3 (13.6 %)
TNC	**Negative**	5 (8.3 %)	2 (6.9 %)	9 (19.1 %)	4 (18.2 %)
	Weak	6 (10 %)	4 (13.8 %)	17 (36.2 %)	12 (54.5 %)
	Moderate/strong	49 (81.7 %)	19 (65.5 %)	16 (34 %)	6 (27.3 %)
	No data	-	4 (13.8 %)	5 (10.6 %)	-
Nucleolin	**≤ 50 %**	4 (6.7 %)	3 (10.3 %)	7 (14.9 %)	2 (9.1 %)
	> 50 %	56 (93.3 %)	23 (79.4 %)	38 (80.9 %)	17 (77.3 %)
	No data	-	3 (10.3 %)	2 (4.2 %)	3 (13.6 %)
Ki-67	**Low (≤ 1 %)**	37 (61.7 %)	13 (44.8 %)	25(53.2 %)	10 (45.5 %)
	Moderate (2-4 %)	13 (21.6 %)	4 (13.8 %)	13 (27.7 %)	8 (36.4 %)
	High (≥ 5 %)	10 (16.7 %)	10 (34.5 %)	7 (14.9 %)	3 (13.6 %)
	No data	-	2 (6.9 %)	2 (4.2 %)	1 (4.5 %)

For both clinical trial cohorts, the number and percentage of tumours comprising each biological marker category is shown. FISH = Fluorescent In Situ Hybridisation, TNC = Tenascin-C, NAV1 = Neuron Navigator 1.

6.3.4 Individual biological marker analysis

The results obtained for each biological marker assessed are now presented.

6.3.4.1 Copy number gain of chromosome 1q25

6.3.4.1.1 Scoring and statistical associations

Analysis of chromosome 1q25 copy number gain was performed by FISH on both clinical trial TMA cohorts after being optimised on tonsil control tissue. As described in Chapter 2 section 2.5, a gain threshold of 15 % was used to define groups (Figure 6.6).

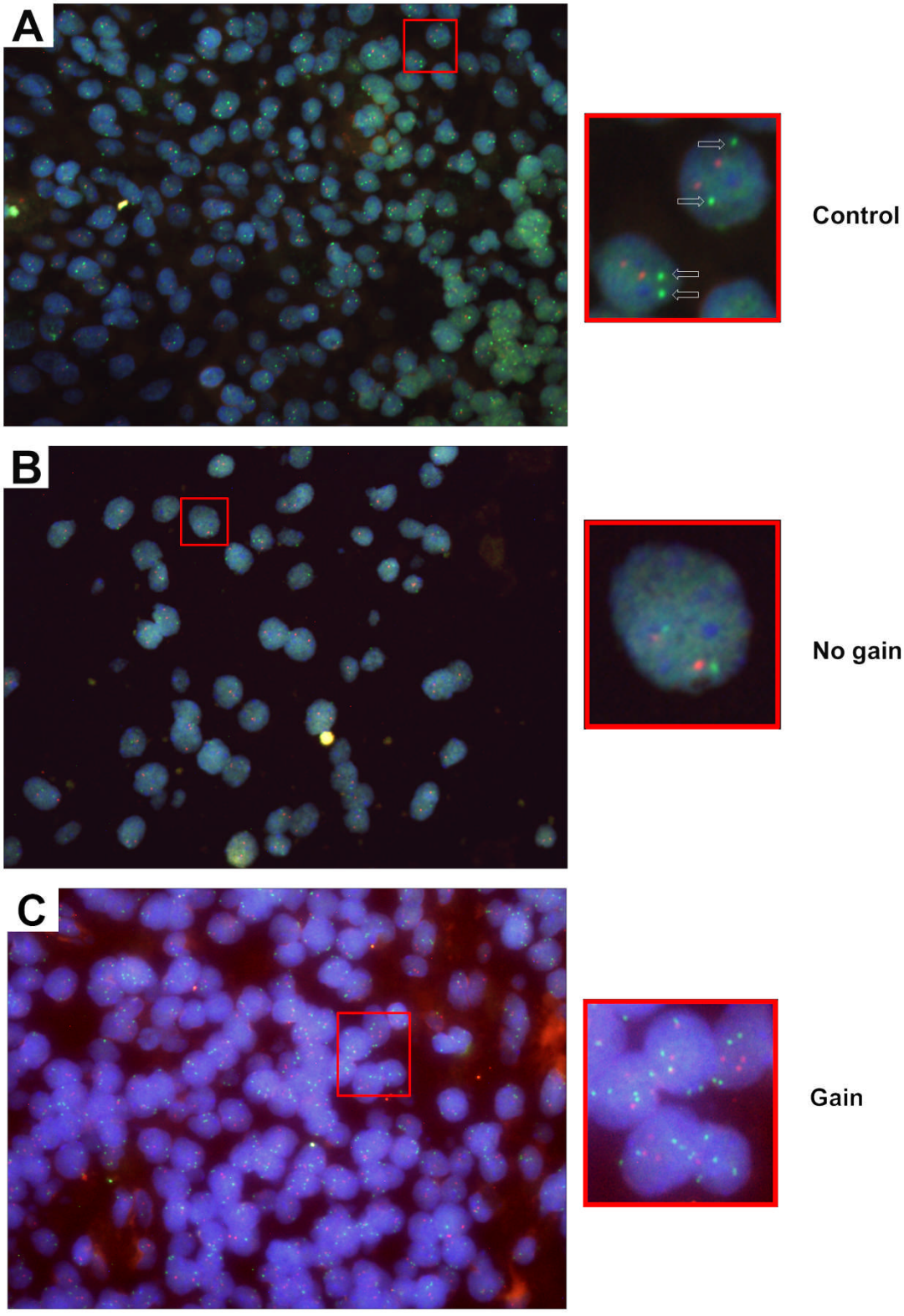

Figure 6.6: Chromosome 1q25 copy number analysis by FISH. This was performed using a commercial LSI 1p36 (red) / LSI 1q25 (green) dual colour probe (Vysis, USA). Main images: objective x 40. (A): The technique was optimised on tonsil control tissue, where fluorescent signals were obtained in over 90 % of nuclei counted and the majority of nuclei had 2 copies of the 1q25 probe (arrow heads) (mean 53.25 %, range 48 – 56 %). The mean score of tonsil nuclei revealing chromosome 1q25 copy number gain was 2.25 % (range 0 – 4 %). (B): Example of a clinical trial TMA specimen without evidence of 1q25 gain (sample shown is 9204 – 66P, average score across the tumour = 7 %, highest core score = 8 %). (C): Example of a clinical trial TMA specimen with 1q25 gain (sample shown is 9904 – 29P, average score across the tumour = 57 %, highest core score = 70 %).

Fluorescent signals were obtained in over 90 % of the control tonsil nuclei counted (mean 93.75 %, range 91 % – 96 %). The mean percentage of nuclei with a diploid copy of 1p36 and 1q25 probes was 39.25 % (range 35 – 43 %), making this the predominant signal combination observed. The majority of nuclei had two copies of the 1q25 probe (mean 53.25 %, range 48 – 56 %), while evidence of 1q25 copy number gain was, as expected, rare (mean 2.25 %, range 0 – 4 %).

Of the 89 intracranial ependymomas comprising the UK CCLG 1992 04 TMA cohort, 21 tumours (eight primary and 13 recurrent) could not be assessed due to either core loss or the presence of non-viable tumour, leaving 52 primary and 16 recurrent tumours available for analysis. Eleven of the primary cases (21.2 %) revealed 1q25 gain (three focal gain, eight widespread gain), while 41 cases did not (78.8 %). Of the recurrent cases, two samples revealed 1q25 gain (12.5 %), while 14 tumours did not (87.5 %). Fisher's exact test found no significant association between gain of chromosome 1q25 in the primary cohort and clinical variables including patient sex ($p = 0.299$), patient age above or below three years (compared against SIOP 1999 04 cohort results; $p = 1.0$), patient age above or below one year ($p = 0.571$), tumour location ($p = 1.0$), WHO grade ($p = 0.09$) and resection status ($p = 1.0$), or tumour recurrence ($p = 0.432$).

Of the 69 intracranial ependymomas comprising the SIOP 1999 04 TMA cohort, 15 tumours (nine primary and seven recurrent) could not be assessed, leaving 38 primary and 15 recurrent tumours available for analysis. Eight of these primary ependymomas (21.1 %) revealed 1q25 gain (one focal gain, seven widespread gain), while 30 cases did not (78.9 %). Of the recurrent cases, five tumours revealed 1q25 gain (33.3 %) and 10 cases did not (66.7 %). Again, Fisher's exact test did not identify an association between gain of chromosome 1q25 in the primary cohort and the clinical factors of patient sex ($p = 0.444$), patient age (see above; $p = 1.0$), tumour location ($p = 0.426$), WHO histological grade ($p = 0.394$) and resection status ($p = 1.0$), or recurrence ($p = 0.241$).

6.3.4.1.2 Comparison of 500K SNP array and FISH results

The correlation of chromosome 1q/1q25 gain as determined by the 500K SNP array and FISH has already been shown in Chapter 3, section 3.3.6.

6.3.4.1.3 Measure of agreement between scorers

To assess the reproducibility of the FISH scoring system adopted for gain of chromosome 1q25, 23 primary ependymomas from the UK CCLG 1992 04 cohort (38 %) and 8 primary ependymomas from the SIOP 1999 04 cohort (17 %) were re-analysed by a blinded second scorer. In total, 72 TMA cores were cross-examined using the 15 % gain threshold (Table 6.6). The Kappa measure of agreement was 0.86, $p = 2.06 \times 10^{-13}$. This result was significantly stronger than if a 10 % gain threshold was adopted (Kappa = 0.18, p = 0.11) (Table 6.7).

Table 6.6: Assessment of chromosome 1q25 gain by two scorers – 15 % gain threshold.

	Scorer 2 (BM) Gain (≥ 15 %)	**Scorer 2 (BM) No gain (< 15 %)**	**Total**
Scorer 1 (JPK) Gain (≥ 15 %)	7 cores	1 cores	8 cores
Scorer 1 (JPK) No gain (< 15 %)	1 cores	63 cores	64 cores
Total	8 cores	64 cores	72 cores

A total of 72 cores were assessed by the two scorers (1 and 2 with ID initials included). The resulting Kappa measure of agreement was 0.86, $p = 2.06 \times 10^{-13}$.

Table 6.7: Assessment of chromosome 1q25 gain by two scorers – 10 % gain threshold.

	Scorer 2 (BM) Gain (≥ 10 %)	**Scorer 2 (BM) No gain (< 10 %)**	**Total**
Scorer 1 (JPK) Gain (≥ 10 %)	11 cores	14 cores	25 cores
Scorer 1 (JPK) No gain (< 10 %)	12 cores	35 cores	47 cores
Total	23 cores	49 cores	72 cores

A total of 72 cores were assessed by the two scorers (1 and 2 with ID initials included). The resulting Kappa measure of agreement was 0.18, p = 0.11.

6.3.4.1.4 Survival analysis

Using the 15 % gain threshold described above, univariate analysis of the UK CCLG 1992 04 primary cohort revealed chromosome 1q25 gain to be associated with a worse event-free patient survival (estimated mean EFS: 2.1 $\pm$ 0.5 years versus 8.1 $\pm$ 1.1 years, p = 2.72 x 10^{-4}) (Figure 6.7). This finding was replicated on multivariate analysis (hazards ratio 3.922 (95% CI 1.414 – 8.758), p = 0.009) (Table 6.8). No association with 1q25 copy number gain and overall survival was observed on either univariate (estimated mean OS: 5.6 $\pm$ 0.9 years (tumours with gain) versus 9.9 $\pm$ 0.9 years (tumours without gain), p = 0.413) or multivariate analysis (p = 0.998) (Table 6.9).

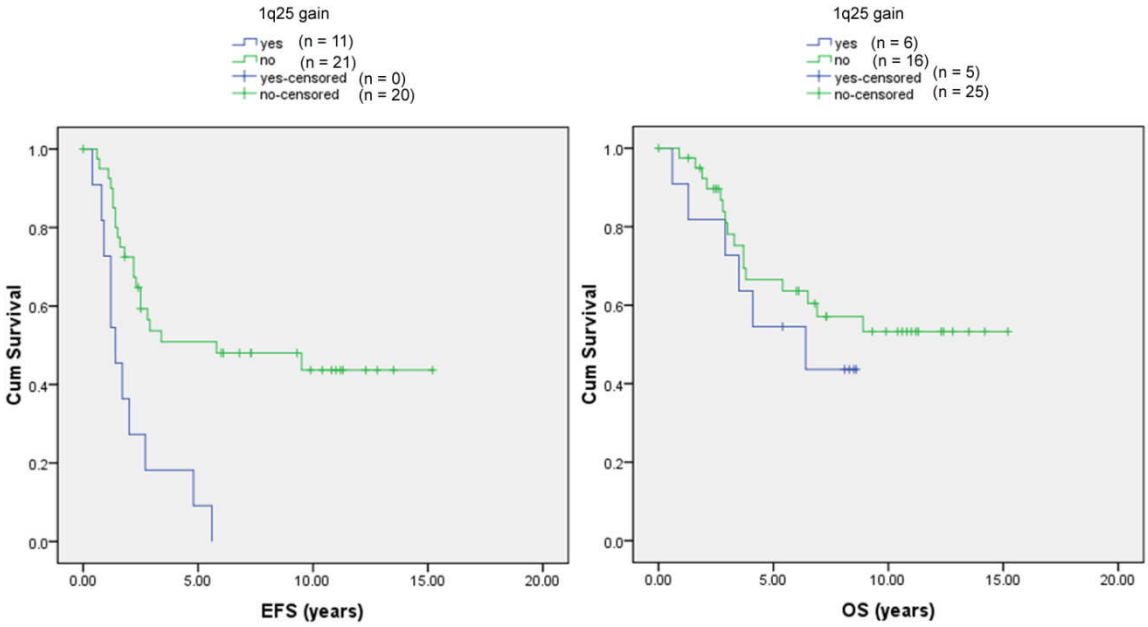

Figure 6.7**:** Kaplan-Meier EFS and OS curves, comparing UK CCLG 1992 04 primary ependymomas demonstrating chromosome 1q25 gain (equal to or above 15 % - blue lines) against those not demonstrating gain (green lines). Gain was associated with a worse EFS (percentage attaining 5 year EFS = 9.1 $\pm$ 8.7 % (gain group) versus 50.9 $\pm$ 8.1 % (no gain group), p = 2.72 x 10^{-4}). However this was not seen for OS (percentage attaining 5 year OS = 54.5 $\pm$ 15 % (gain group) versus 66.5 $\pm$ 7.9 % (no gain group), p = 0.413). Cum survival = cumulative survival.

Table 6.8: Multivariate event-free survival analysis of the UK CCLG 1992 04 cohort.

Cox regression multivariate analysis (n=41 – samples missing data removed)	**Event-Free Survival**		
Factor	**Hazards ratio**	**95 % CI**	**P value**
Histology (WHO grade II vs III)	1.628	0.592 – 4.481	0.345
Tumour location (PF vs ST)	2.333	0.492 – 11.064	0.286
Resection status (incomplete vs complete)	1.485	0.645 – 3.42	0.353
TNC expression (mod/strong vs neg/weak)	1.891	0.366 – 9.711	0.447
1q25 gain (no gain vs gain)	3.922	1.414 – 8.758	0.009
PRUNE expression (strong vs not strong)	3.575	1.459 – 8.025	0.005

Biological markers associated with poor event-free or overall survival on univariate analysis included. ST = supratentorial, PF = posterior fossa, TNC = Tenascin-C, mod = moderate, neg = negative, 95 % CI = 95 % confidence interval. Significant results are highlighted in yellow.

Table 6.9: Multivariate overall survival analysis of the UK CCLG 1992 04 cohort.

Cox regression multivariate analysis (n=41 – samples missing data removed)	Overall Survival		
Factor	**Hazards ratio**	**95 % CI**	**P value**
Histology (WHO grade II vs III)	0.9	0.296 – 2.733	0.852
Tumour location (PF vs ST)	Not computed by SPSS		0.973
Resection status (incomplete vs complete)	1.289	0.485 – 3.427	0.611
TNC expression (mod/strong vs neg/weak)	3.126	0.399 – 24.494	0.278
1q25 gain (no gain vs gain)	1.001	0.305 – 3.29	0.998
PRUNE expression (strong vs not strong)	5.072	1.556 – 16.537	0.007

Biological markers associated with poor event-free or overall survival on univariate analysis included. ST = supratentorial, PF = posterior fossa, TNC = Tenascin-C, mod = moderate, neg = negative, 95 % CI = 95 % confidence interval. Significant results are highlighted in yellow.

Univariate event-free and overall survival analysis of the SIOP 1999 04 primary cohort did not identify gain of chromosome 1q25 as an adverse marker of outcome (estimated mean EFS: 4.1 $\pm$ 1.2 years (tumours with gain) versus 5.7 $\pm$ 0.8 years (tumours without gain), p = 0.397; estimated mean OS: 6.5 $\pm$ 0.8 years (tumours with gain) versus 7.1 $\pm$ 0.6 years (tumours without gain), p = 0.799) (Figure 6.8). This was also evident from multivariate analysis (EFS; p = 0.469, OS; p = 0.560).

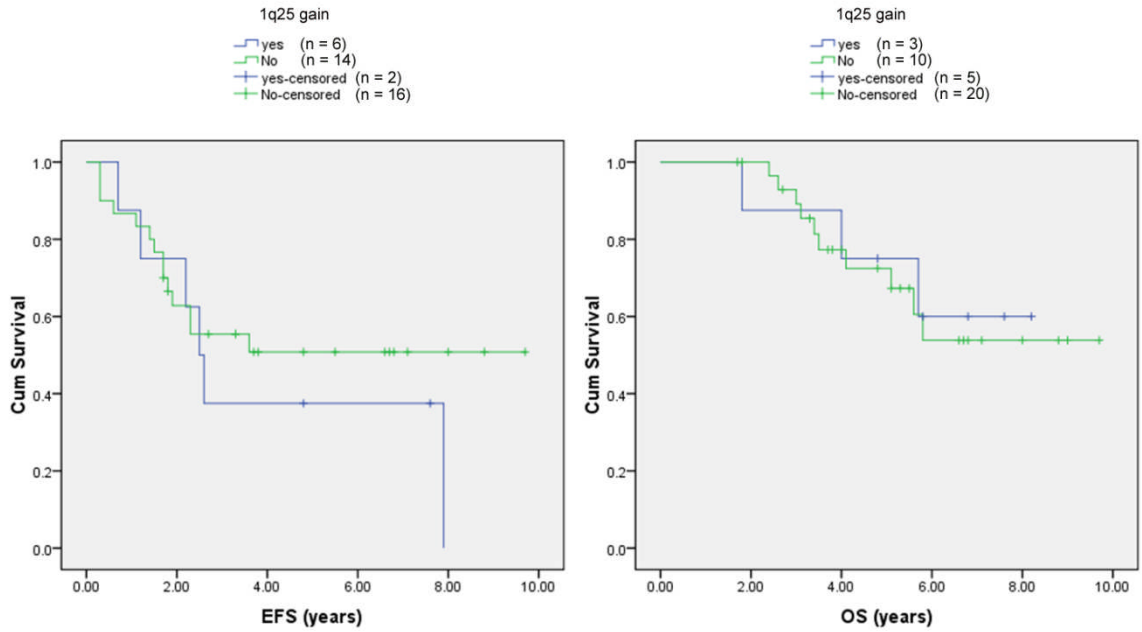

Figure 6.8: Kaplan-Meier EFS and OS curves, comparing SIOP 1999 04 primary ependymomas demonstrating chromosome 1q25 gain (equal to or above 15 % - blue lines) against those not demonstrating gain (green lines). Gain was not associated with a worse EFS (percentage attaining 5 year EFS = 37.5 $\pm$ 17.1 % (gain group) versus 50.8 $\pm$ 9.6 % (no gain group), p = 0.397). Gain was also not associated with a poorer OS (percentage attaining 5 year OS = 75 $\pm$ 15.3 % (1q25 gain group) versus 72.5 $\pm$ 9 % (no gain group), p = 0.799). Cum survival = cumulative survival.

6.3.4.2 PRUNE expression

6.3.4.2.1 Staining patterns and statistical associations

Analysis of PRUNE expression was performed by immunohistochemistry on both clinical trial TMA cohorts. As described in Chapter 2, section 2.6, ependymomas were categorised as demonstrating negative, weak, moderate or strong expression (Figure 6.9).

Of the 89 intracranial ependymomas comprising the UK CCLG 1992 04 TMA cohort, eight tumours (three primary and five recurrent) could not be assessed due to either core loss or the presence of non-viable tumour, leaving 57 primary and 24 recurrent tumours available for analysis. Of these primary tumours, three did not express PRUNE (5.3 %), five revealed weak expression (8.8 %), 20 exhibited moderate expression (35.1 %) and 29 demonstrated strong expression (50.9 %). Of the recurrent tumours, one case showed no PRUNE expression (4.2 %), five revealed weak expression (20.8 %), two exhibited moderate expression (8.3 %), while 16 demonstrated strong expression (66.7 %). Fisher's exact test did not identify a significant association between strong PRUNE expression levels in the primary cohort and clinical factors including patient sex ($p = 0.783$), patient age above or below three years (compared against SIOP 1999 04 cohort results; $p = 0.302$), patient age above or below one year ($p = 0.67$), tumour location ($p = 0.409$), WHO histological grade ($p = 0.593$) or resection status ($p = 1.0$). Expression was also not significantly increased at recurrence ($p = 0.308$, Fisher's exact test).

Of the 69 intracranial ependymomas comprising the SIOP 1999 04 TMA cohort, nine tumours (seven primary, two recurrent) could not be assessed due to core loss, leaving 40 primary and 20 recurrent tumours available for analysis. Of these primary ependymomas, five revealed weak PRUNE expression (12.5 %), ten exhibited moderate expression (25 %) and 25 demonstrated strong expression (62.5 %). Within the recurrent tumour cohort, negative, weak and moderate expression were each seen in two cases (10 %), while 14 tumours demonstrated strong expression (70 %). As with the UK CCLG 1992 04 cohort, PRUNE expression was not significantly higher at recurrence ($p = 1.0$, two-tailed Fisher's exact test).

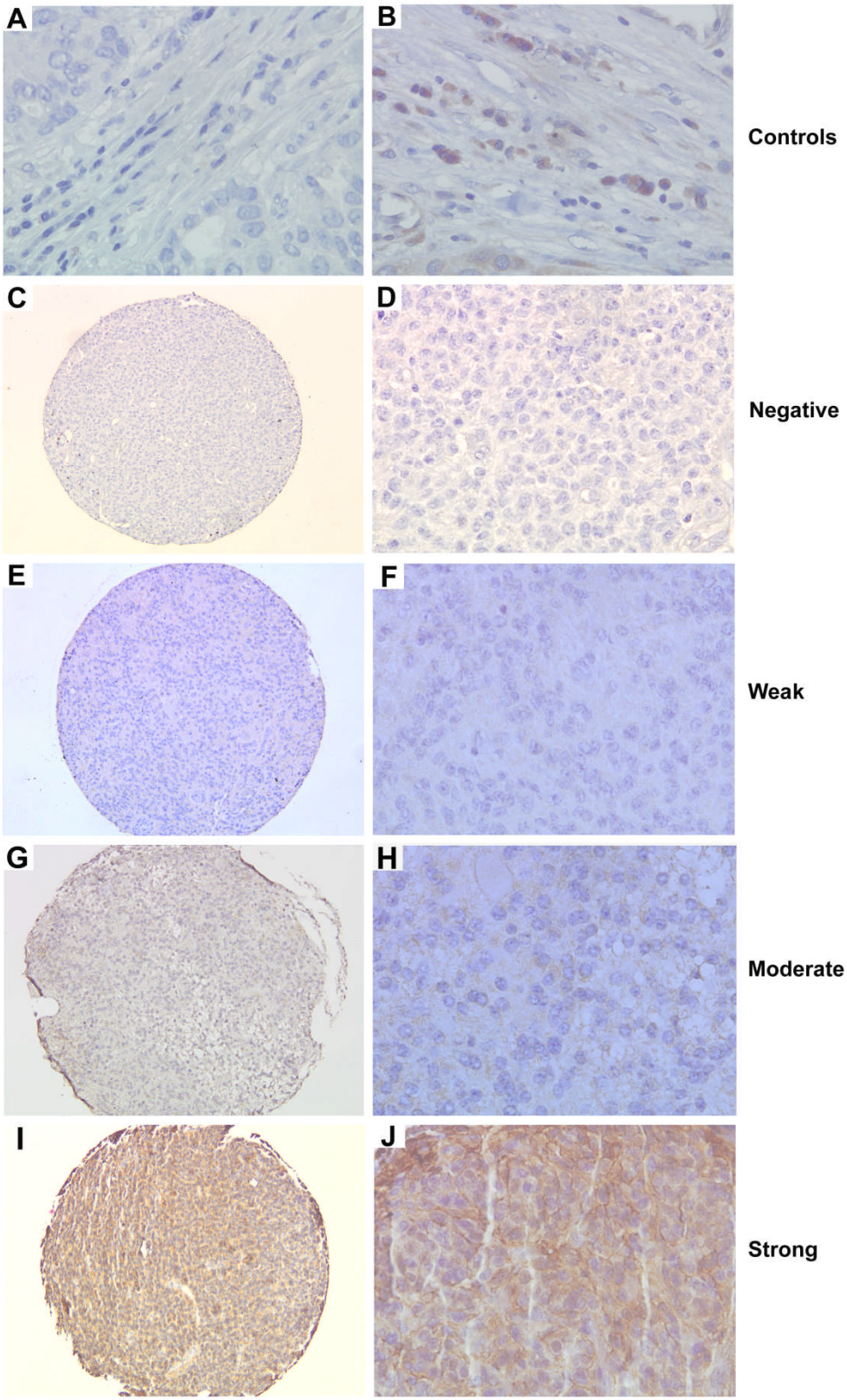

Figure 6.9: PRUNE immunohistochemistry performed using the HPA028411 monoclonal antibody at a 1:800 dilution (Sigma-Aldrich, UK). (A and B): Negative and positive control tissue (breast carcinoma stroma) x 40 objective. (C and D): Example of a clinical trial TMA specimen demonstrating negative PRUNE expression at x10 and x40 objectives respectively (sample shown is 9904 – 30R1). (E and F): Example of a clinical trial TMA core specimen demonstrating weak PRUNE expression at x10 and x40 objectives respectively (sample shown is 9904 – 9R1. (G and H): Example of a clinical trial TMA specimen demonstrating moderate PRUNE expression at x10 and x40 objectives respectively (sample shown is 9204 – 6P). (I and J): Example of a clinical trial TMA specimen demonstrating strong PRUNE expression at x10 and x40 objectives respectively (sample shown is 9904 – 18R1).

In addition, Fisher's exact test did not identify a significant association between strong PRUNE expression levels in the primary cohort and clinical factors including patient sex ($p = 1.0$), patient age above or below three years (see above; $p = 0.689$), tumour location ($p = 0.749$), WHO histological grade ($p = 0.284$) or resection status ($p = 1.0$).

6.3.4.2.2 Comparison of 500K SNP array and IHC results

Twenty-seven paediatric ependymomas that had been subjected to both the 500K SNP array analysis (Chapters 3 – 5) and this immunohistochemical study were examined to verify whether copy number gain of *PRUNE* was associated with a corresponding increase in expression of its encoded protein (Table 6.10).

Table 6.10: Results of *PRUNE* copy number gain as identified by the 500K SNP array analysis and corresponding PRUNE IHC expression for 27 paediatric ependymomas.

Tumour sample ID (500K SNP array ID)	**Evidence of *PRUNE* copy number increase/gain from SNP array**	**Level of PRUNE expression on immunohistochemistry**
1P	No gain	Strong
3P	No gain	Strong
4P	No gain	Weak
6P	No gain	Weak
8P	No gain	Moderate
9P	No gain	Moderate
9R3	No gain	Strong
13P	No gain	Moderate
14P	Gain	Strong
16P	No gain	Moderate
16R1	Gain	Focal strong
17P	No gain	Strong
17R1	No gain	Weak
17R2	No gain	Strong
18P	Gain	Strong
18R1	No gain	Strong
20R1	Gain	Strong
21P	Gain	Strong
22P	No gain	Moderate
23P	Gain	Strong
27P	No gain	Negative
30P	Gain	Strong
32P	No gain	Focal strong
34P	No gain	Strong
36P	No gain	Moderate
41P	No gain	Strong
45P	No gain	Moderate

P = primary, R1 – R3 = 1^{st} – 3^{rd} recurrence.

Seven ependymomas (26 %) had evidence of *PRUNE* copy number gain from the SNP array, while 20 did not (74 %). All seven tumours with genomic gain of *PRUNE* demonstrated strong PRUNE expression with IHC (Tables 6.10 and 6.11). Of the 20 tumours without *PRUNE* copy number gain, nine (45 %) also had evidence of strong expression. Fisher's exact test identified a significant association between copy number increase in *PRUNE* and a strong level of PRUNE expression (p = 0.022, two-tailed Fisher's exact test) (Table 6.11).

Table 6.11: Comparison of *PRUNE* copy number results and PRUNE expression results for 27 paediatric ependymomas.

	PRUNE IHC Strong	**PRUNE IHC Negative/moderate/weak**	**Total**
500K SNP array *PRUNE* gain	7 tumours	0 tumours	7 tumours
500K SNP array No *PRUNE* gain	9 tumours	11 tumours	20 tumours
Total	16 tumours	11 tumours	27 tumours

SNP = Single Nucleotide Polymorphism, IHC = immunohistochemistry. p = 0.022, two-tailed Fisher's exact test.

6.3.4.2.3 Measure of agreement between scorers

To assess the reproducibility of the IHC scoring system used to identify strong PRUNE expression, 40 ependymomas from the UK CCLG 1992 04 cohort (45 %) and 30 tumours from the SIOP 1999 04 cohort (43 %) were re-analysed by a blinded second scorer. In total, 121 TMA cores were cross-examined and classified as either demonstrating, or not demonstrating strong PRUNE expression (Table 6.12). The resulting Kappa measure of agreement was 0.938, $p = 5.65 \times 10^{-25}$.

Table 6.12: Assessment of PRUNE expression by two scorers.

	Scorer 2 (JB) Strong	**Scorer 2 (JB) Not strong**	**Total**
Scorer 1 (JPK) Strong	86 cores	2 cores	88 cores
Scorer 1 (JPK) Not strong	1 cores	32 cores	33 cores
Total	87 cores	34 cores	121 cores

A total of 121 cores were assessed by the two scorers (1 and 2 with ID initials included). The resulting Kappa measure of agreement was 0.938, $p = 5.65 \times 10^{-25}$.

6.3.4.2.4 Survival analysis

Univariate analysis of the UK CCLG 1992 04 primary cohort revealed ependymomas with strong PRUNE expression were associated with a worse event-free and overall patient survival when compared against tumours demonstrating negative, weak and moderate expression (estimated mean EFS: 4 ± 1 year versus 7.6 ± 1.1 years, p = 0.013 and estimated mean OS: 7.5 ± 1.1 years versus 11 ± 0.9 years, p = 0.012) (Figure 6.10). This finding was replicated on multivariate analysis (EFS: hazards ratio 3.575 (95% CI 1.459 – 8.025), p = 0.005; OS: hazards ratio 5.072 (95% CI 1.556 – 16.537), p = 0.007) (Tables 6.8 and 6.9).

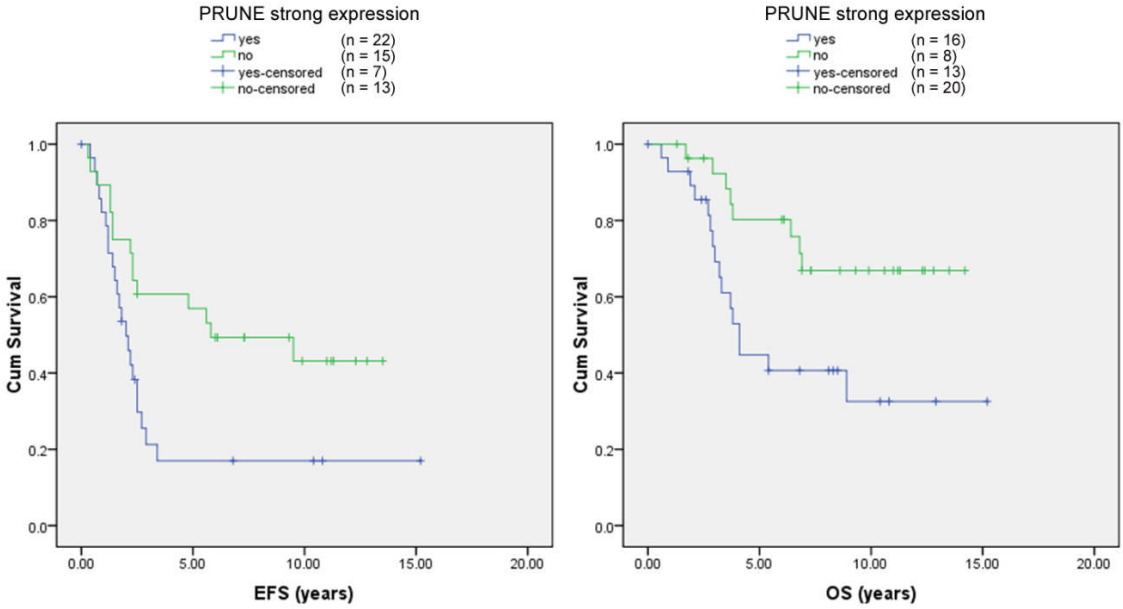

Figure 6.10: Kaplan-Meier EFS and OS curves, comparing UK CCLG 1992 04 primary ependymomas with strong PRUNE expression (blue lines) against those demonstrating negative, weak and moderate expression (green lines). Tumours with strong PRUNE expression were associated with a worse patient EFS (percentage from each group attaining 5 year EFS = 17 ± 7.6 % (strong expression group) versus 56.9 ± 9.4 % (remaining cohort), p = 0.013) and OS (percentage from each group attaining 5 year OS = 44.7 ± 10 % (strong expression group) versus 80.2 ± 7.9 % (remaining cohort), p = 0.012). Cum survival = cumulative survival.

Univariate analysis of the SIOP 1999 04 primary cohort found that strong PRUNE tumour expression was associated with a worse overall patient survival when compared against negative, weak and moderate expression (estimated mean OS: 5.1 ± 0.5 years versus 8.3 ± 0.7 years, p = 0.02) (Figure 6.11). This finding reached a trend towards significance on multivariate OS analysis (hazards ratio 3.787 (95% CI 0.785 – 18.273), p = 0.097) (Table 6.13). No adverse effect of strong PRUNE expression on event-free survival was established from univariate analysis (estimated mean EFS: 3.9 ± 0.7 years

(strong expression group) versus 6.3 ± 1.1 years (remaining cohort), p = 0.156) (Figure 6.11) or multivariate analysis (p = 0.181).

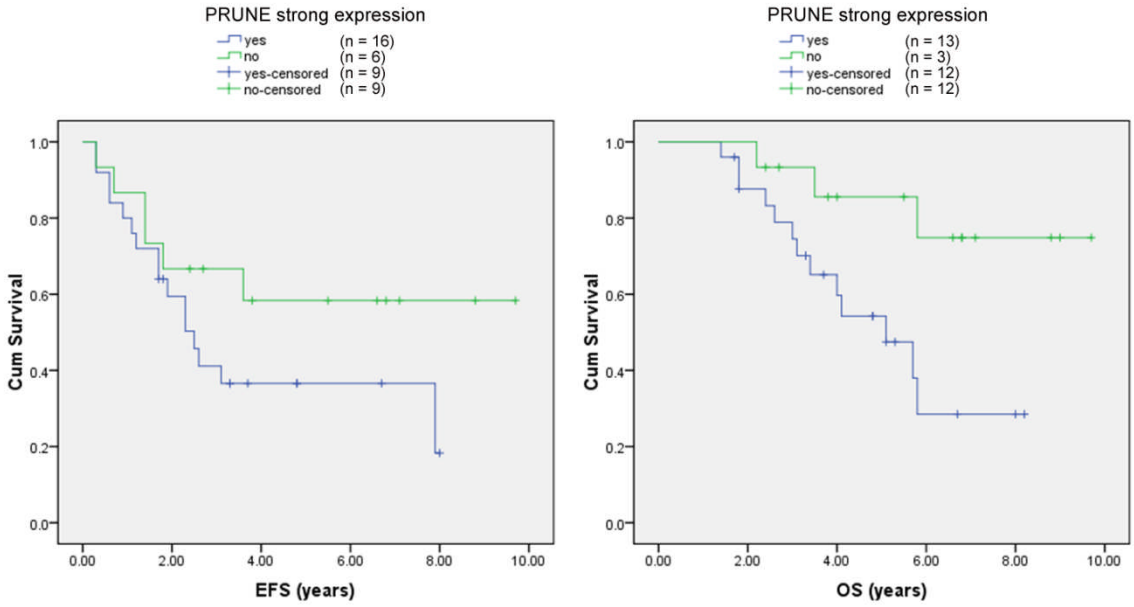

Figure 6.11: Kaplan-Meier EFS and OS curves, comparing SIOP 1999 04 primary ependymomas with strong PRUNE expression (blue lines) against those demonstrating negative, weak and moderate expression (green lines). Tumours with strong PRUNE expression were associated with a worse patient OS (percentage from each group attaining 5 year OS = 54.3 ± 10.9 % (strong expression group) versus 85.6 ± 9.5 % (remaining cohort), p = 0.02). However strong PRUNE expression was not associated with a worse patient EFS (percentage from each group attaining 5 year EFS = 36.6 ± 10.1 % (strong expression group) versus 58.3 ± 13.2 % (remaining cohort), p = 0.156). Cum survival = cumulative survival.

Table 6.13: Multivariate overall survival analysis of the SIOP 1999 04 cohort.

Cox regression multivariate analysis (n=33 – samples missing data removed)	**Overall Survival**		
Factor	**Hazards ratio**	**95 % CI**	**P value**
Histology (WHO grade II vs III)	2.287	0.573 – 9.123	0.241
Tumour location (PF vs ST)	1.248	0.362 – 4.309	0.725
Resection status (incomplete vs complete)	1.523	0.400 – 5.796	0.538
PRUNE expression (strong vs not strong)	3.787	0.785 – 18.273	0.097

No other biological markers were included as only PRUNE was associated with poor event-free or overall survival on univariate analysis. ST = supratentorial, PF = posterior fossa, 95 % CI = 95 % confidence interval. Results with a trend toward statistical significance are highlighted in pale yellow.

6.3.4.3 NAV1 expression

6.3.4.3.1 Staining patterns and statistical associations

Analysis of Neuron Navigator 1 (NAV1) expression was performed by immunohistochemistry on both clinical trial TMA cohorts. As described in Chapter 2,

section 2.6, ependymomas were categorised as demonstrating negative, weak, moderate or strong expression (Figure 6.12).

Of the 89 intracranial ependymomas comprising the UK CCLG 1992 04 TMA cohort, seven tumours (two primary and five recurrent) could not be assessed due to either core loss or the presence of non-viable tumour, leaving 58 primary and 24 recurrent tumours available for analysis. Of the primary tumours, five did not express NAV1 (8.6 %), 12 revealed weak expression (20.7 %), 10 exhibited moderate expression (17.2 %) and 31 demonstrated strong expression (53.4 %). Of the recurrent tumours, six cases showed no NAV1 expression (25 %), four revealed weak expression (16.7 %), five exhibited moderate expression (20.8 %), while nine tumours demonstrated strong expression (37.5 %). Fisher's exact test did not identify a significant association between strong NAV1 expression levels in the primary cohort and clinical factors including patient sex ($p = 0.583$), patient age above or below three years (compared against SIOP 1999 04 cohort results; $p = 0.689$), patient age above or below one year ($p = 0.09$), tumour location ($p = 1.0$), WHO histological grade ($p = 0.429$) or resection status ($p = 0.124$). Expression was also not significantly increased at recurrence ($p = 0.437$, two-tailed Fisher's exact test).

Of the 69 intracranial ependymomas comprising the SIOP 1999 04 TMA cohort, six tumours (four primary, two recurrent) could not be assessed due to core loss or the presence of non-viable tumour, leaving 43 primary and 20 recurrent tumours available for analysis. Of these primary tumours, four did not express NAV1 (9.3 %), eight revealed weak expression (18.6 %), six exhibited moderate expression (14 %) and 25 demonstrated strong expression (53.4 %). Within the recurrent tumour cohort, negative, weak and moderate expression were each seen in two (10 %), one (5.3 %), and five (26.3 %) cases respectively, while 14 demonstrated strong expression (70 %). As with the UK CCLG 1992 04 cohort, NAV1 expression was not significantly higher at recurrence ($p = 0.744$, two-tailed Fisher's exact test). In addition, Fisher's exact test did not identify a significant association between strong NAV1 expression levels in the primary cohort and clinical factors including patient sex ($p = 1.0$), patient age above or below three years (see above; $p = 0.689$), tumour location ($p = 0.765$), WHO histological grade ($p = 0.501$) or resection status ($p = 1.0$).

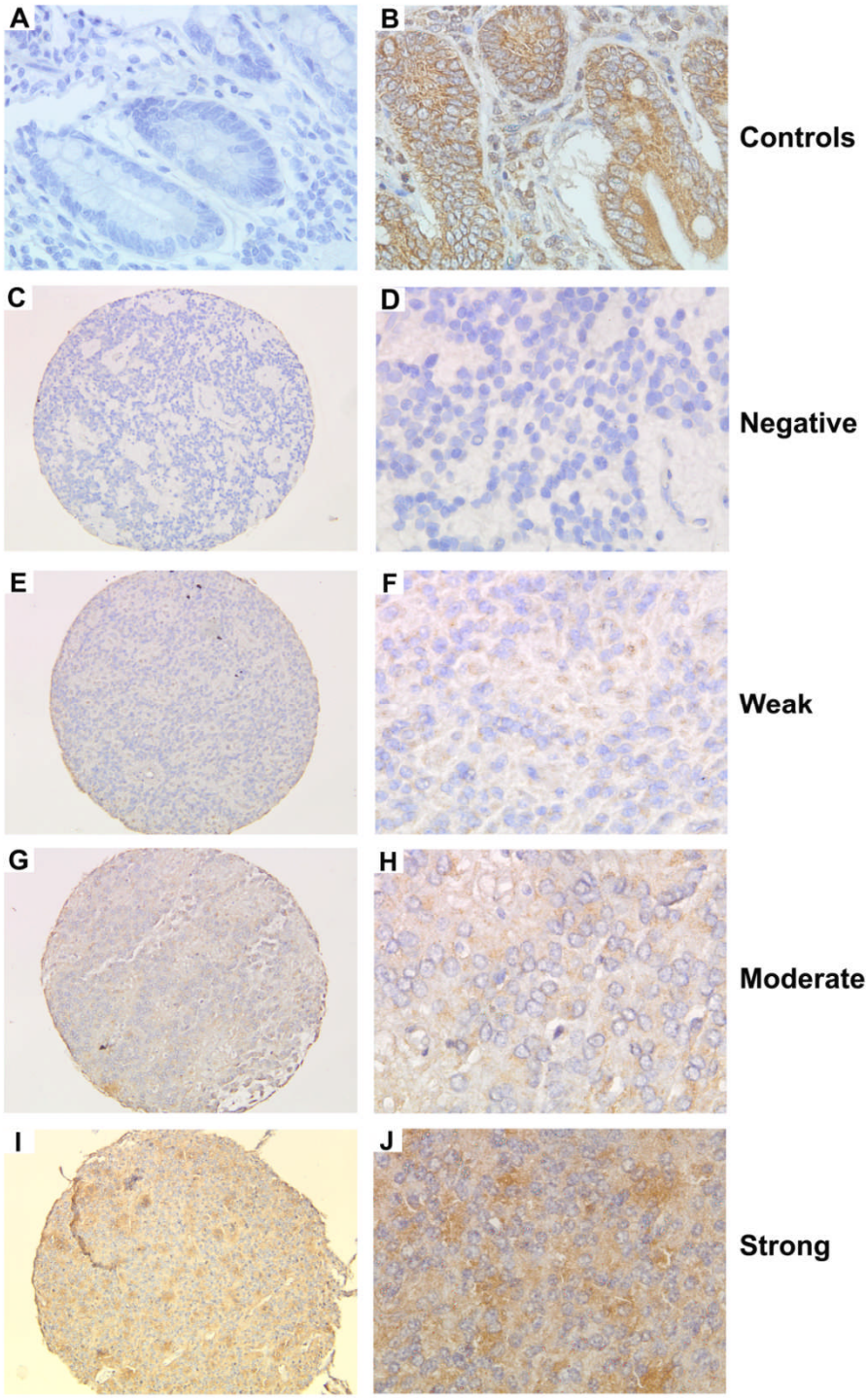

Figure 6.12: Neuron Navigator 1 (NAV1) immunohistochemistry performed using the HPA018127 monoclonal antibody at a 1:350 dilution (Sigma-Aldrich, UK). (A and B): Negative and positive control tissue (small intestine) x40 objective. (C and D): Example of a clinical trial TMA specimen demonstrating negative NAV1 expression at x10 and x40 objectives respectively (sample shown is 9904 – 18R1). (E and F): Example of a clinical trial TMA core specimen demonstrating weak NAV1 expression at x10 and x40 objectives respectively (sample shown is 9904 – 34P; although this core scored weak, sample overall scored moderate). (G and H): Example of a clinical trial TMA specimen demonstrating moderate NAV1 expression at x10 and x40 objectives respectively (sample shown is 9204 – 4R1). (I and J): Example of a clinical trial TMA specimen demonstrating strong NAV1 expression at x10 and x40 objectives respectively (sample shown is 9204 – 47P).

6.3.4.3.2 Comparison of 500K SNP array and IHC results

Twenty-eight paediatric ependymomas that had been subjected to both the 500K SNP array analysis and this immunohistochemical study were examined to verify whether copy number gain of *NAV1* was associated with a corresponding increase in expression of its encoded protein (Table 6.14).

Table 6.14: Results of *NAV1* copy number gain as identified by the 500K SNP array analysis and corresponding NAV1 IHC expression for 28 paediatric ependymomas.

Tumour sample ID (500K SNP array ID)	**Evidence of *NAV1* copy number increase/gain from SNP array**	**Level of NAV1 expression on immunohistochemistry**
1P	No gain	Negative
2P	No gain	Strong
3P	No gain	Weak
4P	No gain	Negative
8P	No gain	Strong
9R3	No gain	Focal strong
13P	Gain	Strong
14P	Gain	Strong
17P	No gain	Strong
17R1	No gain	Weak
17R2	No gain	Moderate
18P	Gain	Strong
18R1	No gain	Negative
20P	No gain	Focal strong
20R1	Gain	Moderate
21P	Gain	Strong
22P	No gain	Strong
23P	No gain	Moderate
26P	Gain	Focal strong
30P	Gain	Moderate
34P	No gain	Focal strong
35P	Gain	Strong
35R1	Gain	Strong
36P	No gain	Focal strong
39P	Gain	Strong
41P	No gain	Strong
43R2	No gain	Weak
43R3	No gain	Focal moderate

P = primary, R1 – R3 = 1st – 3rd recurrence.

Ten ependymomas (36 %) had evidence of *NAV1* copy number gain from the SNP array, while 18 did not (64 %). All ten tumours with genomic *NAV1* gain from the array data demonstrated moderate (n = 2) or strong (n = 8) NAV1 expression (Tables 6.14 and 6.15). However, of the 18 tumours without *NAV1* copy number gain, 12 (67 %) also had evidence of moderate or strong expression. Fisher's exact test found a trend towards

significance for the association between copy number increase in *NAV1* and a moderate or strong level of NAV1 expression (p = 0.062, two-tailed Fisher's exact test) (Table 6.15), although this was lost when considering strong expression alone (p = 0.226).

Table 6.15: Comparison of *NAV1* copy number results and NAV1 expression results for 28 paediatric ependymomas.

	NAV1 IHC Moderate/strong	**NAV1 IHC Negative/weak**	**Total**
500K SNP array *NAV1* gain	10 tumours	0 tumours	10 tumours
500K SNP array No *NAV1* gain	12 tumours	6 tumours	18 tumours
Total	22 tumours	6 tumours	28 tumours

SNP = Single Nucleotide Polymorphism, IHC = immunohistochemistry. p = 0.062, two-tailed Fisher's exact test.

6.3.4.3.3 Measure of agreement between scorers

To assess the reproducibility of the IHC scoring system used to identify strong NAV1 expression, 17 tumours from the UK CCLG 1992 04 cohort (19 %) and 10 ependymomas from the SIOP 1999 04 cohort (15 %) were re-analysed by a blinded second scorer. In total, 61 TMA cores were cross-examined and classified as either demonstrating, or not demonstrating strong NAV1 expression (Table 6.16). The resulting Kappa measure of agreement was 0.865, $p = 9.19 \times 10^{-12}$.

Table 6.16: Assessment of NAV1 expression by two scorers.

	Scorer 2 (JB) Strong	**Scorer 2 (JB) Not strong**	**Total**
Scorer 1 (JPK) Strong	23 cores	4 cores	27 cores
Scorer 1 (JPK) Not strong	0 cores	34 cores	34 cores
Total	23 cores	38 cores	61 cores

A total of 61 cores were assessed by the two scorers (1 and 2 with ID initials included). The resulting Kappa measure of agreement was 0.865, $p = 9.19 \times 10^{-12}$.

6.3.4.3.4 Survival analysis

Univariate event-free and overall survival analysis of the UK CCLG 1992 04 primary cohort did not identify a significantly worse patient prognosis for tumours demonstrating strong NAV1 expression when compared against tumours demonstrating negative, weak and moderate expression (estimated mean EFS: 6.5 $\pm$ 1.1 years versus 5.2 $\pm$ 1 year, p = 0.633; estimated mean OS: 10.2 $\pm$ 1.1 years versus 8.3 $\pm$ 1.1 years, p = 0.343) (Figure 6.13). This was also evident from multivariate analysis (EFS; p = 0.13, OS; p = 0.206).

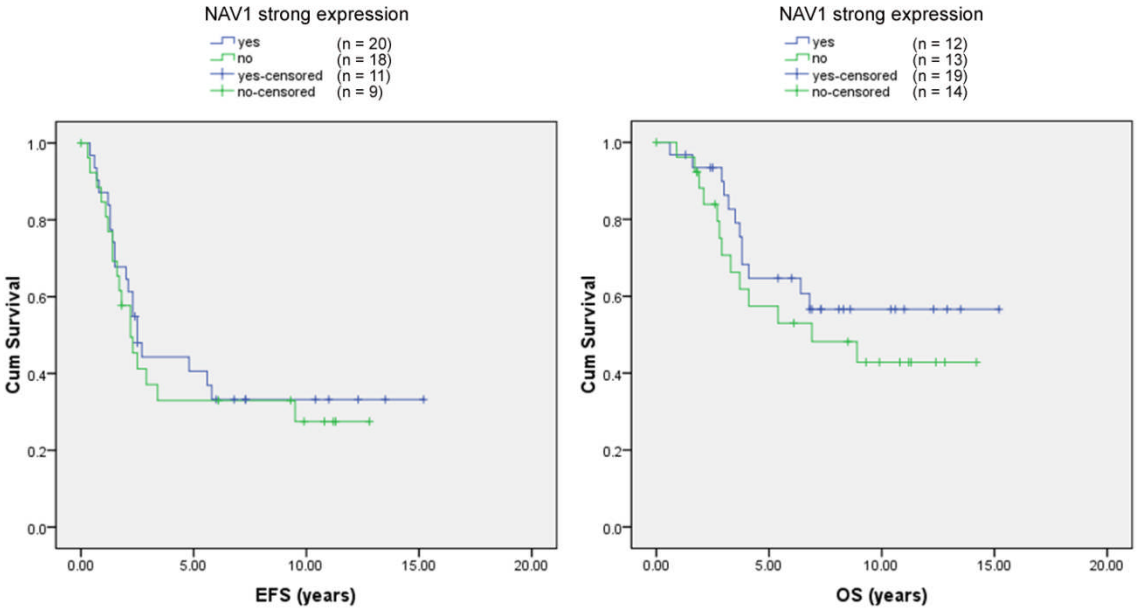

Figure 6.13: Kaplan-Meier EFS and OS curves, comparing UK CCLG 1992 04 primary ependymomas with strong NAV1 expression (blue lines) against those demonstrating negative, weak and moderate expression (green lines). No significant associations were identified for EFS (percentage from each group attaining 5 year EFS = 40.6 $\pm$ 9 % (strong expression group) versus 33 $\pm$ 9.4 % (remaining cohort), p = 0.633) or OS (percentage from each group attaining 5 year OS = 64.7 $\pm$ 9 % (strong expression group) versus 57.4 $\pm$ 10.3 % (remaining cohort), p = 0.343). Cum survival = cumulative survival.

Univariate analysis of the SIOP 1999 04 primary cohort found that tumours with strong NAV1 expression had a trend towards a worse event-free patient survival when compared against those exhibiting negative, weak and moderate expression (estimated mean EFS: 3.8 $\pm$ 0.8 years versus 5.7 $\pm$ 0.8 years, p = 0.057) (Figure 6.14). Strong expression did not significantly influence overall survival however (estimated mean OS: 6 $\pm$ 0.7 years (strong expression group) versus 6.8 $\pm$ 0.7 years (remaining cohort), p = 0.266). Multivariate analysis did not find strong NAV1 expression to be an adverse prognostic marker (EFS; p = 0.225, OS; p = 0.512).

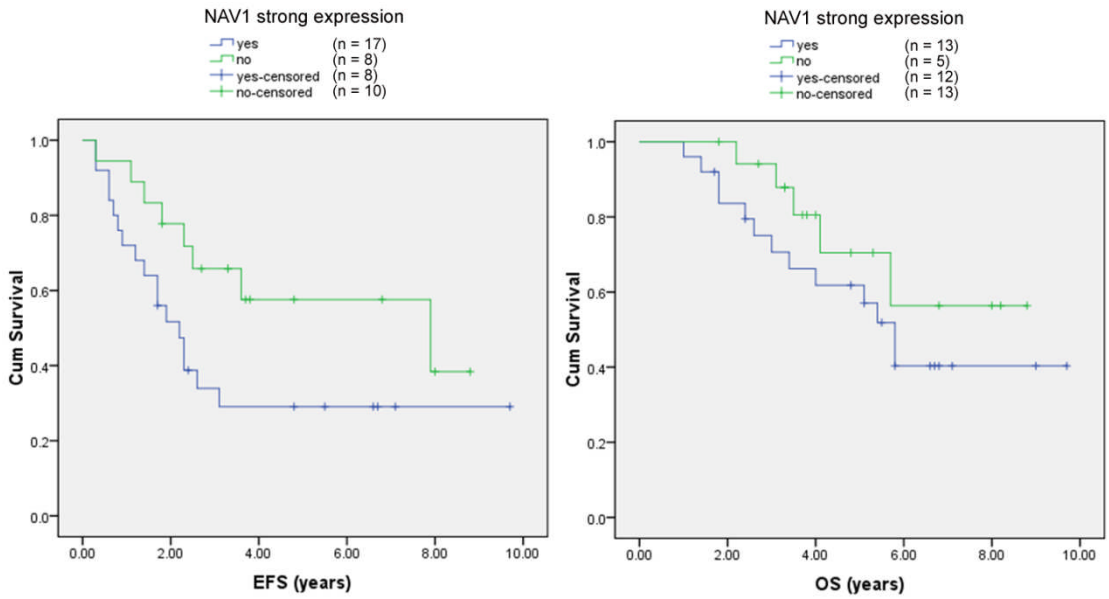

Figure 6.14: Kaplan Meier EFS and OS curves, comparing SIOP 1999 04 primary ependymomas with strong NAV1 expression (blue lines) against those demonstrating negative, weak and moderate expression (green lines). Tumours demonstrating strong expression had a trend towards a worse event-free survival when compared against the remainder of the cohort (percentage attaining 5 year EFS = 29.1 % $\pm$ 9.5 % (strong expression group) versus 57.6 % $\pm$ 12.6 % (remaining cohort), p = 0.057). However this was not seen for OS (percentage attaining 5 year OS = 61.8 % $\pm$ 10.1 % (strong expression group) versus 70.5 % $\pm$ 13 % (remaining cohort), p = 0.266). Cum survival = cumulative survival.

6.3.4.4 Tenascin–C expression

6.3.4.4.1 Staining patterns and statistical associations

Analysis of Tenascin-C (TNC) expression was performed by immunohistochemistry on both clinical trial TMA cohorts. As described in Chapter 2, section 2.6, ependymomas were categorised as demonstrating negative, weak or moderate/strong expression (Figure 6.15).

Of the 89 intracranial ependymomas comprising the UK CCLG 1992 04 TMA cohort, four recurrent tumours could not be assessed due to core loss, leaving 60 primary and 25 recurrent tumours available for analysis. Of the primary tumours, five did not express TNC (8.3 %), six revealed weak expression (10 %), while 49 demonstrated moderate/strong expression (81.7 %).

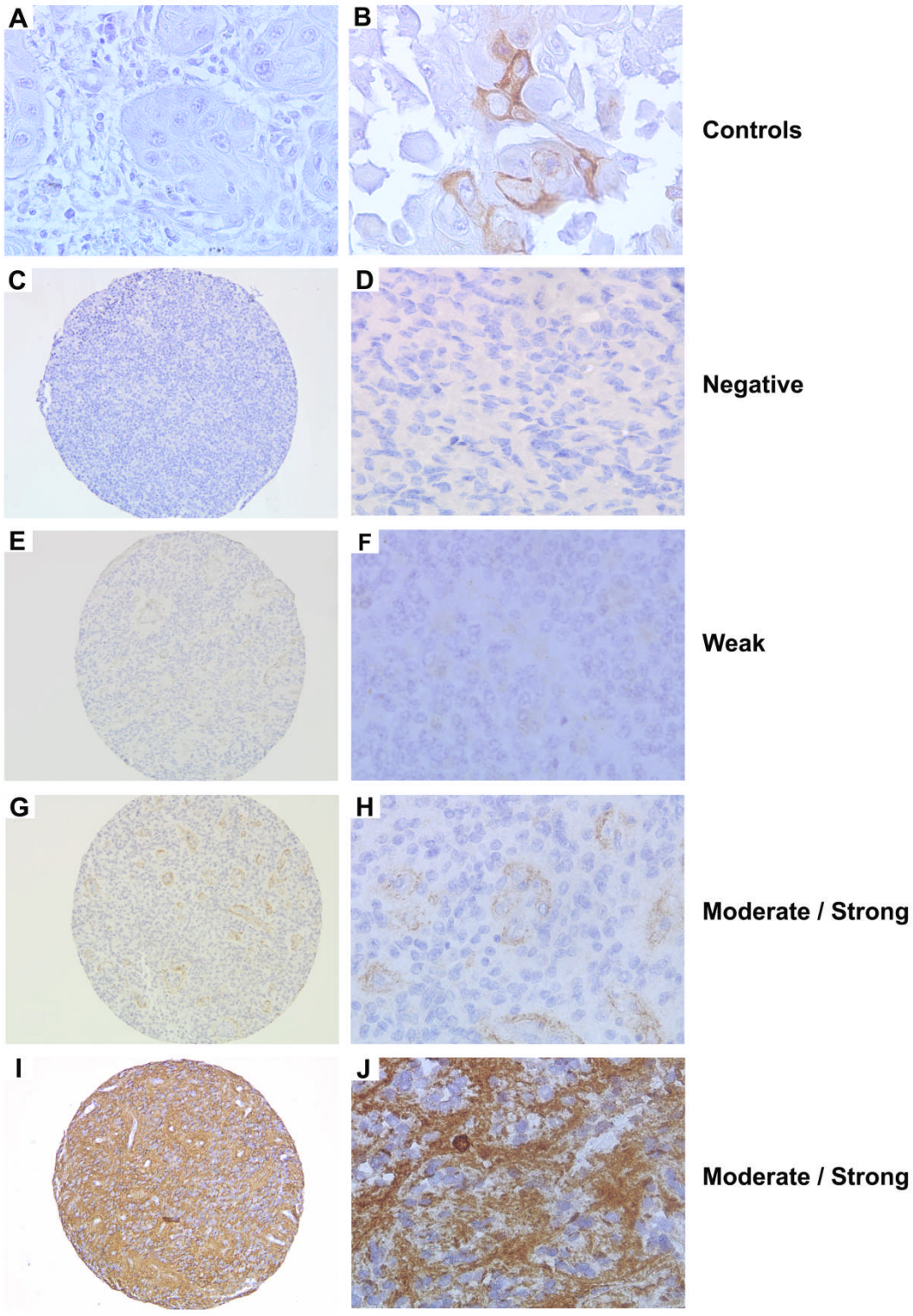

Figure 6.15: Tenascin-C (TNC) immunohistochemistry performed using the sc-25328 monoclonal antibody at a 1:50 dilution (Santa Cruz, USA). (A and B)**:** Negative and positive control tissue (epidermoid carcinoma) x40 objective. (C and D): Example of a clinical trial TMA specimen demonstrating negative TNC expression at x10 and x40 objectives respectively (sample shown is 9904 – 39P). (E and F): Example of a clinical trial TMA specimen demonstrating weak TNC expression at x10 and x40 objectives respectively (sample shown is 9204 – 47P). (G and H): Example of a clinical trial TMA specimen demonstrating lower level moderate/strong TNC expression at x10 and x40 objectives respectively (sample shown is 9904 – 35P). (I and J): Example of a clinical trial TMA specimen demonstrating higher level moderate/strong TNC expression at x10 and x40 objectives respectively (sample shown is 9204 – 62P).

The distribution of staining patterns was similar in the recurrent cohort. Two tumours did not express TNC (8 %), four revealed weak expression (16 %) and 19 demonstrated moderate/strong expression (76 %). Moderate/strong TNC expression in the primary cohort was associated with posterior fossa tumours ($p = 0.01$, two tailed Fisher's exact test) and patients aged equal to or below three years of age when compared to results from the SIOP 1999 04 cohort ($p = 8.979 \times 10^{-6}$, two-tailed Fisher's exact test). No association was identified between altered expression and the clinical variables of patient sex ($p = 1.0$), patient age above or below one year ($p = 0.302$), tumour WHO grade ($p = 0.32$) and resection status ($p = 1.0$), or tumour recurrence ($p = 0.751$).

Of the 69 intracranial ependymomas comprising the SIOP 1999 04 TMA cohort, five primary tumours could not be assessed due to core loss or the presence of non-viable tumour, leaving 42 primary and all 22 recurrent tumours available for analysis. As stated above, in comparison to the younger patient trial cohort, the proportion of primary tumours exhibiting moderate/strong expression in the SIOP 1999 04 cohort was reduced. Nine cases did not express TNC (21.4 %), 17 revealed weak expression (40.6 %), while only 16 demonstrated moderate/strong expression (38 %). Likewise within the recurrent cohort, four tumours had absent expression (18.2 %), 12 exhibited weak expression (54.5 %) and only six cases demonstrated moderate/strong expression (27.3 %). In addition to the aforementioned age association, moderate/high TNC expression in the SIOP 1999 04 cohort was associated again with posterior fossa ependymomas ($p = 0.05$, two-tailed Fisher's exact test) and also with incompletely resected tumours ($p = 0.01$, two-tailed Fisher's exact test). No association was identified for altered expression and patient sex ($p = 0.177$), tumour WHO grade ($p = 0.152$) or recurrence ($p = 1.0$).

6.3.4.4.2 Measure of agreement between scorers

To assess the reproducibility of the IHC technique and scoring system adopted for TNC expression, all 60 primary ependymomas from the UK CCLG 1992 04 cohort and 34 primary tumours from the SIOP 1999 04 cohort (81 %) were assessed for TNC expression by IHC twice, with each attempt independently analysed by a blinded scorer (Table 6.17). The resulting Kappa measure of agreement was 0.91, $p = 1.12 \times 10^{-18}$.

Table 6.17: Assessment of TNC expression by two scorers.

	Scorer 2 (FA) Negative/weak	Scorer 2 (FA) Moderate/Strong	Total
Scorer 1 (JPK) Negative/weak	33 tumours	3 tumours	36 tumours
Scorer 1 (JPK) Moderate/strong	3 tumours	57 tumours	60 tumours
Total	36 tumours	60 tumours	94 tumours

A total of 158 paediatric ependymomas were assessed by the two scorers (1 and 2 with ID initials included). The resulting Kappa measure of agreement was 0.823, $p = 1.12 \times 10^{-13}$. As the assessment undertaken was large, concordance data in relation to tumours, rather than cores is presented.

6.3.4.4.3 Survival analysis

Univariate analysis of the UK CCLG 1992 04 primary cohort revealed moderate to strong TNC tumour expression to be associated with a worse overall patient survival when compared to negative and weak expression (estimated mean OS: 8.4 $\pm$ 0.9 years versus 11.2 $\pm$ 1.1 years, p = 0.044). Ependymomas demonstrating moderate/strong TNC expression also a trend towards an adverse event-free survival (estimated mean EFS: 5.3 $\pm$ 0.9 years (moderate/strong expression group) versus 7.7 $\pm$ 1.5 years (remaining cohort), p = 0.087) (Figure 6.16). However, this finding was not replicated on multivariate analysis (EFS; p = 0.447, OS; p = 0.278) (Tables 6.8 and 6.9).

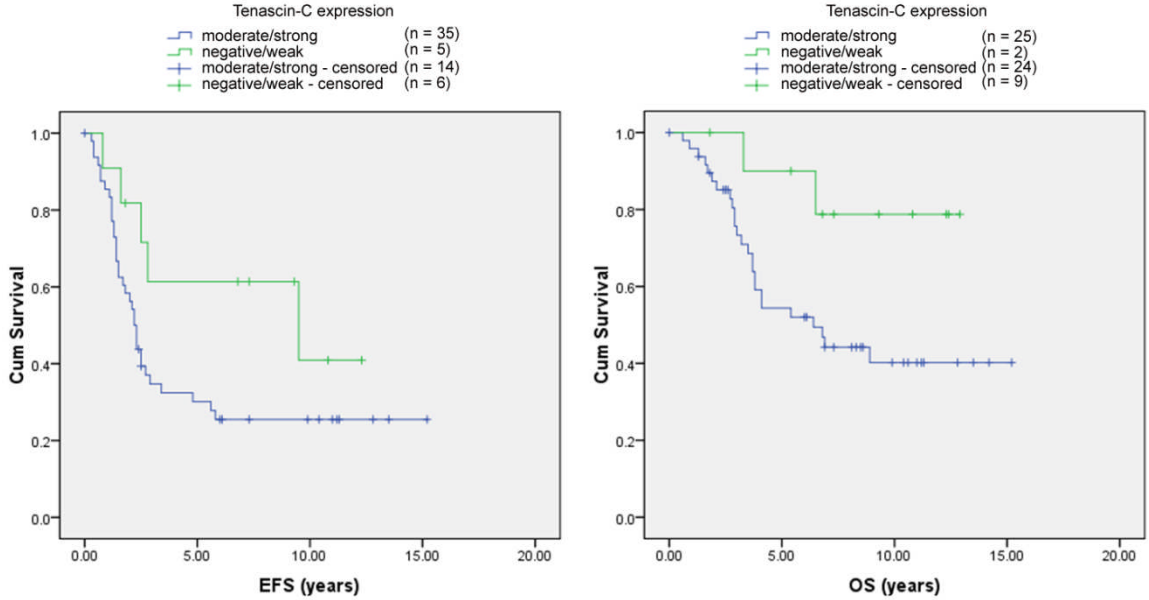

Figure 6.16: Kaplan- Meier EFS and OS curves, comparing UK CCLG 1992 04 primary ependymomas with moderate to strong TNC expression (blue lines) against those demonstrating negative or weak expression (green lines). Moderate/strong expression was significantly associated with an adverse OS (percentage from each group attaining 5 year OS = 54.4 $\pm$ 7.6 % (moderate/strong expression group) versus 90 $\pm$ 9.5 % (negative/weak expression group), p = 0.044). Moderate/strong expression also revealed a trend towards a worse event-free survival (percentage from each group attaining 5 year EFS = 30.1 $\pm$ 6.8 % (moderate/strong expression group) versus 61.4 $\pm$ 15.3 % (negative/weak expression group), p = 0.087). Cum survival = cumulative survival.

Univariate event-free and overall survival analysis of the SIOP 1999 04 primary cohort did not identify a significantly worse prognosis for tumours demonstrating moderate/strong TNC expression when compared to those exhibiting negative or weak expression (estimated mean EFS: 4.1 $\pm$ 0.9 years versus 5.5 $\pm$ 0.8 years, p = 0.289; estimated mean OS: 5.8 $\pm$ 0.8 years versus 7.2 $\pm$ 0.7 years, p = 0.315) (Figure 6.17). This lack of association was also evident from multivariate analysis (EFS; p = 0.872, OS; p = 0.156).

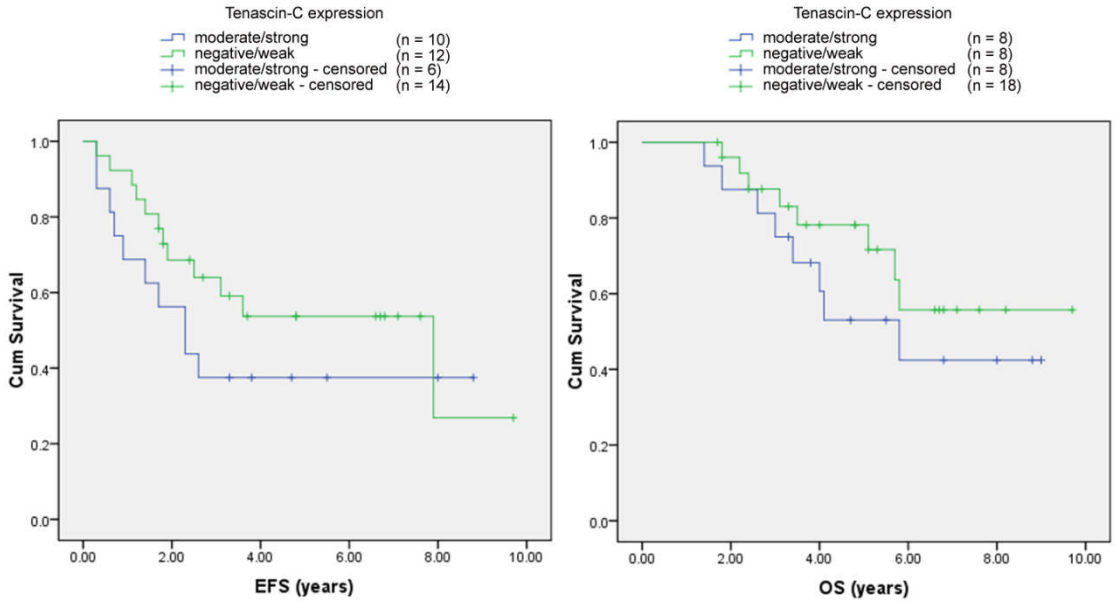

Figure 6.17: Kaplan-Meier EFS and OS curves, comparing SIOP 1999 04 primary ependymomas with moderate to strong TNC expression (blue lines) against those demonstrating negative or weak expression (green lines). No significant associations were identified for EFS (percentage from each group attaining 5 year EFS = 37.5 $\pm$ 12.1 % (moderate/strong expression group) versus 53.7 $\pm$ 10.5 % (negative/weak expression group), p = 0.289) or OS (percentage from each group attaining 5 year OS = 53 $\pm$ 13.2 % (moderate/strong expression group) versus 78.2 $\pm$ 8.7 % (negative/weak expression group), p = 0.315). Cum survival = cumulative survival.

6.3.4.5 Nucleolin expression

6.3.4.5.1 Staining patterns and statistical associations

Analysis of Nucleolin expression was performed by immunohistochemistry on both clinical trial TMA cohorts. As described in Chapter 2, section 2.6, a Nucleolin labelling index (LI) threshold of 50 % was used to define low and high expression tumour groups (Figure 6.18).

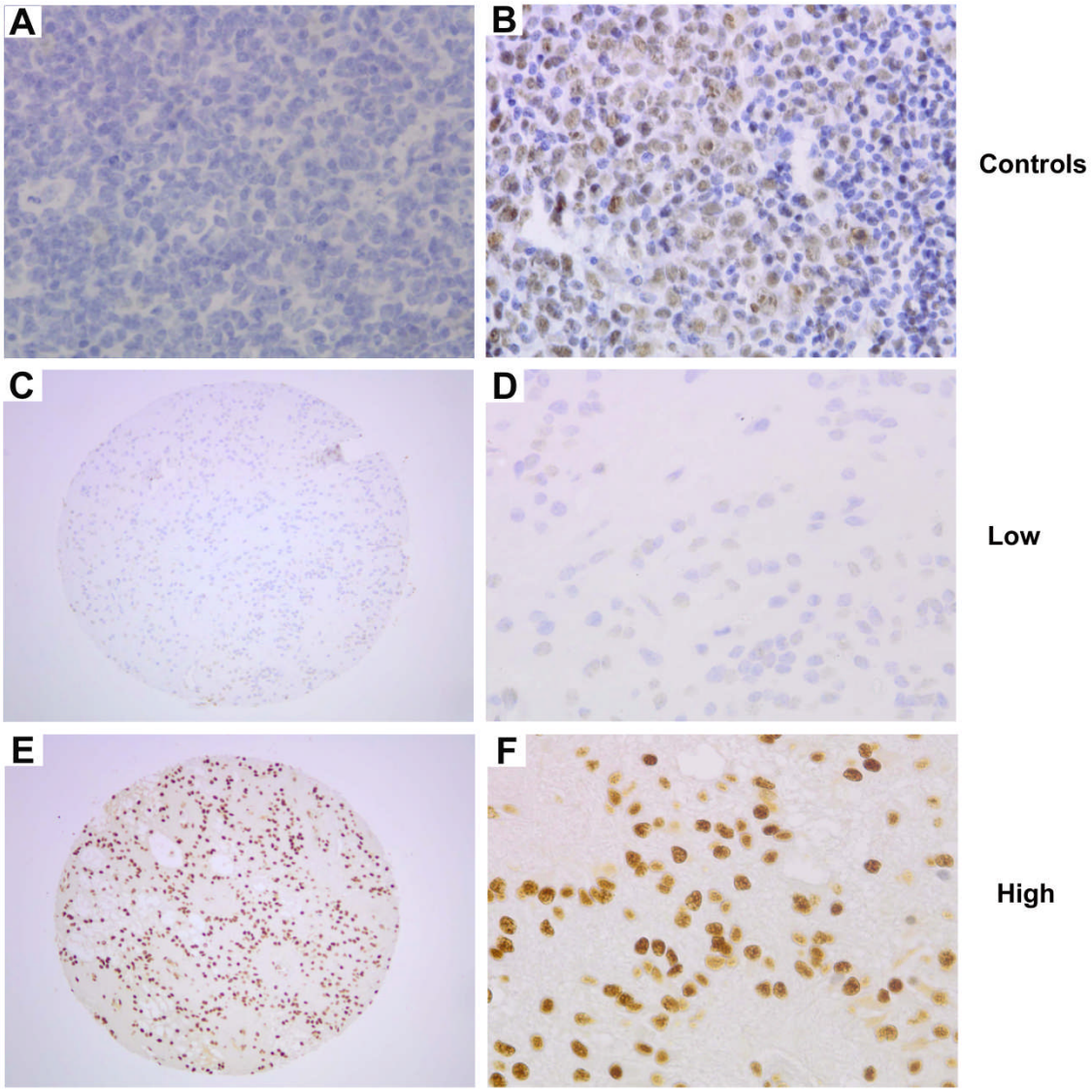

Figure 6.18: Nucleolin immunohistochemistry performed using the ab13541 monoclonal antibody at a 1:400 dilution (Abcam, UK). (A and B): Negative and positive tonsil control tissue at x40 objective. (C and D): Example of a clinical trial TMA specimen demonstrating low Nucleolin expression (Labelling Index $\leq$ 50 %) at x10 and x40 objectives respectively (sample shown is 9204 – 24P: Labelling Index 37 %). (E and F): Example of a clinical trial TMA specimen demonstrating high Nucleolin expression (Labelling Index > 50 %) at x10 and x40 objectives respectively (sample shown is 9904 – 26P: Labelling Index 95 %).

Of the 89 intracranial ependymomas comprising the UK CCLG 1992 04 TMA cohort, three recurrent tumours could not be assessed due to either core loss or the presence of non-viable tumour, leaving 60 primary and 26 recurrent ependymomas available for analysis. Fifty-six primary tumours (93.3 %) demonstrated high Nucleolin expression (mean LI 86.8 % $\pm$ 1.6 %), while four cases (6.7 %) exhibited low expression (mean LI 29.3 % $\pm$ 6.4 %). Of the recurrent cohort, 23 tumours (88.5 %) demonstrated high Nucleolin expression (mean LI 90 % $\pm$ 1.9 %) and three cases (11.5 %) revealed low expression (mean LI 25.3 % $\pm$ 7.3 %). Fisher's exact test did not identify a significant association between altered Nucleolin expression in the primary cohort and clinical

factors including patient sex (p = 0.606), patient age above or below three years (compared against SIOP 1999 04 cohort results; p = 0.199), patient age above or below 1 year (p = 1.0), tumour location (p = 1.0), WHO histological grade (p = 0.614) and resection status (p = 0.616), or tumour recurrence (p = 0.647). Nevertheless, a comparison of Nucleolin values between tumours from these clinical subgroups identified a higher expression value for anaplastic compared to classic ependymomas (mean 88.5 $\pm$ 2.6 % versus 78.9 $\pm$ 3.8 %; p = 0.043, independent t-test) and completely resected tumours when compared to those incompletely resected (mean 88.1 $\pm$ 2.6 % versus 78.4 % $\pm$ 3.8 %; p = 0.04, independent t-test).

Of the 69 intracranial ependymomas comprising the SIOP 1999 04 TMA cohort, five tumours (two primary and three recurrent) could not be assessed due to core loss, leaving 45 primary and 19 recurrent tumours available for analysis. Thirty-eight of these primary cases (73.3 %) demonstrated high Nucleolin expression (mean LI 87.2 $\pm$ 1.9), while seven cases (26.7 %) exhibited low expression (mean LI 39.6 $\pm$ 2.2). Of the recurrent cohort, 17 tumours (89.5 %) demonstrated high Nucleolin expression (mean LI 91.8 $\pm$ 1.6) and two cases (10.5 %) revealed low expression (mean LI 41.5 $\pm$ 1.5). Again, Fisher's exact test did not identify an association between altered Nucleolin expression in the primary cohort and the clinical factors of patient sex (p = 0.419), patient age (see above; p = 0.199), tumour location (p = 0.693), WHO histological grade (p = 0.344) and resection status (p = 1.0), or recurrence (p = 1.0). Similarly, independent t-tests of Nucleolin expression values found no associations between altered expression and these clinical subgroups.

6.3.4.5.2 Measure of agreement between scorers

To assess the reproducibility of the IHC scoring system adopted for Nucleolin expression, 58 ependymomas from the UK CCLG 1992 04 cohort (65 %) and 33 ependymomas from the SIOP 1999 04 cohort (48 %) were re-analysed by a blinded second scorer. In total, 209 TMA cores were cross-examined using the 50 % LI threshold (Table 6.18). The resulting Kappa measure of agreement was 0.823, $p = 1.12 \times 10^{-13}$.

Table 6.18: Assessment of Nucleolin expression by two scorers.

	Scorer 2 (LR) > 50 %	**Scorer 2 (LR) ≤ 50 %**	**Total**
Scorer 1 (JPK) > 50 %	195 cores	4 cores	199 cores
Scorer 1 (JPK) ≤ 50 %	0 cores	10 cores	10 cores
Total	195 cores	14 cores	209 cores

A total of 209 cores were assessed by the two scorers (1 and 2 with ID initials included). The resulting Kappa measure of agreement was 0.823, $p = 1.12 \times 10^{-13}$.

6.3.4.5.3 Survival analysis

Univariate event-free and overall survival analysis of the UK CCLG 1992 04 primary cohort did not identify a prognostic advantage for tumours with a Nucleolin expression below the 50% LI threshold when compared against tumours demonstrating high expression (estimated mean EFS: 2.5 ± 0.4 years (low expression group) versus 6.2 ± 0.8 years (high expression group), p = 0.9; estimated mean OS: 5.2 ± 0.8 years (low expression group) versus 9.5 ± 0.9 years (high expression group), p = 0.249) (Figure 6.19). The lack of association was also evident from multivariate analysis (EFS; p = 0.277, OS; p = 0.49).

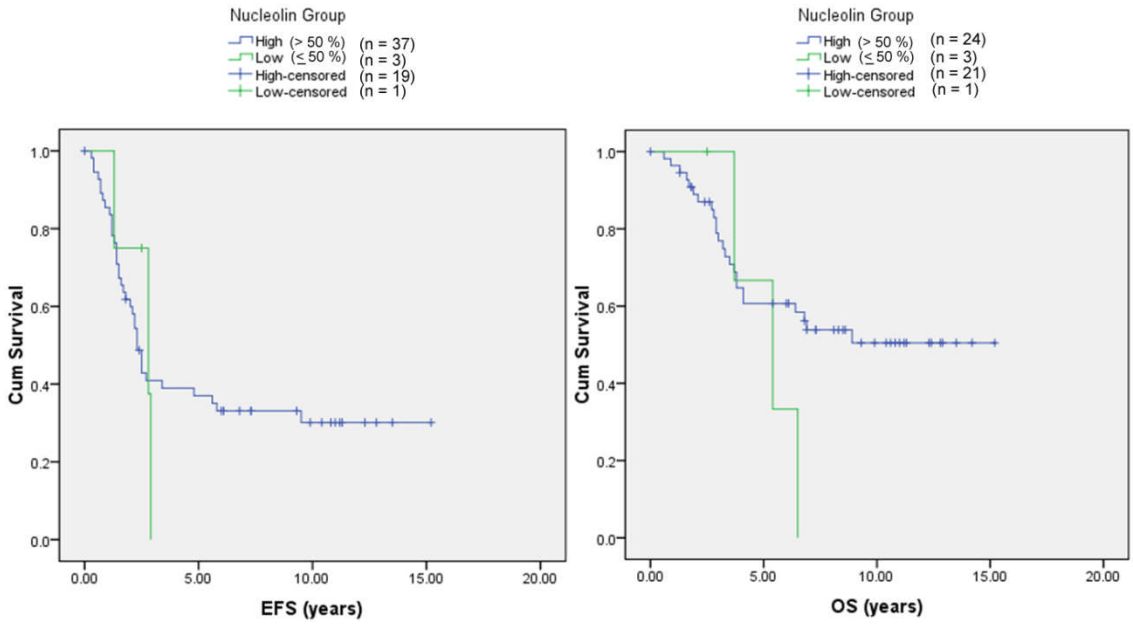

Figure 6.19: Kaplan-Meier EFS and OS curves, comparing UK CCLG 1992 04 primary ependymomas with high Nucleolin expression (above 50 % LI threshold – blue lines) against those demonstrating low expression (below 50 % LI threshold – green lines). No significant associations were identified for EFS (percentage from each group attaining 5 year EFS = 0 % (low Nucleolin expression group) versus 40 ± 7 % (high expression group), p = 0.9) or OS (percentage from each group attaining 5 year OS = 66.7 ± 2.7 % (low expression group) versus 60.7 ± 6.9 % (high expression group), p = 0.249). Cum survival = cumulative survival.

Since only four ependymomas were recognised as having a Nucleolin expression below the LI cut-off of 50 %, the threshold was subsequently adjusted to 60 % in order to identify and re-assess more primary tumours demonstrating low expression. As a result 8/60 cases (13.4 %) were categorised in the modified low Nucleolin expression group, compared to 52/60 tumours demonstrating high expression (86.6 %). However, no prognostic benefit was conferred to this low expression group either. Indeed, ependymomas demonstrating a Nucleolin expression below the 60 % LI threshold had a significantly poorer overall survival on univariate analysis than corresponding tumours exhibiting a higher expression (mean OS: low expression = 4.6 $\pm$ 0.8 years versus high expression = 9.8 $\pm$ 0.9 years, p = 0.041).

As with the UK CCLG 1992 04 cohort, univariate event-free and overall survival analysis of the SIOP 1999 04 primary cohort did not identify a prognostic advantage for tumours with a Nucleolin expression below the 50% LI threshold when compared to tumours demonstrating high expression (estimated mean EFS: 4.4 $\pm$ 1.3 years (low expression group) versus 5.2 $\pm$ 0.7 years (high expression group), p = 0.682; estimated mean OS: 6.2 $\pm$ 0.9 years (low expression group) versus 6.7 $\pm$ 0.6 years (high expression group), p = 0.86) (Figure 6.20). The lack of association was confirmed on multivariate analysis (EFS; p = 0.287, OS; p = 0.595). Adjusting the Nucleolin LI threshold to 60 % failed to identify low expression as a beneficial prognostic marker.

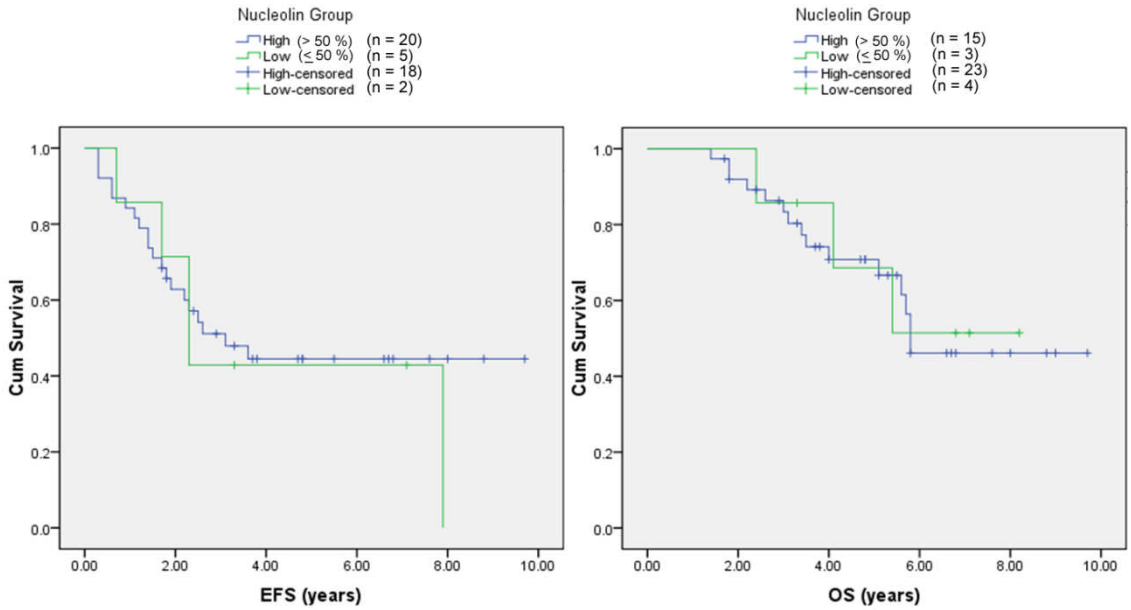

Figure 6.20: Kaplan-Meier EFS and OS curves, comparing SIOP 1999 04 primary ependymomas with high Nucleolin expression (above 50 % LI threshold – blue lines) against those demonstrating low expression (below 50 % LI threshold – green lines). No significant associations were identified for EFS (percentage from each group attaining 5 year EFS = 42.9 $\pm$ 18.7 % (low Nucleolin expression group) versus 44.5 $\pm$ 8.5 % (high expression group), p = 0.682) or OS (percentage from each group attaining 5 year OS = 68.6 $\pm$ 18.6 % (low expression group) versus 70.8 $\pm$ 7.8 % (high expression group), p = 0.86). Cum survival = cumulative survival.

6.3.4.6 Ki-67 expression

6.3.4.6.1 Staining patterns and statistical associations

Analysis of Ki-67 expression was performed by immunohistochemistry on both clinical trial TMA cohorts. The Ki-67 labelling index (LI) was calculated as described in Chapter 2, section 2.6, in order to categorise tumour expression as low (LI $\leq$1 %), moderate (LI 2 – 4 %) or high (LI $\geq$ 5 %) (Figure 6.21).

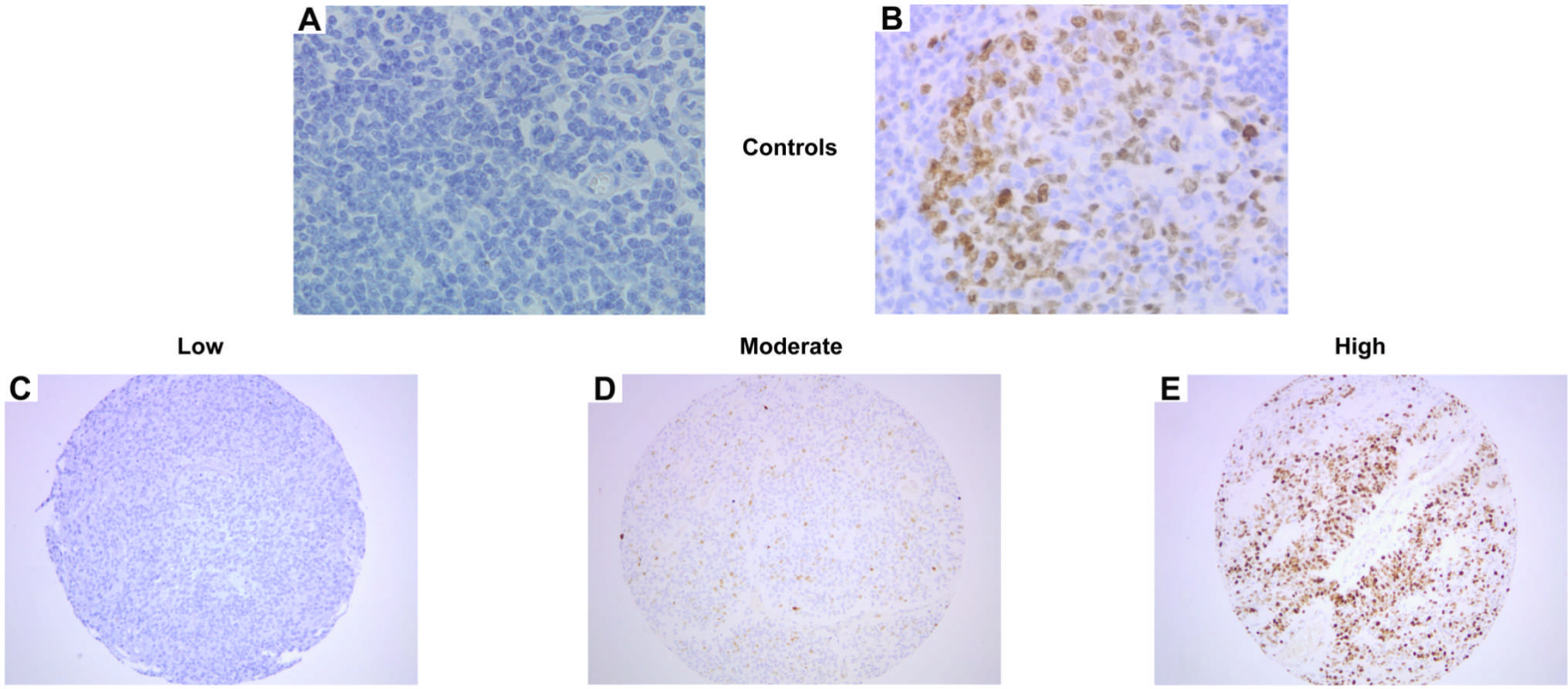

Figure 6.21: Ki-67 immunohistochemistry performed using the M7240 monoclonal antibody at 1:50 dilution (DAKO, UK). (A and B): Negative and positive tonsil control tissue at x40 objective. (C): Example of a clinical trial TMA specimen demonstrating low Ki-67 expression (Labelling Index $\leq$ 1 %) at x10 objective (sample shown is 9204 – 13P: Labelling Index < 1 %). (D): Example of a clinical trial TMA specimen demonstrating moderate Ki-67 expression (Labelling Index 2 – 4 %) at x10 objective (sample shown is 9904 – 26R2: Labelling Index 4 %). (E): Example of a clinical trial TMA specimen demonstrating high Ki-67 expression (Labelling Index $\geq$ 5 %) at x10 objective (sample shown is 9204 – 2R1: Labelling Index 65 %).

Of the 89 intracranial ependymomas comprising the UK CCLG 1992 04 TMA cohort, two recurrent tumours could not be assessed due to core loss, leaving 60 primary and 27 recurrent tumours available for analysis. Of the primary cohort, 37 tumours demonstrated low expression (61.7 %), 13 exhibited moderate expression (21.6 %) and 10 revealed high expression (16.7 %). Contrasting expression was present in the recurrent cohort. While 13 tumours demonstrated low expression (48.1 %) and four revealed moderate expression (14.8 %), 10 cases (37.1 %) revealed high expression. Consequently, high Ki-67 expression was associated with recurrent tumours ($p = 0.036$, two-tailed Fisher's exact test) and the mean Ki-67 expression value for the recurrent cohort was significantly higher than that of the primary cohort (primary tumours mean LI = 2.61 ± 0.45 (range < 1 – 17), recurrent tumours mean LI = 8.41 ± 2.75 (range < 1 – 65), $p = 0.047$, independent t-test). This association was accentuated when only the 15 pairs of patient-matched primary and recurrent ependymomas were assessed ($p = 0.018$, two-tailed Fisher's exact test; primary cohort mean LI = 2.07 ± 0.45, paired recurrent cohort mean LI = 7.76 ± 2.39, $p = 0.031$, independent t-test).

High Ki-67 expression ($\geq$ 5 %) also correlated with a patient age equal to or below one year ($p = 0.05$, two-tailed Fisher's exact test; mean LI for patients aged equal to or below 1 year = 6 ± 2.1, mean LI for children aged above 1 year – 3 years = 2.2 ± 0.42, $p = 0.011$, independent t-test). A higher level of expression was also found in WHO grade III ependymomas (mean LI for grade II tumours = 1.45 ± 0.31, mean LI for grade III tumours = 3.95 ± 0.84, $p = 0.009$, independent t-test). Further assessment by Fisher's exact test or independent t-tests on the primary cohort did not identify associations between altered Ki-67 expression and other clinical factors including patient sex, patient age above or below three years, tumour location or tumour resection status.

Of the 69 intracranial ependymomas comprising the SIOP 1999 04 TMA cohort, three tumours (two primary and one recurrent) could not be assessed due to either core loss or the presence of non-viable tumour, leaving 45 primary 21 recurrent tumours available for analysis. Of the primary ependymomas, 25 demonstrated low expression (53.2 %), 13 exhibited moderate expression (27.7 %) and seven revealed high expression (14.9 %). A similar distribution was evident in the recurrent cohort. Ten tumours (47.6 %) demonstrated low expression, eight showed moderate expression (38.1 %), and three revealed high expression (14.3 %). Consequently, no significant differences in

expression were identified between primary and recurrent tumour groups (primary tumours mean LI = 2.4 $\pm$ 0.39 (range <1 – 10), recurrent cohort mean LI = 2.9 $\pm$ 0.69 (range <1 – 3), p = 0.476, independent t-test). Indeed, Fisher's exact test did not associate altered Ki-67 expression in the primary cohort with any clinical factors including patient sex (p = 1.0), patient age above or below three years (see above; p = 0.929), tumour location (p = 0.767), resection status (p = 0.554) or WHO histological grade (p = 0.188). Similarly, independent t-tests of Ki-67 expression values found no associations between altered expression and these clinical subgroups.

6.3.4.6.2 Measure of agreement between scorers

To assess the reproducibility of the IHC scoring system adopted for Ki-67 expression, 12 ependymomas from the UK CCLG 1992 04 cohort (13 %) and six ependymomas from the SIOP 1999 04 cohort (9 %) were re-analysed by a blinded second scorer. In total, 70 TMA cores were cross-examined using the pre-defined method of calculating the Ki-67 labelling index (Chapter 2, section 2.6). Cores were classified as either demonstrating high (LI $\geq$ 5 %) or low/moderate (LI < 5 %) expression (Table 6.19). The resulting Kappa measure of agreement was 0.85, p = 1.12 x 10^{-12}.

Table 6.19: Assessment of Ki-67 expression by two scorers.

	Scorer 2 (LR) $\geq$ 5% (High)	**Scorer 2 (LR) < 5 % (Low/mod)**	**Total**
Scorer 1 (JPK) $\geq$ 5% (High)	50 cores	2 cores	52 cores
Scorer 1 (JPK) < 5 % (Low/mod)	2 cores	16 cores	18 cores
Total	52 cores	18 cores	70 cores

A total of 70 cores were assessed by the two scorers (1 and 2 with ID initials included). The resulting Kappa measure of agreement was 0.85, p = 1.12 x 10^{-12}. Low/mod = low or moderate Ki-67 expression.

6.3.4.6.3 Survival analysis

Univariate event-free and overall survival analysis of the UK CCLG 1992 04 primary cohort did not find elevated Ki-67 expression to be significantly associated with adverse prognosis, when accounting for high versus low expression (EFS; p = 0.19, OS; p = 0.239), high versus low/moderate expression (EFS p = 0.299, OS p = 0.452) or

moderate/high versus low expression (estimated mean EFS = 4.9 ± 1.2 years versus 6.1 ± 0.9 years, p = 0.146; estimated mean OS = 7.9 ± 1.4 years versus 9.5 ± 0.9 years, p = 0.138) (Figure 6.22). The lack of association was also evident from multivariate analysis (e.g. moderate/high versus low/negative expression EFS; p = 0.623, OS; p = 0.312).

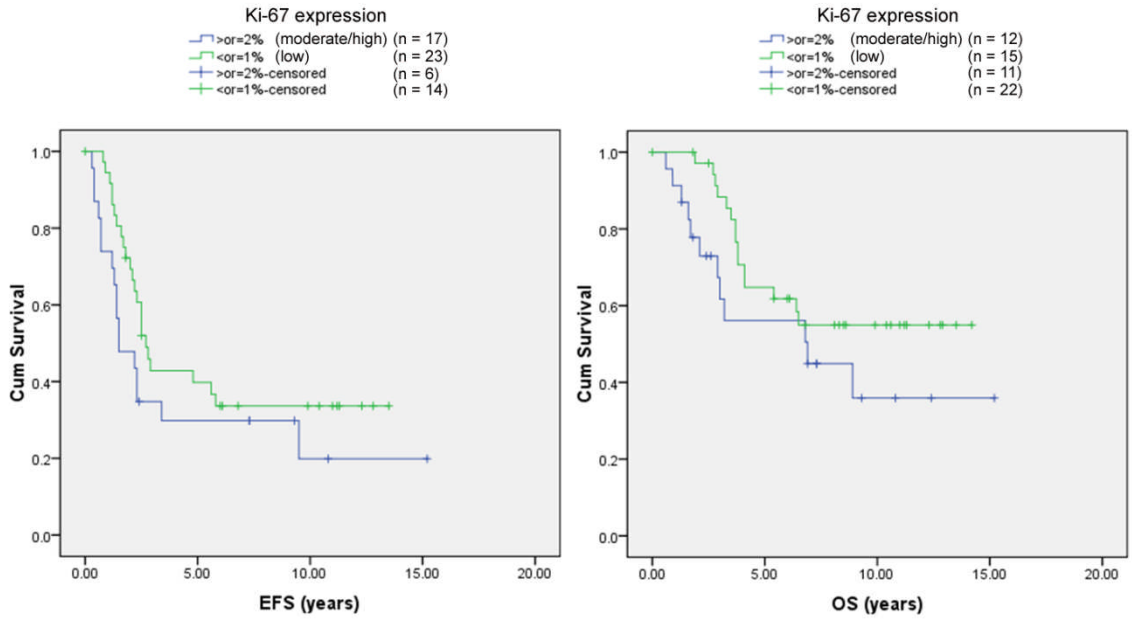

Figure 6.22: Kaplan-Meier EFS and OS curves, comparing UK CCLG 1992 04 primary ependymomas with moderate to high Ki-67 expression (equal or above labelling index of 2 % - blue lines) against those demonstrating low expression (equal to or below labelling index of 1 % - green lines). No significant associations were identified for EFS (percentage from each group attaining 5 year EFS = 29.8 ± 9.7 % (moderate/high expression group) versus 39.8 ± 8.4 % (low expression group), p = 0.146) or OS (percentage from each group attaining 5 year OS = 56.1 ± 11.2 % (moderate/high expression group) versus 64.8 ± 8.2 % (low expression group), p = 0.138). Cum survival = cumulative survival.

As with the UK CCLG 1992 04 cohort, univariate event-free and overall survival analysis of the SIOP 1999 04 primary cohort did not identify a worse prognosis for tumours with elevated Ki-67 expression, despite considering high versus low expression (EFS; p = 0.345, OS; p = 0.969), high versus low/moderate expression (EFS p = 0.385, OS p = 0.937) or moderate/high versus low expression (estimated mean EFS = 5.8 ± 0.9 years versus 4.1 ± 0.6 years, p = 0.373; estimated mean OS = 6.9 ± 0.8 years versus 6.3 ± 0.6 years, p = 0.827) (Figure 6.23). This lack of association was also evident from multivariate analysis (moderate/high versus low/negative expression EFS; p = 0.608, OS; p = 0.622).

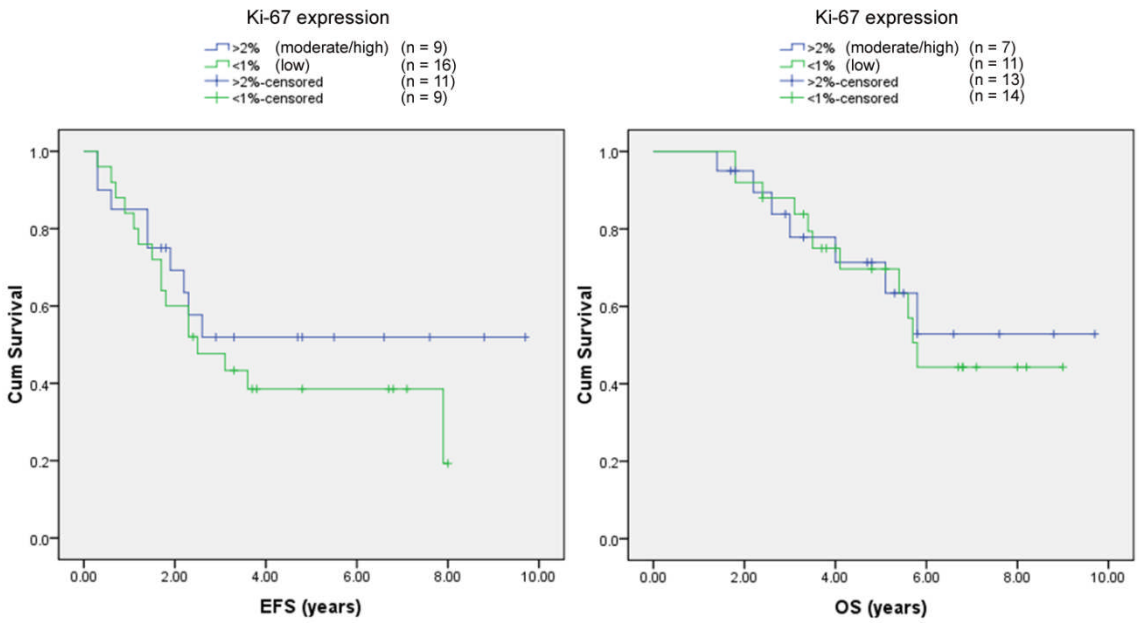

Figure 6.23: Kaplan-Meier EFS and OS curves, comparing SIOP 1999 04 primary ependymomas with moderate to high Ki-67 expression (equal or above labelling index of 2 % - blue lines) against those demonstrating low expression (equal to or below labelling index of 1 % - green lines). No significant associations were identified for EFS (percentage from each group attaining 5 year EFS = 51.9 $\pm$ 11.7 % (moderate/high expression group) versus 38.5 $\pm$ 10 % (low expression group), p = 0.373) or OS (percentage from each group attaining 5 year OS = 71.3 $\pm$ 10.9 % (moderate/high expression group) versus 69.6 $\pm$ 9.7 % (low expression group), p = 0.827). Cum survival = cumulative survival.

6.3.5 Summary of statistically significant results

The tables below summarise the significant clinical and prognostic associations for the six putative biological markers assessed across primary ependymomas from the CCLG UK 1992 04 clinical trial cohort (Table 6.20) and the SIOP 1999 04 clinical trial cohort (Table 6.21).

Table 6.20: Clinical and prognostic associations for the analysed biological markers – UK CCLG 1992 04 primary cohort.

Marker	Patients (%)	Association with clinical variables (p-value)	5 year EFS (%) (SE)	Univariate (log rank) p-value	Multivariate (cox regression) p-value	5 year OS (%) (SE)	Univariate (log rank) p-value	Multivariate (cox regression) p-value
Chromosome 1q25 gain	11 (21.2)		9.1 ± 8.7 %	2.72×10^{-4}	0.009	54.5 ± 15 %	0.413	0.998
No chromosome 1q25 gain	41 (78.8)		50.9 ± 8.1 %			66.5 ± 7.9 %		
Strong PRUNE expression	29 (50.9)		17 ± 7.6 %	0.013	0.005	44.7 ± 10 %	0.012	0.007
Negative/weak/moderate PRUNE expression	28 (49.1)		56.9 ± 9.4 %			80.2 ± 7.9 %		
Strong NAV1 expression	31 (53.4)		40.6 ± 9 %	0.633	0.13	64.7 ± 9 %	0.343	0.206
Negative/weak/moderate NAV1 expression	27 (46.6)		33 ± 9.4 %			57.4 ± 10.3 %		
Moderate/strong TNC expression	49 (81.7)	Posterior fossa tumours and patients aged equal to or below three years ($p = 0.01$ & 8.979×10^{-6})	30.1 ± 6.8 %	0.087	0.447	54.4 ± 7.6 %	0.044	0.278
Negative/weak TNC expression	11 (18.3)		61.4 ± 15.3 %			90 ± 9.5 %		
High Nucleolin expression	56 (93.3)	Grade III and CR tumours ($p = 0.043$ & 0.04)	40 ± 7 %	0.9	0.277	60.7 ± 6.9 %	0.249	0.49
Low Nucleolin expression	4 (6.7)		0 %			66.7 ± 2.7 %		
Moderate/high Ki-67 expression	23 (53.7)	High expression - recurrence, patients aged equal to or below one year, grade III tumours ($p = 0.047$, 0.011 & 0.009)	29.8 ± 9.7 %	0.146	0.623	56.1 ± 11.2 %	0.138	0.312
Low Ki-67 expression	37 (46.3)		39.8 ± 8.4 %			64.8 ± 8.2 %		

Clinical associations were determined either by two-tailed Fisher's exact tests or independent t-tests. Results with statistical significance highlighted yellow. Results with a trend towards significance are highlighted pale yellow. TNC = Tenascin-C, NAV1 = Neuron Navigator 1, CR = completely resected, SE = standard error.

Table 6.21: Clinical and prognostic associations for the analysed biological markers - SIOP 1999 04 primary cohort.

Marker	Patients (%)	Association with clinical variables (p-value)	5 year EFS (%) (SE)	Univariate (log rank) p-value	Multivariate (cox regression) p-value	5 year OS (%) (SE)	Univariate (log rank) p-value	Multivariate (cox regression) p-value
Chromosome 1q25 gain	8 (21.1)		37.5 ± 17.1 %	0.397	0.469	75 ± 15.3 %	0.799	0.560
No chromosome 1q25 gain	30 (78.9)		50.8 ± 9.6 %			72.5 ± 9 %		
Strong PRUNE expression	25 (62.5)		36.6 ± 10.1 %	0.156	0.181	54.3 ± 10.9 %	0.02	0.097
Negative/weak/moderate PRUNE expression	15 (37.5)		58.3 ± 13.2 %			85.6 ± 9.5 %		
Strong NAV1 expression	18 (46.6)		29.1 ± 9.5 %	0.057	0.225	61.8 ± 10.1 %	0.266	0.512
Negative/weak/moderate NAV1 expression	25 (53.4)		57.6 ± 12.6 %			70.5 ± 13 %		
Moderate/strong TNC expression	16 (38)	Posterior fossa tumours, IR tumours, patients aged equal to or below three years ($p = 0.05$, 0.01 & 8.979×10^{-6})	37.5 ± 12.1 %	0.289	0.872	53 ± 13.2 %	0.315	0.156
Negative/weak TNC expression	26 (62)		53.7 ± 10.5 %			78.2 ± 8.7 %		
High Nucleolin expression	38 (73.3)		44.5 ± 8.5 %	0.682	0.287	70.8 ± 7.8 %	0.86	0.595
Low Nucleolin expression	7 (26.7)		42.9 ± 18.7 %			68.6 ± 18.6 %		
Moderate/high Ki-67 expression	20 (46.8)		51.9 ± 11.7 %	0.373	0.608	71.3 ± 10.9 %	0.827	0.622
Low Ki-67 expression	25 (53.2)		38.5 ± 10 %			69.6 ± 9.7 %		

Clinical associations were determined either by two-tailed Fisher's exact tests or independent t-tests. Results with statistical significance highlighted yellow. Results with a trend towards significance are highlighted pale yellow. TNC = Tenascin-C, NAV1 = Neuron Navigator 1, IR = incompletely resected, SE = standard error.

6.4 Discussion

This tissue microarray analysis is the first study to assess putative biological prognostic markers on intracranial ependymomas obtained exclusively from paediatric patients who have been treated uniformly within the confines of two age-dependent clinical trials.

The clinical profiles of the UK CCLG 1992 04 and SIOP 1999 04 cohorts were generally representative of that reported for paediatric ependymoma patients. A bias towards male patients was present in both groups (male: female ratios of 2.1:1 (1992 04) and 1.3:1 (1999 04)), in keeping with other large series (Goldwein, Leahy et al. 1990; Horn, Heideman et al. 1999; Jaing, Wang et al. 2004; Shu, Sall et al. 2007). The observation that approximately two-thirds of paediatric intracranial ependymomas occur in the posterior fossa (Goldwein, Leahy et al. 1990; Ernestus, Schroder et al. 1996; Perilongo, Massimino et al. 1997; Ellison 1998; Robertson, Zeltzer et al. 1998) was supported by the location of primary tumours within the SIOP 1999 04 cohort (60 % infratentorial, 40 % supratentorial). However the relatively higher proportion of primary posterior fossa tumours (77 %) compared to supratentorial tumours (10 %) in the UK CCLG 1992 04 cohort, reinforced the observed propensity for infant ependymomas to originate from an infratentorial location (Rousseau, Habrand et al. 1994; Duffner, Krischer et al. 1998; Sala, Talacchi et al. 1998; Grill, Le Deley et al. 2001; Grundy, Wilne et al. 2007). The majority of primary tumours in both cohorts were of a classic histology, supporting preceding sizeable studies (Rousseau, Habrand et al. 1994; Perilongo, Massimino et al. 1997; Horn, Heideman et al. 1999). Similarly, complete surgical resection was reported in approximately half of all primary cases from both groups, within the 31 – 85 % range defined previously in Chapter 3 (Rousseau, Habrand et al. 1994; Bouffet, Perilongo et al. 1998; Merchant and Fouladi 2005).

The five year event-free survival (35.7 $\pm$ 6.4 % (1992 04); 44.2 $\pm$ 7.6 % (1999 04)), mean time to relapse (2.1 $\pm$ 0.3 years (1992 04); 1.9 $\pm$ 0.3 years (1999 04)) and five year overall survival (61.1 $\pm$ 6.7 % (1992 04); 69 $\pm$ 7.3 % (1999 04)) for both cohorts were also comparable with the survival findings of previous work (Pollack, Gerszten et al. 1995; Perilongo, Massimino et al. 1997; Robertson, Zeltzer et al. 1998; Horn, Heideman

et al. 1999; Agaoglu, Ayan et al. 2005; Zacharoulis, Levy et al. 2007). The association of posterior fossa ependymomas with an adverse overall survival in the UK CCLG 1992 04 cohort upholds evidence that infratentorial ependymomas convey a worse prognosis in children, particularly the very young (Sutton, Goldwein et al. 1990; Heidemann RL 1997; Perilongo, Massimino et al. 1997; Sala, Talacchi et al. 1998), while the impact of incomplete surgical resection on event-free survival in the SIOP 1999 04 cohort supports complete resection as a consistently reported favourable clinical prognostic factor (reviewed by (Bouffet, Perilongo et al. 1998)); (Duffner, Krischer et al. 1998; Robertson, Zeltzer et al. 1998; Horn, Heideman et al. 1999; Grill, Le Deley et al. 2001; Merchant, Li et al. 2009). The negligible prognostic effect of resection status in the younger UK CCLG 1992 04 group contrasted with findings from other studies of intracranial ependymomas in young children (Duffner, Krischer et al. 1998; Grill, Le Deley et al. 2001) and may reflect the sample size or tumour biology of the cohort, or the accuracy of resection status reporting in the young (Bouffet, Perilongo et al. 1998).

Of the six putative biological markers analysed, three were associated with adverse patient prognosis, either in the UK CCLG 1992 04 cohort alone or across both trial groups. These were gain of chromosome 1q25 as detected by FISH, strong PRUNE expression and moderate/strong Tenascin-C expression.

Gain of chromosome 1q25 using FISH was identified as an independent marker of adverse event-free survival in the younger UK CCLG 1992 04 cohort (Table 6.8). While this lends support to the findings of two large retrospective ependymoma FISH analyses reporting 1q25 gain as a marker of event-free and overall survival on mixed age cohorts (Mendrzyk, Korshunov et al. 2006; Korshunov, Witt et al. 2010), this is the first study to demonstrate a prognostic role for 1q25 FISH gain in a prospective paediatric clinical trial setting. The percentage threshold used to define gain in this study (15 % of nuclei counted) was higher than that of the two retrospective series (10 % of nuclei), as the lower threshold did not yield a significant event-free survival difference for the resulting stratified groups, nor did it allow a satisfactory measure of agreement between independent scorers (section 6.3.4.1.3). Nevertheless, the proportion of cases demonstrating 1q25 gain in the 1992 04 cohort (21 %) was similar to the 20 – 25 % reported in these preceding studies (Mendrzyk, Korshunov et al. 2006; Korshunov, Witt et al. 2010).

In contrast to the retrospective analyses, 1q25 FISH gain was not associated with increased mortality for the UK CCLG 1992 04 ependymoma trial patients. While the precise reason for this remains unclear, it could reflect the beneficial influence of a chemotherapy-predominant treatment regime in this age group, or a potential favourable effect of introducing radiotherapy at relapse. Nevertheless, it is plausible that with continued surveillance, a reduction in overall survival may eventually become realised for those with tumours demonstrating gain. In support of this, evidence from paediatric ependymoma patients in the United Kingdom suggests that over 70 % of children below three years of age who suffer tumour recurrence will not survive long term (Messahel, Ashley et al. 2009), affirming that the identification of a prognostic marker that only detects relapse risk in these young children remains an important discovery upon which therapy may be stratified.

The proportion of tumours exhibiting chromosome 1q25 FISH gain in the older SIOP 1999 04 cohort (21 %) was comparable with that observed in both the UK CCLG 1992 04 group and retrospective analyses discussed (Mendrzyk, Korshunov et al. 2006; Korshunov, Witt et al. 2010). However, in contrast to these studies, no association with adverse outcome was identified for 1q25 gain in the 1999 04 cohort. Possible contributory factors to explain this include different cohort sizes between studies, disparate tumour biology and tissue microenvironments for ependymomas from different patient age groups and, particularly, the variety and efficacy of adjuvant therapeutic interventions adopted in each analysis. Indeed, complete surgical tumour resection was identified in the SIOP 1999 04 patient group, but not the UK CCLG 1992 04 cohort, as the only clinical factor to improve univariate event-free survival (section 6.2.1.2), although this did not retain clinical significance on multivariate survival analysis. Moreover, the standardised, focal administration of cranial irradiation to all patients is unique to the 1999 04 trial when compared to the treatment regimes of the other analyses. Radiotherapy was not administered uniformly in the Mendrzyk study (Mendrzyk, Korshunov et al. 2006) and was only reserved for WHO grade III ependymomas or cases of incomplete resection in the Korshunov analysis (Korshunov, Witt et al. 2010), while the 1992 04 trial aimed to avoid or delay radiotherapy using the chemotherapy based strategy discussed (Grundy, Wilne et al. 2007).

Therefore, 1q25 copy number gain does not appear an effective prognostic marker for older paediatric ependymoma patients treated with surgical resection and standardised, focal cranial radiotherapy. Whether the radiation sensitivity of ependymomas per se counteracts a potential negative impact on survival conferred by gain of either 1q25 or the entire 1q arm is unknown, as is whether copy number gain of another region on chromosome 1q could prove a more appropriate and robust prognostic marker for this patient group. A variety of solid malignancies from older patients have revealed 1q21-22 gain as a frequent aberration including sarcomas, ovarian cancers and hepatocellular carcinoma (Forus, Weghuis et al. 1995; Forus, Weghuis et al. 1995; Tapper, Sarantaus et al. 1998; Wong, Lai et al. 1999; Zimonjic, Keck et al. 1999), while gain of 1q21-25 has been associated with adverse prognosis in high grade Ewing sarcoma and neuroblastoma (Hirai, Yoshida et al. 1999; Tarkkanen, Kiuru-Kuhlefelt et al. 1999). Indeed, gain of the 1q21.2-21.3 region encompassing *PRUNE* was associated with a poor patient outcome from the 500K SNP array work presented earlier, while other 1q sites were not, such as gain of the 1q32.1 region incorporating *CHI3L1* (Chapter 4, section 4.3.2). Copy number analysis of regions including 1q21.3 in the SIOP 1999 04 TMA cohort would require design of appropriate FISH probes, a future project discussed further in the final chapter.

In this study, strong PRUNE expression was an independent marker of adverse event-free and overall survival in the young UK CCLG 1992 04 cohort (Tables 6.8 and 6.9), while in the SIOP 1999 04 group it was associated with reduced overall survival on univariate analysis (section 6.3.4.2.4). This confirmed the hypothesis evolving from the preceding SNP array work, suggesting a prognostic role for copy number gain of P*RUNE* (1q21.3) in paediatric ependymoma (Chapter 4, section 4.3.2). *PRUNE* is the human homologue of the *DRES17* gene involved with eye development in *Drosophila* (Banfi, Borsani et al. 1996). The encoded human PRUNE protein is a member of the DHH (Asp-His-His motif) superfamily of phosphodiesterases which hydrolyse cyclic nucleotides and contribute to maintaining cellular homeostasis (Aravind and Koonin 1998; Marino and Zollo 2007).

PRUNE is known to interact with several proteins involved in regulating cell motility, invasiveness and proliferation (D'Angelo and Zollo 2004; Marino and Zollo 2007). It has been shown to bind to and potentially inhibit NM23-H1, a putative metastasis-

suppressor protein, to promote cell motility *in vitro* (D'Angelo, Garzia et al. 2004), while the interaction of PRUNE and Gelsolin, a protein involved in cytoskeleton remodelling, has been purported to enhance the invasive properties of neoplastic cells (Garzia, Roma et al. 2006). Another complex formed by PRUNE binding to the serine-threonine kinase GSK-3β has been reported to regulate the disassembly of cellular focal adhesions, thereby also promoting *in vitro* cell migration (Kobayashi, Hino et al. 2006). Clinically, increased PRUNE expression has been demonstrated in several solid malignancies such as sarcomas and numerous carcinomas (Forus, D'Angelo et al. 2001; Zollo, Andre et al. 2005; Kobayashi, Hino et al. 2006; Oue, Yoshida et al. 2007; Noguchi, Oue et al. 2009). For instance, overexpression has been associated with increased metastatic potential, disease progression and poor patient survival in breast, oesophageal and gastric cancer (Zollo, Andre et al. 2005; Oue, Yoshida et al. 2007; Noguchi, Oue et al. 2009). Moreover, high PRUNE expression has been correlated with an increased depth of neoplastic invasiveness in tumours of the stomach, oesophagus, pancreas and colon (Kobayashi, Hino et al. 2006; Oue, Yoshida et al. 2007; Noguchi, Oue et al. 2009). This latter finding could explain why increased PRUNE expression is often seen in tumours such as well differentiated liposarcomas that frequently relapse locally but rarely metastasise (Forus, D'Angelo et al. 2001), an attribute shared by paediatric ependymomas.

While such evidence suggests *PRUNE* may be a potential proto-oncogene, it remains contentious as to whether PRUNE protein expression is gene copy number dependent. In this study, a comparison of 500K SNP array data and immunohistochemistry results for 27 paediatric ependymomas identified a correlation between strong PRUNE protein expression and genomic gain of the encoding *PRUNE* gene, despite nine tumours (33 %) demonstrating strong expression without *PRUNE* gain (section 6.3.4.2.2). In contrast, PRUNE expression did not correlate with chromosome 1q copy number gain (as detected by 1q25 FISH) in the primary ependymomas from both TMA clinical trial cohorts (1992 04 cohort, $p = 0.299$; 1999 04 cohort, $p = 0.179$), while multivariate survival analysis of the 1992 04 cohort identified strong PRUNE protein expression as an independent adverse prognostic marker from 1q25 FISH gain (Tables 6.8 and 6.9). The disparity in correlation may simply reflect the genomic analysis of incongruent regions within chromosome 1q, questioning whether the 1q25 FISH probe is truly representative of the entire chromosome arm. This is accentuated with a FISH gain

threshold of only 15 % nuclei counted as this is below the 30 % limit that appears required for detection as copy number gain on the SNP array platform (discussed in Chapter 3, section 3.4.4). Insufficient sample numbers prevented a feasible correlation of PRUNE expression with such high-level 1q25 FISH gain, exceeding the 30 % gain threshold (Chapter 3, section 3.4.4).

Other comparative genomic and expression analyses of *PRUNE* as a candidate oncogene in tumours, including ependymoma, have contributed to the uncertainty. While *PRUNE* amplification regularly correlated with high mRNA and protein expression in a study of invasive sarcoma sub-types (Forus, D'Angelo et al. 2001), an analysis of breast carcinomas demonstrating increased PRUNE expression found fewer than seven percent were associated with increased gene copy number (Zollo, Andre et al. 2005). Similarly, the integrative SNP and gene expression ependymoma array analysis performed by Johnson and colleagues (Chapter 4, section 4.1), whilst identifying the 1q21.3 region encompassing *PRUNE* as a site of amplification in this tumour group, did not establish copy number driven *PRUNE* expression. However, this assessment was performed on a mixed age cohort where only 40 % of the tumours analysed on the SNP array were also examined on the expression array, thereby potentially under-representing tumours with genomic gain and corresponding overexpression of the gene.

The presented data suggests that, while PRUNE expression may be driven by increased gene copy number, alternative undefined pathways could activate protein expression. Irrespective of this, the present study has identified high PRUNE expression as an independent marker of event-free and overall survival in intracranial ependymomas from young children treated uniformly according to a clinical trial. PRUNE overexpression may be a distinct prognostic marker from 1q25 FISH gain for ependymomas in this age group since it not only identifies independently patients at risk of relapse, but also those young children who will not survive five years from diagnosis regardless of current treatment strategies (section 6.3.4.2.4, Table 6.20). In the older SIOP 1999 04 paediatric trial cohort, strong PRUNE expression was associated with a reduced overall patient survival on univariate analysis, reaching a trend towards significance on multivariate analysis. Patients with ependymomas demonstrating strong PRUNE expression also had a reduced event-free survival compared to the remaining

cohort, although this was not statistically significant (section 6.3.4.2.4, Table 6.21). While the influence on survival of standardised cranial radiotherapy must be considered in this age group, it could be argued that expanding the cohort size may prove valuable in ascertaining an independent prognostic role for PRUNE in older children with intracranial ependymoma.

Tenascin-C (TNC) is an extracellular matrix glycoprotein, thought to play a role in normal neurodevelopment by contributing to neural stem cell niche formation through the regulation of progenitor cell self-renewal and differentiation (Garcion, Halilagic et al. 2004). While TNC expression in normal adult brain tissue is relatively low (Puget, Grill et al. 2009; Sivasankaran, Degen et al. 2009), overexpression has been reported in certain malignant brain tumours including high grade gliomas and ependymomas, where it has been associated with poor prognosis using either adult or mixed age cohorts (Herold-Mende, Mueller et al. 2002; Korshunov, Golanov et al. 2002; Sivasankaran, Degen et al. 2009).

In this paediatric clinical trial cohort analysis, increased TNC protein expression was more prevalent in patients aged three years or younger (section 6.3.4.4.1). This supports the results of an integrated aCGH and gene expression analysis of 24 intracranial ependymomas which reported upregulation of the encoding *TNC* gene and copy number gain of chromosome 9qter in infant ependymoma (Modena, Lualdi et al. 2006). The present analysis also correlated higher TNC expression with posterior fossa ependymomas, irrespective of patient age. This replicated the finding of TNC overexpression in infratentorial ependymomas from a comparative immunohistochemical analysis of 66 primary paediatric intracranial ependymomas divided according to CNS location (Andreiuolo, Puget et al. 2010). Moreover, analysis of the SIOP 1999 04 cohort in this work found increased TNC expression in cases of incomplete tumour resection, suggesting a role for TNC in tumour invasiveness and hence recurrence. Indeed, strong expression of TNC, like PRUNE, has been identified at the invasive front of several tumour types (Orend and Chiquet-Ehrismann 2006) where it is potentially mediated by Notch signalling (Sivasankaran, Degen et al. 2009), while higher TNC expression has been reported in recurrent childhood intracranial ependymoma when compared to primary tumours (Puget, Grill et al. 2009).

As a prognostic marker, increased TNC expression was associated with a worse overall patient survival on univariate analysis of the UK CCLG 1992 04 cohort, while correlation with a reduced event-free survival reached a trend toward significance (section 6.3.4.4.3). This result gives some support to a retrospective immunohistochemical study of 88 ependymomas from children and adults that identified TNC staining as a marker of reduced event-free survival on multivariate analysis (Korshunov, Golanov et al. 2002). However, while warranting further consideration, TNC expression was not an independent correlate of adverse event-free or overall survival in the 1992 04 analysis.

Unlike the UK CCLG 1992 04 study, no association with higher TNC expression and either relapse or reduced survival was identified in the older SIOP 1999 04 cohort (section 6.3.4.4.3). As with 1q25 copy number gain, potential explanations for this discrepancy include the smaller size of the 1999 04 cohort, different ependymoma biology and tissue microenvironments between tumours from young and old children, and the contrasting therapeutic strategies used for each trial. Alternatively, it has been postulated that either a range of specific TNC receptors or various TNC isoforms created by alternative splicing may be responsible for inconsistent and contradictory effects of TNC on the migratory and invasive properties of cells (Deryugina and Bourdon 1996; Giese, Loo et al. 1996; Kiernan, Gotz et al. 1996; Phillips, Krushel et al. 1998; Treasurywala and Berens 1998; Herold-Mende, Mueller et al. 2002). However, an *in vitro* analysis of glioma cells counteracts this suggestion, reporting the promotion of cell proliferation and migration by all endogenous TNC isoforms (Herold-Mende, Mueller et al. 2002).

This prospective analysis has identified three biological markers of adverse prognosis in paediatric ependymoma. The reproducibility of these findings, particularly those from the UK CCLG 1992 04 analysis, is currently being assessed on an independent clinical trial TMA cohort of ependymomas from young children, treated with an alternative post-surgical chemotherapy-based regimen from SFOP (Société Française d'Oncologie Pédiatrique) (Grill, Le Deley et al. 2001). This is discussed further in the final chapter. The remaining biological prognostic markers examined across the two clinical trial TMA cohorts included Nucleolin, Ki-67 and NAV1 expression.

Nucleolin is a major eukaryotic nucleolar phosphoprotein thought to be implicated in a vast range of cellular processes including ribosomal component formation, maturation and transport between cytoplasm and nucleolus (reviewed by (Tuteja and Tuteja 1998)). Reports of Nucleolin as a proliferation marker in tumour cells (Derenzini, Sirri et al. 1995) and as a nuclear chaperone for hTERT (Khurts, Masutomi et al. 2004; Ridley, Rahman et al. 2008) have suggested a potential role in oncogenesis. Indeed, a previous retrospective IHC analysis of 80 paediatric intracranial ependymomas undertaken by the CBTRC found that low Nucleolin expression was an independent marker of improved event-free survival (Ridley, Rahman et al. 2008). The present study, in part, assessed the reproducibility of this preceding work, incorporating and re-evaluating 27 intracranial ependymomas analysed by Ridley et al. However, despite high Nucleolin expression being associated with an anaplastic histology in the UK CCLG 1992 04 cohort, no association between expression and patient outcome was established in either the 1992 04 or SIOP 1999 04 trial groups (section 6.3.4.5.3).

Several explanations may account for such disparate findings. In the study by Ridley et al., the dilution of the monoclonal Nucleolin antibody used (ab13541; Abcam, UK) was 1:2000 (Ridley, Rahman et al. 2008). However, to eradicate subtle, weakly staining cores in this analysis and thus define tumour samples with a low Nucleolin expression labelling index more confidently, the same antibody was used at a dilution of 1:400. Despite this higher concentration, optimisation remained readily achievable with tonsil control tissue (Figure 6.24A and B). It could be argued that increasing the antibody concentration in this manner potentially decreased the numbers of low Nucleolin expression cases available for the analysis, thereby reducing the prospect of ascertaining a significant beneficial result for this subgroup. Nevertheless, the definitive tumours with low expression that were identified actually conferred a survival disadvantage on their respective patients (section 6.3.4.5.3), suggesting that any impact of the altered antibody concentration on findings obtained was negligible.

By contrast, the influence of standardised adjuvant therapy on subsequent survival data, irrespective of the putative marker assessed, should be taken into consideration as this differed significantly between the two studies, potentially contributing to the different outcome findings for Nucleolin expression. The work of Ridley et al. was retrospective, assessing ependymomas from children of any age up to 16 years who had either

undergone surgery in isolation or in combination with a range of chemotherapeutic agents and/or radiotherapy. Indeed, while trying to account for some of this therapeutic diversity using multivariate analysis, low Nucleolin expression was only identified as an independent marker of improved event-free, not overall survival (Ridley, Rahman et al. 2008). In contrast, the current study was prospective with paediatric ependymoma patients treated uniformly within the confines of the two age-defined clinical trials. This allowed clarity in assessing the survival impact of a selected prognostic marker for a particular paediatric age group, without the bias and interference introduced by dysregulated multi-modal treatments as found in the retrospective study.

Tumour tissue heterogeneity also provides a more feasible rationale for the conflicting results of the two studies. This is highlighted by case 9204 – 68P (Figure 6.24C – E), an ependymoma originally classified as demonstrating low Nucleolin expression from triplicate core analysis by the Ridley study (Figure 6.24C) (Ridley, Rahman et al. 2008). Using the Nucleolin antibody at a dilution of 1:400 on these tumour tissue cores did not substantially increase the nuclear staining pattern (Figure 6.24D), in keeping with the previous suggestion that expression results were not influenced significantly by increasing the antibody concentration. However, novel tissue cores from a different region of this tumour were also examined in the current prospective analysis, demonstrating very high Nucleolin expression with the 1:400 antibody dilution (Figure 6.24E). This suggests that cellular heterogeneity between different ependymoma tissue cores, or individual sections from the same core, could significantly alter the expression results for any given marker assessed by IHC and produce contradictory findings such as those discussed. While the current standardised neuropathological identification of representative tumour regions for TMA coring attempts to minimise such difficulties, it is not foolproof and reinforces the importance of obtaining sizeable tumour tissue sections for analysis and reproducing potentially significant IHC findings on independent ependymoma cohorts.

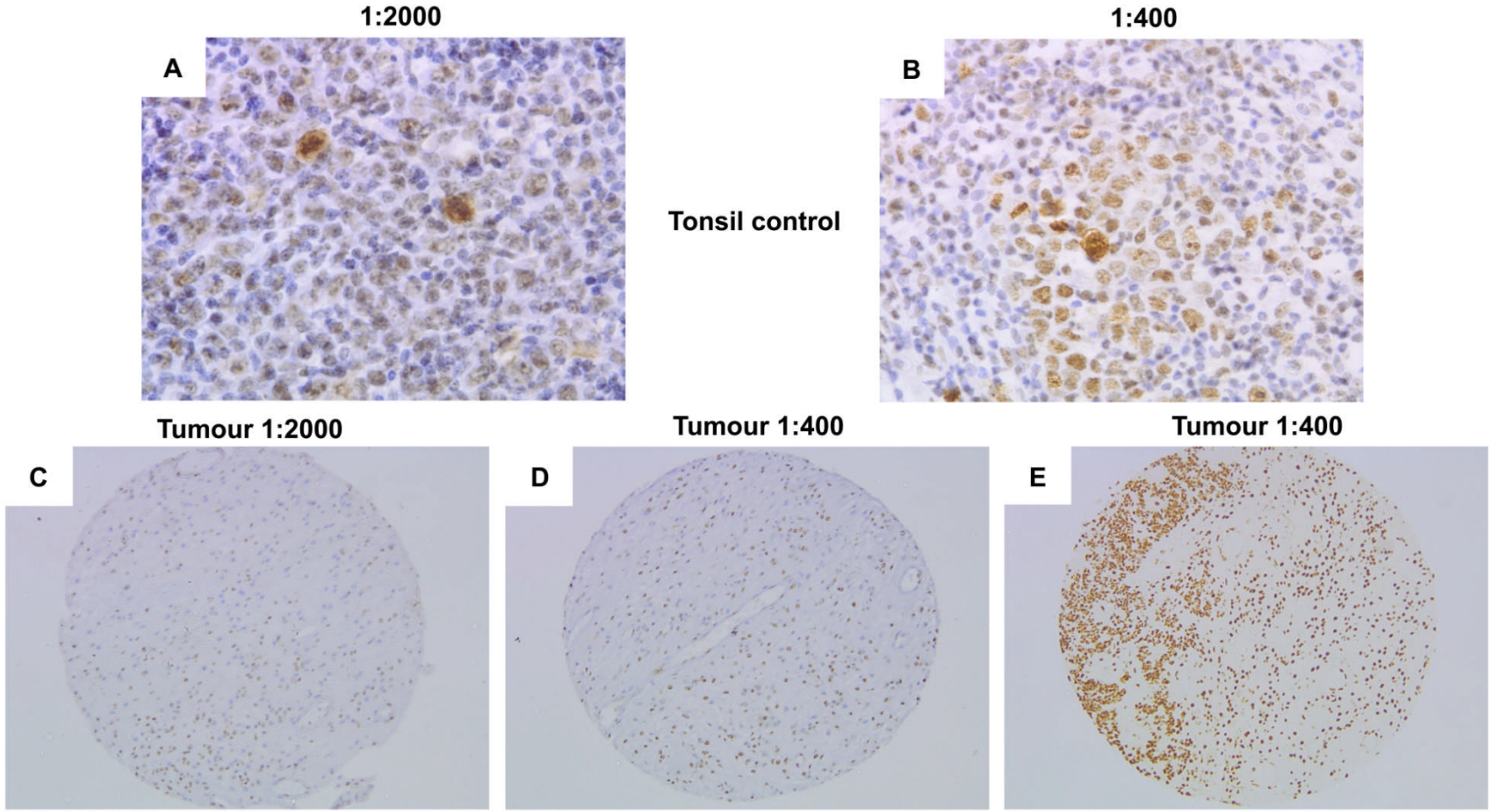

Figure 6.24: Nucleolin immunohistochemistry performed using the ab13541 monoclonal antibody (Abcam, UK) at 1:2000 and 1:400 dilutions. (A and B): Positive tonsil control tissue at x40 objective. Note the slight, but not substantial increase in nuclear staining at the higher concentration. (C): Example of an ependymoma that was initially analysed for Nucleolin expression as part of a retrospective cohort analysed by Ridley et al. (Ridley, Rahman et al. 2008), using the Nucleolin antibody at a 1:2000 dilution. (D): This ependymoma was then incorporated into the current prospective clinical trial study (sample 9204 – 68P), using the Nucleolin antibody at a 1:400 dilution. As can be seen, increasing the Nucleolin antibody concentration from 1:2000 to 1:400 only refines the nuclear staining of the tumour core. (E): A different core to that shown in picture D cut from the same tumour, 9204 – 68P (Nucleolin antibody 1:400), highlighting the potential influence of tumour heterogeneity on Nucleolin IHC results. In figures C – E only one core of a triplicate is shown as this was representative. Figures C – E taken at x10 objective.

Irrespective of potential explanations to account for the contrasting results of the two studies, this analysis rejects the hypothesis that low Nucleolin expression is a beneficial prognostic marker for children with intracranial ependymomas. Indeed, the numerous cellular housekeeping functions attributed to Nucleolin make it difficult to ascertain its precise role and importance in paediatric ependymoma (Tuteja and Tuteja 1998). This does not, however, exclude a prognostic role for hTERT expression in childhood ependymoma, because telomerase activation is present in most malignant cells (Shay and Bacchetti 1997), whilst telomere maintenance is a valuable prognostic marker in other CNS tumours (Hakin-Smith, Jellinek et al. 2003; Didiano, Shalaby et al. 2004). Nevertheless, the accurate detection of hTERT at an immunohistochemical level remains a technical dilemma.

Ki-67 is a nuclear protein, expression of which is present in proliferating cells and absent in non-dividing cells (Gerdes, Lemke et al. 1984). This study found increased Ki-67 expression was associated with ependymomas from infant patients (section 6.3.4.6.1), reflecting the increased proliferative activity which naturally occurs in the immature, developing central nervous system (Scotting, Walker et al. 2005). Within the younger UK CCLG 1992 04 patient cohort, a higher Ki-67 labelling index was also associated with tumour recurrence, corroborating results from the retrospective IHC study performed by Ridley et al. (Ridley, Rahman et al. 2008) and suggesting proliferation in ependymoma increases with disease progression. Moreover Ki-67 expression was shown to correlate with tumour grade in the young, supporting the view from other reports that increased proliferation appears a feature of histological anaplasia (Rushing, Brown et al. 1998; Suzuki, Oka et al. 2001; Suri, Tatke et al. 2004; Ridley, Rahman et al. 2008). Associations with ependymoma grade and recurrence were not evident in the analysis of the older SIOP 1999 04 patient cohort, again suggesting biological disparity exists between ependymomas from the two age categories.

High Ki-67 expression was not established as an adverse prognostic marker in either clinical trial age group of the current analysis (section 6.3.4.6.3). While this finding supported the results of the large study by Ridley et al. (Ridley, Rahman et al. 2008), it contradicted the findings of two other sizeable paediatric ependymoma analyses identifying increased Ki-67 expression as a putative outcome marker (Bennetto, Foreman et al. 1998; Gilbertson, Bentley et al. 2002). However, the labelling index

threshold used to define prognostic groups in these latter two studies was 25 %. This was significantly higher than those used in both the present analysis and the work of Ridley et al. (<1 %, 2 – 4 % and 5 %) and provides a potential explanation for the contrasting results. Nevertheless, no primary tumour in either clinical trial cohort of this study exceeded a Ki-67 labelling index of 25 %, rendering the higher threshold inappropriate. Moreover, the relatively lower range of Ki-67 expression results obtained were comparable with several reports of ependymomas from children or mixed age cohorts (Prayson 1999; Figarella-Branger, Civatte et al. 2000; Verstegen, Leenstra et al. 2002; Ridley, Rahman et al. 2008). Confidence in the current results is also strengthened by the aforementioned prospective nature of this study, removing the potential biases introduced by therapeutic diversity and allowing a more accurate assessment of the survival impact of Ki-67 expression compared to all preceding retrospective analyses on paediatric ependymomas.

The present analysis also failed to accredit strong NAV1 expression as a poor prognostic marker in paediatric ependymoma (section 6.3.4.3.4). The NAV1 protein has been implicated in the promotion of neurite outgrowth from mammalian cells (van Haren, Draegestein et al. 2009), while the encoding *NAV1* gene has homology to an axonal guidance gene in the roundworm (Maes, Barcelo et al. 2002). Results from the preceding 500K SNP array analysis had identified gain of *NAV1* to be a frequent imbalance in intracranial recurrent ependymomas and an indicator of adverse event-free patient survival on multivariate analysis of the primary tumour cohort (Chapter 4, section 4.3.2).

However, no significant correlation was established between gene copy number alteration and protein expression for 28 ependymomas analysed on both SNP array and IHC platforms (section 6.3.4.3.2), indicating that NAV1 protein expression appears independent of genomic alteration and is not copy number driven. Whilst no significant association of NAV1 expression with patient survival was ascertained from the IHC analysis, the relationship between strong expression and adverse EFS in the older SIOP 1999 04 cohort reached a trend toward significance, again suggesting that expanding the size of this age defined group for assessment of NAV1 overexpression could be considered.

6.5 Summary

Identifying biological correlates of outcome in paediatric ependymoma should enable a more precise understanding and prediction of tumour behaviour and subsequent clinical sequelae which, in turn, could lead to modifications in the current therapeutic strategies. In summary, this analysis was the first to examine a panel of six putative biological prognostic markers in paediatric ependymoma from patients treated according to two age-dependent clinical trials (UK CCLG 1992 04 and SIOP 1999 04). The markers analysed were PRUNE, NAV1, TNC, Nucleolin and Ki-67 expression, together with gain of chromosome 1q25 as detected by FISH.

Within the younger UK CCLG 1992 04 cohort, copy number gain of chromosome 1q25 and PRUNE overexpression were independently associated with an increased risk of disease progression, while strong PRUNE expression was also an independent marker of worse overall survival. In addition, increased expression of Tenascin-C correlated with a reduced overall survival on univariate analysis. These are the first biological markers to be identified prospectively in a cohort of paediatric ependymoma patients treated uniformly within the confines of a clinical trial, where chemotherapy was the primary adjuvant treatment administered. To ensure validity, the reproducibility of these findings is now being evaluated on an independent, yet corresponding clinical trial cohort of paediatric ependymomas supplied by the French Society of Paediatric Oncology. This is discussed further in the final chapter.

Assessment of the UK CCLG 1992 04 cohort also revealed anaplastic ependymomas were characterised by strong Nucleolin expression and an increased Ki-67 labelling index. Moreover, high Ki-67 expression was a feature of recurrent disease. Increased Tenascin-C expression was found to be associated with posterior fossa tumours and patients aged under three years of age, the latter when results were compared with those from the SIOP 1999 04 cohort of older patients. Exclusive analysis of the 1999 04 group revealed increased Tenascin-C expression was again associated with posterior fossa ependymomas in addition to incomplete tumour resection at surgery.

The only identified biological correlate of outcome for the SIOP 1999 04 cohort was PRUNE overexpression, associated with adverse overall survival on univariate analysis. Expansion of the current SIOP 1999 04 TMA tumour collection is currently being investigated. If successful, this may help establish PRUNE as an independent prognostic marker of survival for ependymoma in this age group and identify a marker that discerns older children with an increased relapse risk which, at present, remains elusive.

Results from this study have identified an adverse prognostic role for PRUNE overexpression across both paediatric ependymoma trial cohorts. The functional assessment of PRUNE for a role in childhood ependymoma pathogenesis and consideration of its feasibility as a therapeutic target in this tumour group are discussed in the final chapter.

CHAPTER 7

FINAL DISCUSSION

7.1 Overview and conclusions

The management of paediatric ependymoma remains a distinct challenge, on account of several reasons. The variable surgical accessibility of tumours from particular CNS locations is one such factor, especially as ependymomas have a predilection for invading through the foramen of Luschka to involve the lower cranial nerves or indeed the brainstem. Other compounding issues include the European avoidance of craniospinal irradiation in patients below three years of age due to neurotoxicity concerns, the limited delivery of chemotherapy to the CNS from the innate blood-brain barrier and the subjectivity of histological classification systems which influence current treatment protocols. This is underpinned by an inadequate understanding of the biology of these heterogeneous tumours in children and the subsequent influence this has on the therapeutic modalities in use. As a result, patient survival rates for ependymoma are poor in relation to other childhood malignancies, while a substantial proportion of survivors experience significant co-morbidity.

It is thereby hoped that an improved knowledge of ependymoma biology will encourage novel prognostic stratifications that improve patient outcome whilst minimising toxicity and facilitate the development of targeted therapy to supplement or replace the generic approaches presently adopted. This approach has proved fruitful in medulloblastoma (Pomeroy, Tamayo et al. 2002; Gajjar, Hernan et al. 2004; Ellison, Onilude et al. 2005; Thompson, Fuller et al. 2006; Kool, Koster et al. 2008) and haematological malignancies such as paediatric ALL (Chessels, Swansbury et al. 1997; Pui, Sandlund et al. 2004) and CML (Roy, Guilhot et al. 2006) such that these conditions could serve as potential templates for structuring future paediatric ependymoma management.

This study has initially demonstrated the range and nature of genomic and epigenetic aberrations in paediatric ependymoma at varying degrees of resolution, using Affymetrix® 500K SNP array and Illumina® GoldenGate® Cancer Panel I methylation array platforms on individual tumour cohorts which had undergone central histopathological confirmation. Selected genomic aberrations were validated by either FISH or qPCR. While collective assessment revealed the most common anomalies, specific aberrations were characteristic of certain ependymoma subgroups, particularly

those relating to tumour location, patient age, disease recurrence and patient prognosis, each of which will be discussed in turn. Indeed, genomic gain involving regions of chromosome 1q was a feature of tumour relapse and correlated with an unfavourable patient outcome (Chapters 3 and 4). This adverse prognostic association was upheld in the subsequent part of this work, where chromosome 1q25 gain and strong PRUNE protein expression, together with increased Tenascin-C expression, were identified as adverse prognostic markers from a prospective analysis of paediatric ependymomas where patients had been treated according to age-dependent clinical trials (Chapter 6). By combining copy number and LOH data, this work also identified acquired uniparental disomy as a plausible, albeit infrequent mechanism in ependymoma pathogenesis affecting approximately five percent of cases (Chapter 4).

The array studies revealed distinct global biological profiles and candidate signature genes for paediatric ependymomas differentially located within the central nervous system. Spinal tumours were characterised by numerous arm or whole chromosome genomic anomalies (Chapter 3, section 3.3.4) and a unique methylation profile, clearly distinguishable from intracranial ependymomas (Chapter 3, section 3.3.7). Posterior fossa tumours were associated with chromosome 1q gain whereas supratentorial tumours demonstrated few broad genomic imbalances (Chapter 3, section 3.3.4). Moreover, epigenetic differences were evident between and within ependymomas from these intracranial locations (Chapter 3, section 3.3.7), supporting gene expression array evidence that further biological subgroups of ependymoma exist (Taylor, Poppleton et al. 2005; Johnson, Wright et al. 2010) and suggesting that the various expression profiles identified may in part be a manifestation of dysregulated gene methylation mechanisms.

At higher genomic resolution (Chapter 4, section 4.3), spinal ependymomas were associated with hypomethylation of the putative oncogene *EYA4* (6q23), while intracranial tumours frequently demonstrated gain and amplification of pathogenic candidate genes such as *NSL1* (1q32.3) and *DNAJC25* (9q31.3), together with deletion or hypermethylation of proposed tumour suppressor genes including *FILIP1* (6q14.1), *FRK/RAK* (6q22.1) and *RASSF1A* (3p21.3). As stated above, posterior fossa tumours within the intracranial compartment were associated with genomic gain of numerous genes within chromosome 1q loci, such as *CHI3L1* (1q32.1). Indeed, dysregulation

observed in other chitinase genes (*CHIT1* gain and *CHI3L2* hypomethylation) suggest involvement of this family in infratentorial paediatric ependymoma pathogenesis. Posterior fossa tumours were also characterised by the hypomethylation of putative signature genes outwith chromosome 1q, including *PPARG* (3p25), *SPDEF* (6p21.3) and *BCR* (22q11.23), whereas supratentorial tumours demonstrated relative hypomethylation of genes such as *WNT10B* (12q13) and *HCK* (20q11).

The location-specific findings of the methylation array data analysis, performed within the time constraints of this project, have subsequently been developed to show that spinal and supratentorial ependymomas display a higher proportion of hypermethylated genes than infratentorial tumours (Rogers, Kilday et al. 2011, paper submitted), akin to the 'CpG island methylator phenotype' observed in colon carcinoma (Toyota, Ahuja et al. 1999; Weisenberger, Siegmund et al. 2006) and glioma (Noushmehr, Weisenberger et al. 2010; Laffaire, Everhard et al. 2011). By integrating the results with ependymoma mRNA expression array data originally published by Johnson et al. (Johnson, Wright et al. 2010), this hypermethylation profile was associated with the upregulation of genes implicated in DNA methylation (*DNMT1*, *DNMT3A* and *DNMT3B*), implying an underlying mechanism. Further integrative analysis identified methylation induced alteration in the expression of genes associated with the regulation of apoptosis and cell growth, such as *PPARG* and members of *JNK* pathway, together with genes of the immune system. The methylation array findings for selected candidate genes were validated by bisulphite sequencing (Rodgers, Kilday et al. 2011, paper submitted).

Evidence suggests that such disparity between histologically similar ependymomas from different CNS locations may be the result of malignant transformation in regionally defined neural progenitor cells of the developing CNS, thought most likely to be radial glia, which is potentially initiated by an inter-relationship between specific tissue microenvironments and dysregulated cellular mechanisms (Taylor, Poppleton et al. 2005; Johnson, Wright et al. 2010). Such regionally discrete progenitor cell populations have also been identified in medulloblastoma subgroups (Gibson, Tong et al. 2010). It remains unclear what proportion of the biological aberrations observed in ependymomas from different CNS sites are tumour specific and not merely a reflection of the normal underlying cell of origin before tumourigenic conversion. Comparing the

gene expression profiles of location-matched human radial glia and ependymomas may help elucidate this.

Data presented from the array work also confirmed biological disparity between paediatric ependymomas from different patient age groups. In the ependymoma array cohorts and clinical trial groups, posterior fossa tumours were associated with younger patients, while spinal tumours occurred in older children. Irrespective of tumour location, the number and size of identified genomic aberrations increased with age such that intracranial ependymomas with a 'balanced' genomic profile were associated with children under three years of age, while numerous and broad imbalances were evident in ependymomas from older children (Chapter 3, section 3.3.4). This latter finding could not be attributed solely to the presence of spinal ependymomas in the older cohort, since almost half of the tumours demonstrating multiple, large anomalies were located in the posterior fossa (Chapter 3, Figure 3.7B). Indeed, when comparing posterior fossa tumours exclusively, thereby removing any bias introduced by tumour location, focal genomic aberrations remained evident between age categories, such as the confirmed gain of *TXN* (9q31.3) in children aged above three years (Chapter 4, section 4.3.1.10).

Explanations for the biological disparity between ependymomas from patients of different ages remain unclear. Work by Johnson et al., correlating the transcriptomic profile of human ependymomas to those of location-matched radial glia and adult neural stem cells in mice, has suggested that the diversity may be accounted for developmentally distinct cells of origin undergoing malignant transformation (Johnson, Wright et al. 2010). Indeed, spinal ependymomas, which are more prevalent in older children and adults, had an expression profile which corresponded better with the murine adult neural stem cells than embryonic radial glia while, in contrast, the transcriptome of paediatric supratentorial tumours more closely resembled that of the foetal radial glia (Johnson, Wright et al. 2010). Nevertheless, the study failed to establish such developmentally defined putative cells of origin for posterior fossa ependymomas.

The nature of the biological insult initiating tumourigenesis must also be considered. The observation of ependymomas with a 'balanced' genome in very young children suggests that these tumours may result from mutagenic 'hits', such as point mutations,

which are sufficient to limit the number of subsequent aberrations required for tumour formation or possess a tumourigenic effect that is restricted to a developmentally defined stage, cell type or environment. Recent murine work analysing medulloblastoma supports this by demonstrating that oncogenic *PTCH* mutation in multipotent neural stem cells, when compared to more mature progenitors, can result in rapid expansion of the stem cell pool and a consequent earlier onet of tumour formation (Yang, Ellis et al. 2008). An alternative possibility is that epigenetic dysregulation may alter normal cell differentiation and self-renewal programmes without genetic disruption in 'balanced' tumours. Whilst, in general, the work presented suggests that a methylation induced mechanism may not be responsible in ependymoma, the relatively low resolution and design of the GoldenGate® array for pre-selected genes cannot exclude it nor preclude alternative epigenetic processes such as histone deacetylation. The use of novel high density epigenetic arrays, together with expression array profiling and high throughput sequencing on larger cohorts of such 'balanced' ependymomas from particularly young children may help to explore these hypotheses further.

If, as discussed, regionally, developmentally or genetically restricted neural progenitor cells are purported to undergo malignant transformation into the ependymoma-initiating cells of varying tumour subgroups (Taylor, Poppleton et al. 2005; Johnson, Wright et al. 2010) and evidence suggests that only a minority of these cells are required for tumourigenesis (Singh, Hawkins et al. 2004; Taylor, Poppleton et al. 2005), a hypothesis for ependymoma recurrence is conceivable. Since standard therapeutic approaches are generally expected to remove tumour bulk, they could potentially fail to eradicate these small ependymoma-initiating cell populations which may have inherent resistance to conventional treatments or acquire resistance through mutation. If correct, future curative strategies for paediatric ependymoma would have to incorporate novel agents which target such dysregulation once identified.

Further to the independent analyses of intracranial primary and recurrent ependymomas (Chapters 3 and 4), the comparative SNP array assessment of patient-matched primary and relapsed tumour sets enabled regions of genomic imbalance to be detected which were either sustained into recurrence or present only at relapse, thereby incorporating candidate genes potentially implicated in tumour maintenance or disease progression (Chapter 5, section 5.3). Gain of chromosome 1q was a feature of relapsed intracranial

tumours. Indeed, focal regions of gain across this chromosome arm were among the most common maintained and acquired genomic alterations identified across the tumour sets, encompassing genes such as *CENPF* (1q41), *CDC42BPA* (1q42.13), *HAX1*(1q21.3) and *CNTN2/TAX1* (1q32.1). The comparative analysis of the tumour sets also revealed other loci demonstrating maintained or acquired imbalances, harbouring established tumour suppressor genes (*CDKN2A* (9p21.3), *PTEN* (10q23.2)), candidates identified from other analyses of ependymoma (*COL27A1* (9q32), *DNM1* (9q34.11), *TIMP3* (22q12.3)) and novel genes including *PAXIP1/PTIP* (7q36.2) and *KCNK9* (8q24.3). In addition, enrichment at relapse for imbalanced regions encompassing genes implicated in neurite growth, guidance and cell migration was observed.

Although awaiting validation, two of the gained candidate genes on chromosome 1q, *CENPF* and *CNTN2*/*TAX1*, have also been identified from gene expression work as upregulated signature genes of childhood ependymoma recurrence (Peyre, Commo et al. 2010). Indeed, *in vitro* work has suggested *CNTN2*/*TAX1* overexpression may contribute to the invasiveness of high grade gliomas by promoting tumour cell migration (Rickman, Tyagi et al. 2001), while increased expression of the kinetochore associated *CENPF* has been associated with glioma progression, potentially reflecting the increased proliferation often observed in tumour recurrence (van den Boom, Wolter et al. 2003). The functional assessment of these candidates for a role in paediatric ependymoma recurrence is thereby warranted, using techniques discussed later in this chapter.

As alluded to above, the time taken to extract and process the DNA samples for both array platforms, accrue accompanying clinical information and manually interpret the vast amounts of data generated on evolving computer software programmes meant that decisions had to be taken regarding which candidate genes and regions were appropriate to validate within the inherent time constraints of the project. This was compounded by sample depletion as discussed in Chapter 4, section 4.4.3. Consequently, validation of imbalanced loci other than those detailed in preceding chapters, such as those identified from analysing the primary and relapsed ependymoma sets, were deferred but will be performed in the future (section 7.2). Despite the protracted nature of the genomic analysis, consideration should be given to expanding the SNP array cohort. Whilst processing more samples would allow a more representative analysis of certain

ependymoma subgroups, continuing the method of analysing patient-matched tumour and constitutional DNA would enable the high resolution assessment of genomic imbalance on the X chromosome across a substantial number of tumours; an analysis not undertaken as yet due to the minority of unpaired cases used for this study.

During the latter stages of this work, alternative statistical means of interpreting the data generated from both types of array became available. However, it was not feasible to assess or incorporate them within the time limits of the study. GISTIC (Genomic Identification of Significant Targets In Cancer) is a computational tool that uses signal intensity ratios from the SNP array to identify genomic regions that are imbalanced more often than would be statistically expected by chance, according to the magnitude of such imbalances (Beroukhim, Getz et al. 2007). The method is weighted in favour of detecting high magnitude aberrations, such as amplifications or homozygous deletions, which potentially drive tumour pathogenesis. A correction module can also be integrated into GISTIC to account for potential 'batch effect' bias introduced by processing groups of arrays at different times or venues, a feature unavailable to other analytical methods. A comparison of results produced by GISTIC and GTYPE/CNAG for the SNP array ependymoma cohort of this study would therefore be a project worthy of consideration. Similarly, the M-value has been proposed as another means of calculating the methylation status of individual CpG sites on Illumina® epigenetic arrays (Du, Zhang et al. 2010). Unlike the Beta-value, the M-value is the $\log_2$ ratio of signal intensities between methylated and unmethylated probes for a particular site and has been reported to improve statistical validity when performing differential methylation analysis between groups (Du, Zhang et al. 2010). Nevertheless, by using the Beta-value derived methylation levels from the array analysis presented in this thesis, subsequent work has established an association between an ependymoma hypermethylation profile and the increased expression of genes responsible for DNA methylation, whilst demonstrating an inverse correlation between the methylation and expression profiles of several selected genes (Rodgers, Kilday et al. 2011, paper submitted). Moreover, the methylation status of various promoter CpG sites identified from this analysis have been successfully validated in a number of paediatric ependymomas (Rodgers, Kilday et al. 2011, paper submitted), all of which suggests that this scoring method reliably reflects underlying tumour biology.

The detrimental impact on patient survival associated with genomic gain involving chromosome 1q was a consistent feature of the entire work. Initially, the SNP array analysis correlated whole arm gain with poor overall survival, reaffirming findings from conventional CGH analysis of paediatric ependymomas (Dyer, Prebble et al. 2002) (Chapter 3, section 3.3.4). Utilising the capacity of the SNP array to examine the ependymoma genome at a higher resolution, this study further identified gain of *NAV1* (1q32.1) across the primary cohort as a marker of shortened event-free survival, while gain of a focal region on 1q21.3, encompassing *PRUNE*, *BNIPL*, *CDC42SE1* and *AF1q*, was associated with an increased risk of mortality on multivariate analysis, together with a reduced event-free survival on univariate analysis (Chapter 4, section 4.3.2). The genomic gain of *PRUNE* correlated with an increased encoded protein expression, as assessed by immunohistochemistry. (Chapter 6, section 6.3.4.2.2).

This association with unfavourable patient outcome continued into the prospective clinical trial TMA cohort analysis, particularly for children aged below three years (Chapter 6, sections 6.3.3 – 6.3.5). Indeed, within the younger UK CCLG 1992 04 cohort, copy number gain of chromosome 1q25 and PRUNE overexpression were independently associated with an increased risk of disease progression, while strong PRUNE expression was also an independent marker of worse overall survival. In addition, increased Tenascin-C expression correlated with a reduced overall survival on univariate analysis. For older children in the SIOP 1999 04 cohort, strong PRUNE expression in ependymomas was again identified as an adverse prognostic marker, correlating with increased mortality on univariate assessment.

As approximately 20 to 45 % of young children with intracranial ependymomas remain free of disease with the UK CCLG 1992 04 chemotherapeutic regime (Grundy, Wilne et al. 2007), 1q25 gain, PRUNE and Tenascin-C expression may help delineate such patients that can be cured with chemotherapy from those who cannot. Infants with tumours demonstrating adverse biological parameters may consequently receive alternative adjuvant intervention up-front, such as new agent therapy. However, before this could be implemented, independent verification of the findings from this work is required. Accordingly, this is now being performed on a clinical trial TMA cohort of ependymomas taken from younger children who have been treated with a corresponding, post-operative chemotherapy-driven protocol formulated by SFOP

(Société Française d'Oncologie Pédiatrique**)** (Grill, Le Deley et al. 2001) (section 7.2). It is hoped that any marker established from this subsequent analysis would be incorporated into future European clinical trial design to enable prognosis-based therapeutic stratification for young children diagnosed with intracranial ependymoma.

A similar classification system for older paediatric patients could aim to assess the benefit of treating ependymomas demonstrating unfavourable biology with post-surgical adjuvant chemotherapy in conjunction with radiotherapy, as opposed to the current SIOP 1999 04 standard practice of post-operative cranial irradiation alone. This work has suggested that strong PRUNE expression may also be of use as an adverse outcome marker in this context, but requires confirmation in a larger cohort.

The distinct survival results for FISH detected 1q25 gain and PRUNE overexpression in both prospective trial cohorts have raised the possibility that biological aberrations within different regions of chromosome 1q may have varying impacts on patient survival and, particularly for older paediatric ependymoma patients, gain of 1q21.3 may represent a more appropriate prognostic marker, a hypothesis to be assessed in the future (section 7.2).

This study identified strong PRUNE protein expression as an adverse prognostic marker across the two paediatric ependymoma clinical trial cohorts and suggests that the encoding *PRUNE* gene may be a potential proto-oncogene. Whilst the precise role of PRUNE in paediatric ependymoma pathogenesis remains unclear, potential mechanisms of action have been proposed from other work (Figure 7.1A and B). Breast cancer *in vitro* analyses have discovered that PRUNE can induce neoplastic cell motility through interactions with the putative metastasis suppressor protein NM23-H1 (D'Angelo, Garzia et al. 2004; Garzia, D'Angelo et al. 2008) PRUNE has also been shown to bind to Gelsolin, a protein thought to regulate cellular adhesion and cytoskeleton remodelling, in a breast cancer cell model (Garzia, Roma et al. 2006). It has been hypothesised that this interaction may lead to invasive properties for neoplastic cells, although this has yet to be established (Garzia, Roma et al. 2006). Other work has shown PRUNE can bind to the serine-threonine kinase GSK-3β to promote cell migration through the modulation of focal adhesion disassembly (Kobayashi, Hino et al. 2006).

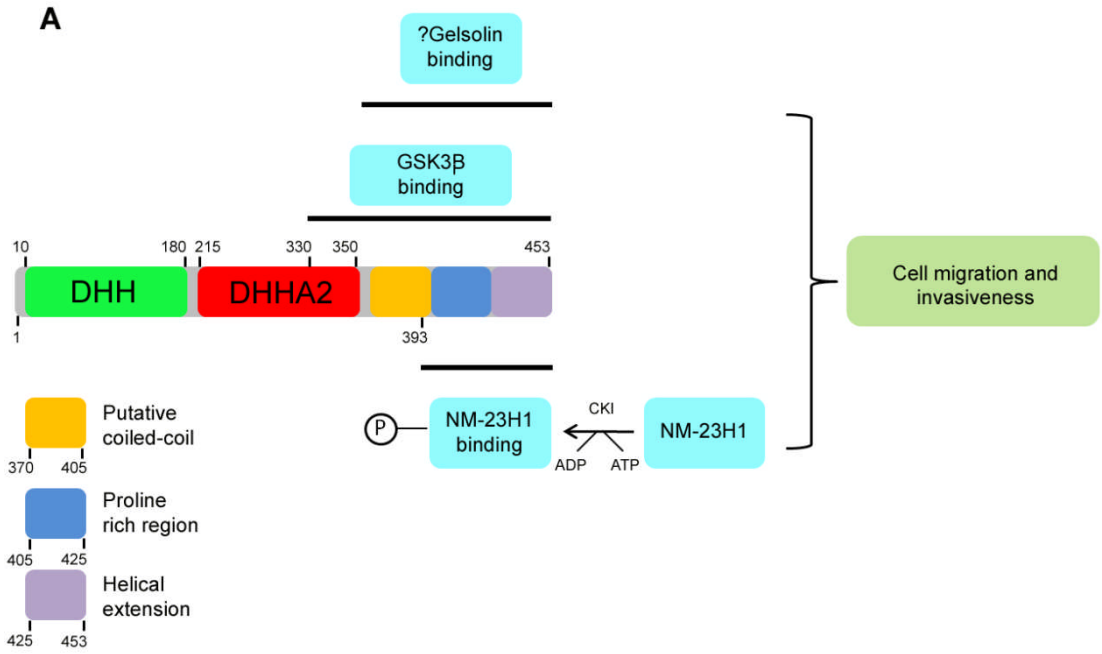

Figure 7.1A: The domains of the PRUNE protein and reported binding partners. PRUNE consists of DHH and DHHA2 domains, adjacent to a C-terminal CHD domain (cortexillin homology domain) with putative coiled-coil and proline rich regions. PRUNE binding to GSK3B and phosphorylated NM-23H1 *in vitro* has been shown to promote neoplastic cell migration (D'Angelo, Garzia et al. 2004; Kobayashi, Hino et al. 2006; Garzia, D'Angelo et al. 2008). PRUNE is also known to interact with Gelsolin, although the precise site is unknown. This interaction has been hypothesised to enhance the invasive properties of cancer cells (Garzia, Roma et al. 2006). Figure adapted from "Understanding h-PRUNE biology in the fight against cancer" (Marino and Zollo 2007).

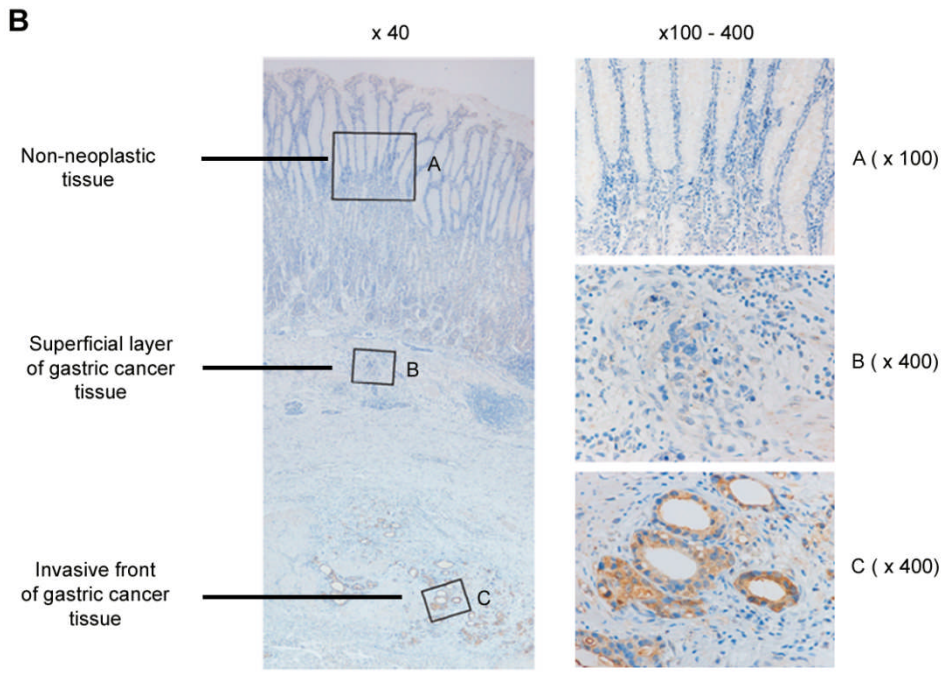

Figure 7.1B: PRUNE expression and tumour invasiveness. A role for PRUNE in tumour invasiveness has been supported by IHC analyses of oesophagus, pancreas, colon and gastric cancers (Kobayashi, Hino et al. 2006; Oue, Yoshida et al. 2007; Noguchi, Oue et al. 2009). In these studies, strong PRUNE expression was associated with depth of tumour invasion and was observed at the tumour invasive front (Region C), as can be seen in the gastric cancer case shown. Picture reproduced from "Increased expression of h-PRUNE is associated tumour progression and poor survival in gastric cancer" (Oue, Yoshida et al. 2007) with objectives as indicated.

PRUNE has also been implicated in stem cell biology, with overexpression reported to maintain the undifferentiated phenotypic properties of murine embryonic progenitor cells despite exposure to differentiating conditions (Pritsker, Ford et al. 2006). The impact of *PRUNE* overexpression on the proliferation, motility and invasiveness of ependymoma cells therefore warrants further consideration and plans to be addressed using in-house generated cell lines, as discussed in section 7.2.

Studies have begun to evaluate PRUNE as an amenable target for therapeutic inhibition, since PRUNE knockdown using RNA interference (RNAi) technology was shown to reduce cell motility in Hela and colorectal cancer cell lines (Kobayashi, Hino et al. 2006). Decreased tumour cell migration has been observed in a breast cancer cell line following the application of IC261, an inhibitor of the casein kinase I protein which promotes PRUNE binding to NM-23H1 (Garzia, Roma et al. 2006). The platelet anti-aggregant drug dipyridamole has also been reported to block the phosphodiesterase activity of PRUNE and inhibit the motility of breast cancer cells *in vitro* (D'Angelo, Garzia et al. 2004). However, this drug is known to impair the activity of other inherent phosphodiesterases, suggesting that dipyridamole analogues with specificity towards PRUNE would have to be developed before pre-clinical studies could be considered (Marino and Zollo 2007). Indeed, a fuller characterisation of the range of cellular activities of PRUNE is required before significant progress can be made in this field.

7.2 Future work and summary

Further validation of certain SNP array results, such as the maintained and acquired focal imbalances identified from the analysis of patient-matched primary and relapsed ependymoma sets, is required. Such aberrations will be verified by performing qPCR on selected cases, as before. A methylation profile analysis of ependymomas from these primary and recurrent sets will also be performed. The generation of gene expression array data for tumours analysed on the SNP and methylation arrays is also an imperative future project, since integrating datasets for each ependymoma would distinguish tumourigenic candidate genes demonstrating a genomic imbalance or epigenetic dysregulation that directly influences the subsequent expression profile. Such an amalgamative approach is now becoming feasible with the advent of analytical

computer software. Data accrued from these array analyses of ependymomas in children could also be supplemented by microRNA profiling and, when technology and funding allows, next generation sequencing of tumour samples.

Specific results arising from this work are also being investigated further. As indicated previously, the reproducibility of the prognostic findings for chromosome 1q25 gain, strong PRUNE expression and increased Tenascin-C expression are being assessed on a clinical trial TMA cohort of 153 ependymomas taken from children aged below five years who have been uniformly treated according to the BBSFOP protocol (Grill, Le Deley et al. 2001). This regime comprised seven cycles of post-surgical chemotherapy administered over one and a half years. Each cycle consisted of three courses of alternating agents (procarbazine and carboplatin, etoposide and cisplatin, vincristine and cyclophosphamide). Initial results from a trial cohort meta-analysis are encouraging, suggesting that 1q25 gain and increased Tenascin-C expression remain independent adverse prognostic markers, while work on PRUNE expression is ongoing.

This work has also suggested that, particularly for older paediatric ependymoma patients, increased copies of the chromosome 1q21.3 locus may prove a more refined adverse prognostic marker than 1q25 gain. This will be assessed on both the UK CCLG 1992 04 and SIOP 1999 04 trial TMA cohorts by FISH, using non-commercial probes derived from BAC clones. Moreover, the protein expression of genes other than *PRUNE*, encompassed within the focal region of 1q21.3 gain identified from the SNP array analysis (*BNIPL*, *CDC42SE1*, *AF1q*; Chapter 4, section 4.3.2), will also be assessed for prognostic significance across the trial cohorts using immunohistochemistry. This IHC analysis will incorporate three further putative prognostic markers proposed from recent studies of ependymomas in paediatric or mixed age cohorts; NEFL, EVI1 (increased expression) and P16/INK4A (loss of expression) (Andreiuolo, Puget et al. 2010; Korshunov, Witt et al. 2010; Koos, Bender et al. 2011).

Generating ependymoma cell lines for the functional assessment of prognostic targets or the development and testing of novel therapies has proved difficult, hampered by the limited capacity of dissociated ependymoma cells to survive and grow in culture. Indeed no commercially available ependymoma cell line exists. Nevertheless, the establishment

of two in-house permanent cell lines (nEPN1 and nEPN2), derived from a primary and recurrent paediatric intracranial ependymoma respectively, has recently been published (Hussein, Punjaruk et al. 2011). Each cell line was fully characterised by confirming a relationship to the tumour of origin, the retention of neural stem cell markers (CD133 and Sox-2) and tumourigenicity in murine xenografts (Hussein, Punjaruk et al. 2011). The functional consequences of overexpression or knockdown of selected genes on tumour cell properties, such as proliferation, migration, invasiveness and drug resistance, will be assessed on both of these cell lines using the techniques of transient transfection and RNAi. The first candidates to be assessed in this way will be *PRUNE*, *CENPF* and *CNTN2*. It is hoped that results from such *in vitro* functional work could elucidate the role of particular genes in ependymoma initiation and pathogenesis. Informative findings could then be extended to future *in vivo* analysis, evaluating the capacity of ependymoma cells demonstrating dysregulated expression of established candidate genes to initiate murine xenograft tumour formation on transplantation, thereby potentially identifying therapeutic targets.

In order to achieve significant advances in outcome for childhood ependymoma, a better appreciation of underlying tumour biology is required through high resolution molecular characterisation. This work has further contributed to the understanding that exists regarding the genomic and epigenetic heterogeneity of ependymomas in children, while suggesting areas for further research that may provide the initial basis for targeted therapy development. Moreover, using clinical trial tumour cohorts, it has defined novel biological prognostic markers for paediatric ependymoma. This represents a significant step towards establishing risk-based therapeutic stratifications to supplement or replace the classification systems that guide current treatment, in order to improve outcome and quality of life for children with this tumour.

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Appendix 1: Commonly used reagents

- DNA lysis buffer (500ml):
  50 mM Trizma® base (Sigma, UK) – pH 8.0
  100 mM di-sodium EDTA (BDH, UK) – pH 8.0
  100 mM sodium chloride solution (Fisher Scientific, UK)
  50 ml of 10 % SDS (w/v) (Sigma, UK)
  Final volume adjusted to 500 ml with distilled water

- 50 x TAE stock solution (1 L):
  2 M Trizma® base (Sigma, UK) – pH 8.0
  5 mM di-sodium EDTA (BDH, UK) – pH 8.0
  57.1 ml glacial acetic acid (Fisher Scientific, UK)
  Final volume adjusted to 1 L with distilled water
  1 x TAE buffer was made from 1 ml of the 50 x stock and 49 ml of distilled water

- Carnoy solution (40 ml):
  30 ml 100 % methanol (v/v) and 10 ml glacial acetic acid (Fisher Scientific, UK)

- Sodium citrate solution (5 L):
  50 mM citric acid monohydrate (Fisher Scientific, UK) in distilled water – pH 6.0

- 1 x PBS solution (500 ml):
  5 PBS tablets (Oxoid, UK) dissolved in 500 ml distilled water

- Pepsin solution (40 ml):
  320 mg pepsin (Dako, UK) dissolved in 40 ml 0.1 M hydrochloric acid (Fisher Scientific, UK)

- 20 x SSC (1 L):

  3 M sodium chloride (Fisher Scientific, UK) and 3.3 M sodium citric acid dehydrate (Fisher Scientific, UK) – pH 6.0

  Final volume adjusted to 1 L with distilled water

  2 x SSC made by diluting 10 ml of 20 x SSC in 90 ml distilled water

Appendix 2: 500K SNP analysis of 45 paediatric blood samples from patients contributing ependymomas to the SNP study.

Blood sample ID	Number of SNPs with diploid copy number	Percentage of total SNPs with diploid copy number
1P bl	489608	99.93
2P bl	489762	99.97
3P bl	489708	99.96
4P bl	489856	99.99
5P bl	489852	99.99
6P bl	489896	99.99
7P bl	489676	99.95
8P bl	489633	99.94
9P, R2 – 5 bl	489748	99.96
10P bl	489768	99.97
11P bl	489633	99.94
12P bl	489891	99.99
13P bl	489604	99.94
14P bl	483916	98.62
15P bl	489690	99.95
16P-R1 bl	489890	99.99
17P – R2 bl	427962	87.35
18P – R1 bl	489400	99.89
19R1 – R2 bl	402953	82.24
20P – R1 bl	489178	99.85
21P bl	489873	99.99
22P bl	489043	99.82
23P bl	489712	99.96
24P bl	489776	99.97
25P bl	489671	99.95
26P – R3 bl	489793	99.97
27P bl	489769	99.97
28P bl	489877	99.99
29P bl	489467	99.91
30P bl	489737	99.96
31P bl	489462	99.91
32P bl	498764	99.97
33R1 bl	489824	99.98
34P bl	489629	99.94
35P – R2 bl	406380	82.94
36P bl	489213	99.86
37P bl	489792	99.97
38P bl	488773	99.77
39P bl	479363	97.84
40P – R1 bl	489768	99.97
41P bl	489729	99.96
42P bl	489631	99.94
43R1 – 3 bl	489655	99.95
44P bl	489737	99.96
45P bl	489691	99.95

P = primary, R1 – R5 =1st – 5th recurrence, bl = blood. Samples highlighted in red represent samples where the percentage of total SNPs with a diploid copy number is below 99 %. Each blood sample was analysed in GTYPE using the batch analysis algorithm BRLMM against the 44 other blood samples in the cohort, followed by CNAG to generate copy numbers for each SNP probe as described in Chapter 2, sections 2.2.3 – 2.2.4.

Appendix 3: SNP call rates for 63 paediatric ependymoma analysed using Affymetrix® 500K SNP arrays.

ID	NspI 250K array (%)	StyI 250K array (%)	Average (%)
1P	97.39	94.73	96.06
2P	98.23	95.99	97.11
3P	98.01	96.05	97.03
4P	98.80	97.41	98.11
5P	87.76	95.18	91.47
6P	97.11	97.05	97.08
7P	91.63	93.05	92.34
8P	96.52	96.55	96.54
9P	96.09	94.43	95.26
9R2	95.40	94.95	95.18
9R3	99.2	97.94	98.57
9R4	98.3	96.81	97.56
9R5	97.01	97.47	97.24
10P	98.38	96.68	97.53
11P	98.67	94.33	96.5
12P	98.14	95.06	96.6
13P	99.16	95.77	97.47
14P	95.56	94.11	94.84
15P	97.99	94.12	96.06
16P	98.84	95.49	97.17
16R1	95.10	96.6	95.85
17P	96.91	94.95	95.93
17R1	98.3	97.07	97.69
17R2	98.29	96.58	97.44
18P	93.46	88.31	90.89
18R1	98.7	96.29	97.5
19R1	91.27	97.97	94.62
19R2	97.29	97.2	97.25
20P	98.53	92.52	95.53
20R1	98.99	96.81	97.9
21P	97.71	90.44	94.08
22P	98.78	97.68	98.23
23P	97.29	95.8	96.55
24P	96.77	95.07	95.92
25P	89.63	94.05	91.84
26P	93.03	87.27	90.15
26R1	95.38	92.85	94.12
26R2	98.09	95.32	96.71
26R3	97.75	94.9	96.33
27P	96.24	95.56	95.9
28P	95.3	96.86	96.08
29P	96.73	97.56	97.15
30P	97.77	97.13	97.45
31P	93.72	95.87	94.80
32P	96.74	96.27	96.51
33R1	95.72	95.81	95.77
34P	93.27	97.13	95.2
35P	95.42	93.09	94.23
35R1	96.61	95.66	96.14
35R2	90.07	88.18	89.13
36P	98.19	96.52	97.36
37P	96.8	96.23	96.52
38P	92.82	96.51	94.67
39P	94.17	90.44	92.31
40P	98.09	97.88	97.99

ID	NspI 250K array (%)	StyI 250K array (%)	Average (%)
40R1	96.92	97.24	97.08
41P	94.67	92.54	93.61
42P	97.55	96.01	96.78
43R1	97.66	93.9	95.78
43R2	98.24	96.38	97.31
43R3	96.01	93.37	94.69
44P	97.03	97.62	97.33
45P	96.61	96.83	96.72

The Affymetrix® NspI 250K array results for the tumour DNA samples had a mean SNP call rate of 96.38 ± 0.31 %, a median of 97.01 % and a range of 87.76 % – 99.2 %. The Affymetrix® StyI 250K array results had a mean SNP call rate of 95.26 ± 0.3 %, a median of 95.87 % and a range of 87.27 % – 97.97 %. P= primary; R1-R5=1st to 5th recurrence respectively.

Appendix 4: SNP call rates for 40 constitutional DNA samples analysed using Affymetrix® 500K SNP arrays.

ID	NspI 250K array (%)	StyI 250K array (%)	Average (%)
1P bl	99.5	97.91	98.71
2P bl	93.29	96.88	95.09
3P bl	93.84	95.86	94.85
4P bl	92.6	97.39	94.99
5P bl	91.26	91.65	91.46
6P bl	98.87	96.18	97.53
7P bl	99.45	98.12	98.79
8P bl	99.45	97.88	98.67
9P, R2-5 bl	96.99	93.53	95.26
10P bl	93.69	95.68	94.69
11P bl	99.32	97.39	98.36
12P bl	88.27	97.47	92.87
13P bl	97.49	96.64	97.07
15P bl	98.37	97.42	97.90
16P-R1 bl	91.75	96.76	94.26
18P-R1 bl	94.65	90.46	92.56
20P-R1 bl	93.87	88.28	91.08
21P bl	96.53	97.76	97.15
22P bl	99.07	97.66	98.37
23P bl	94.06	96.34	95.2
24P bl	98.64	97.61	98.13
25P bl	99.38	97.52	98.45
26P-R3 bl	96.14	98.16	97.15
27P bl	98.73	97.64	98.19
28P bl	95.58	98.32	96.95
29P bl	96.63	94.48	95.56
30P bl	99.21	99.11	99.16
31P bl	97.5	95.75	96.63
32P bl	98.91	97.68	98.3
33R1 bl	98.24	97.84	98.04
34P bl	93.67	95.83	94.75
36P bl	97.96	97.17	97.57
37P bl	95.76	95.34	95.55
38P bl	94.7	96.8	95.75
40P-R1 bl	95.94	98.13	97.04
41P bl	99.44	97.75	98.6
42P bl	97.84	95.48	96.66
43R1-3 bl	99.3	97.52	98.41
44P bl	94.5	96.3	95.4
45P bl	97.47	96.94	97.21

All 40 samples were from patients contributing paediatric ependymomas to the SNP array analysis. The Affymetrix® NspI 250K array results for the blood DNA samples had a mean SNP call rate of 96.4 ± 0.43 %, a median of 97.2 % and a range of 88.27 – 99.5 %. The Affymetrix® StyI 250K array results had a mean SNP call rate of 96.5 ± 0.34 %, a median of 97.3 % and a range of 88.28 % – 99.11 %. Blood samples corresponding to tumour samples 14P, 17P – R2, 19R1 – R2, 35P – R2 and 39P had been removed from the analysis (see section 3.2.1.1 and Appendix 2). P= primary; R1-R5=1st to 5th recurrence respectively.

Appendix 5: Method used to calculate aUPD for an ependymoma sample.

The CNAG output included genotyping data for the test sample (tumour) and the patient-matched reference sample (blood). Several genotype calls were possible:
0 = No call
1 = AA (homozygous)
2 = AB (heterozygous)
3 = BB (homozygous)

To identify LOH in tumour samples, the CNAG output file was opened in an Excel 2007 spreadsheet (Microsoft, USA), where genotype call values of 3 for test and reference samples were converted to 1, while values of 2 were converted to 0. A loss of heterozygosity column was added using the formula:

=IF(test>blood, “1”, “0”)

Three results were possible:

Tumour (Test)	Blood (Reference)	Formula results
1	1	= 0 (no LOH)
1	0	= 1 (LOH)
0	1	= 0 (no LOH)

To identify SNP probes demonstrating copy number neutral LOH, data from the LOH column and copy number column in the spreadsheet were incorporated into the following formula:

=IF(AND(CN=2,LOH=, “1”, “0”))

If five or more consecutive SNP probes were identified by the above equation, the corresponding region, established from the annotation data, was designated one of aUPD.

Appendix 6: An overview of the method used to identify gene copy number aberrations that are associated with a particular clinical subgroup.

Example - to identify high resolution genomic gains associated with posterior fossa tumours:

Spreadsheets were created in Excel 2007 (Microsoft, USA) for the SNP probe copy number values generated by CNAG across both the posterior fossa group and the supratentorial group. Each sample was represented by a column in the spreadsheet. The COUNTIF function (Excel 2007) was then used to calculate the number of samples in each location group that had a copy number greater than two for each SNP probe. This data was initially analysed by Fisher's exact testing (SPSS 16), so that the strongest statistical associations possible for high resolution genomic gain and posterior fossa tumours were established. In this case, from the data:

1st = Gain in 10/24 posterior fossa samples versus 0/11 supratentorial samples; p = 0.015.
2nd = Gain in 9/24 posterior fossa samples versus 0/11 supratentorial samples; p = 0.033.

Excel 2007 was subsequently used to generate a new list comprising a column of COUNTIF values from the posterior fossa group (PF), a column of COUNTIF values from the supratentorial group (ST) and the standard annotation data. The SNP probe order (1-500,000) for both COUNTIF data sets were ensured to be identical before merging into this new list. At this point probes on chromosomes 21p and X were removed. After this, the following calculation in Excel 2007 was performed to identify SNP probes that match the strongest statistical associations identified above. For example the calculation below identified gained probes associated with posterior fossa tumours (p = 0.015):

=IF((PF COUNTIF value = 10)*(AND(ST COUNTIF value = 0)), "gain", " no gain")

While the calculation below identified gained probes associated with posterior fossa tumours (p = 0.033):

=IF((PF COUNTIF value = 9)*(AND(ST COUNTIF value = 0)), "gain", " no gain")

If five or more consecutive SNP probes were identified by the above equation, the corresponding region, established from the annotation data, was incorporated into the gene list of genomic gains associated with posterior fossa tumours. This list was ranked in order of strength of association as deemed by the p-value obtained from the Fisher's exact test shown above.

Appendix 7: An overview of the method used to identify maintained and acquired gene copy number aberrations in a patient-matched set of primary and recurrent paediatric ependymomas

Example – to identify high resolution maintained gains and losses across a primary-recurrent set.

A spreadsheet was created in Excel 2007 (Microsoft, USA) for the SNP probe copy number values generated by CNAG across all tumours of a given set (primary and x number of recurrences). Each tumour sample was represented by a column in the spreadsheet. The COUNTIF function was then used to generate a COUNTIF column on the spreadsheet that identified probes with a copy number $\geq$ 3 across *all* recurrent tumours of the set. Such probes were identified as they had a value in the COUNTIF column that matched the number of recurrent tumours in the set. For instance, if there were four recurrent tumours in a set, the probes of interest had to have a COUNTIF column value of four.

The primary tumour column and the COUNTIF column were then incorporated into an equation which generated a final 'maintained gain' column identifying regions of gain (i.e. copy number $\geq$ 3) in the primary tumour which were maintained through the entire recurrent set also. An example is shown below where, again, there are four recurrent tumours in the set:

IF((PRIMARY COLUMN >=3)*(AND(COUNTIF COLUMN =4)), "3", "2")

Probes demonstrating maintained gain were thereby designated the value three. This was performed for each of the eight sets. The 'maintained gain' columns were then amalgamated and ranked to identify the most frequently shared regions of maintained gain across all eight sets. Regions had to span at least five consecutive SNPs and be >10kb in size. Regions on chromosome X were removed from the analysis as discussed in the thesis.

For maintained loss, the process was the same, except the COUNTIF column identified probes with a copy number < 2 across all recurrent tumours of each set. In addition, the

‘maintained loss’ column was calculated from an equation identifying regions of loss (i.e. copy number $\leq$ 1) in the primary tumour which were maintained through the entire recurrent set. An example of this equation is shown below where, again, there are four recurrent tumours in the set:

IF((PRIMARY COLUMN <=1)*(AND(COUNTIF COLUMN =4)), “1”, “2”)

Regions of maintained loss were thereby designated the value one. This was performed for each of the eight sets. The ‘maintained loss’ columns were then amalgamated and ranked to identify the most frequently shared regions of maintained loss across all eight sets. Regions had to span at least five consecutive SNPs and be >10kb in size. Regions on chromosome X were removed from the analysis.

Example – to identify high resolution acquired gains and losses across a primary-recurrent set.

The process was exactly as per identifying maintained aberrations. The only differences were in the equations used to calculate ‘acquired gain’ and ‘acquired loss’ columns on the spreadsheets. For regions of acquired gain, the primary tumour of each set had to demonstrate either a normal copy number or loss (i.e. copy number $\leq$ 2). For regions of acquired loss, the primary tumour of each set had to demonstrate either a normal copy number or gain (i.e. copy number $\geq$ 2).

Examples of these equations are shown below where, once again, there are four recurrent tumours in a set.

For gain:

IF((PRIMARY COLUMN <=2)*(AND(COUNTIF COLUMN =4)), “3”, “2”)

For loss:

IF((PRIMARY COLUMN >=2)*(AND(COUNTIF COLUMN =4)), “1”, “2”)

Appendix 8: Biological prognostic marker results (FISH and immunohistochemistry) for the CCLG 1992 04 clinical trial cohort

Sample ID	Trial Number	Average FISH Signal absence (%)	Average 1q25 Score (%)	Highest focal 1q25 score (%)	Tenascin-C Score	Mean Nucleolin Score (%)	Mean Ki-67 Score (%)	NAV-1 Score	PRUNE Score
9204 - 1P	170	11/103 (11%)	6/103 (6%)	6/103 (6%)	Negative	93	2	Weak	Moderate
9204 - 2R1	182	Unable to score	Unable to score	Unable to score	Mod/strong	95	65	Weak	Strong
9204 - 3P	89	35/388 (9%)	24/388 (6%)	10/144 (7%)	Mod/strong	93	3	Weak	Strong
9204 - 3R1	89	Unable to score	Unable to score	Unable to score	Mod/strong	95	12	Negative	Negative
9204 - 4R1	159	Unable to score	Unable to score	Unable to score	Mod/strong	80	1	Moderate	Weak
9204 - 5P	158	17/330 (5%)	21/330 (6%)	15/174 (9%)	Negative	93	7	Negative	Negative
9204 - 6P	128	Unable to score	Unable to score	Unable to score	Mod/strong	90	15	Strong	Strong
9204 - 7P	133	42/360 (12%)	48/360 (13%)	31/225 (14%)	Mod/strong	95	1	Moderate	Strong
9204 - 7R1	133	28/382 (7%)	22/382 (6%)	8/127 (6%)	Mod/strong	87	6	Focal strong	Strong
9204 - 8P	210	19/358 (5%)	29/358 (8%)	12/105 (11%)	Mod/strong	62	8	Negative	Strong
9204 - 9R2	96	Unable to score	Unable to score	Unable to score	Negative	95	1	Moderate	Weak
9204 - 9R3	96	x	x	x	x	95	<1	x	x
9204 - 10P	15	25/277 (9%)	52/277 (19%)	24/118 (20%)	Mod/strong	58	<1	Strong	Moderate
9204 - 11P	21	44/549 (8%)	75/549 (14%)	24/173 (14%)	Mod/strong	95	1	Strong	Strong
9204 - 12P	70	Unable to score	Unable to score	Unable to score	Mod/strong	95	1	Strong	Focal strong
9204 - 13P	97	12/130 (9%)	12/130 (9%)	12/130 (9%)	Mod/strong	70	2	Moderate	Strong
9204 - 13R1	97	8/117 (7%)	13/117 (11%)	13/117 (11%)	Mod/strong	95	1	Moderate	Strong
9204 - 14R1	117	14/124 (11%)	6/124 (5%)	6/124 (5%)	Mod/strong	93	<1	Negative	Moderate
9204 - 15P	118	15/163 (9%)	2/163 (1%)	1/54 (2%)	Mod/strong	95	1	Weak	Moderate
9204 - 16P	199	23/260 (9%)	26/260 (10%)	18/154 (12%)	Mod/strong	58	17	Focal strong	x
9204 - 17P	203	17/176 (10%)	4/176 (2%)	2/67 (3%)	Mod/strong	85	3	Negative	Moderate
9204 - 17R1	203	12/113 (11%)	3/113 (3%)	3/113 (3%)	Mod/strong	95	40	Weak	Weak
9204 - 18P	213	Unable to score	Unable to score	Unable to score	Mod/strong	95	6	Focal moderate	Moderate
9204 - 19P	10	25/348 (7%)	29/348 (8%)	17/141 (12%)	Weak	80	5	Focal moderate	Strong
9204 - 20P	30	32/405 (8%)	26/405 (6%)	11/149 (7%)	Negative	95	<1	Strong	Weak
9204 - 21P	137	16/418 (4%)	42/418 (10%)	17/143 (12%)	Mod/strong	53	1	Moderate	Strong
9204 - 22P	168	87/944 (9%)	74/944 (8%)	16/163 (10%)	Weak	95	1	Strong	Focal strong
9204 - 23P	189	21/325 (7%)	39/325 (12%)	22/102 (22%)	Mod/strong	93	13	Strong	Strong
9204 - 24P	201	14/173 (8%)	4/173 (2%)	4/173 (2%)	Mod/strong	37	1	Strong	Weak
9204 - 25P	207	22/125 (18%)	16/125 (13%)	16/125 (13%)	Negative	95	1	Weak	Focal strong
9204 - 26R1	14	25/263 (10%)	15/263 (6%)	10/123 (8%)	Weak	95	<1	Weak	Strong
9204 - 27P	32	55/491 (11%)	58/491 (12%)	27/160 (17%)	Mod/strong	93	<1	Strong	Strong
9204 - 28P	101	46/873 (5%)	56/873 (6%)	16/154 (10%)	Mod/strong	93	<1	Strong	Strong

Sample ID	Trial Number	Average FISH Signal absence (%)	Average 1q25 Score (%)	Highest focal 1q25 score (%)	Tenascin-C Score	Mean Nucleolin Score (%)	Mean Ki-67 Score (%)	NAV-1 Score	PRUNE Score
9204 - 29P	138	54/730 (7%)	100/730 (14%)	24/139 (17%)	Mod/strong	80	1	Strong	Focal strong
9204 - 29R1	138	x	x	x	x	x	10	x	x
9204 - 30P	13	22/186 (12%)	12/186 (7%)	12/186 (7%)	Negative	20	<1	x	Unable to score
9204 - 30R1	13	32/175 (18%)	17/175 (10%)	17/175 (10%)	Mod/strong	35	<1	Moderate	Weak
9204 - 31P	56	26/307 (9%)	28/307 (9%)	16/167 (10%)	Mod/Strong	90	<1	Negative	Focal strong
9204 - 31R1	56	51/316 (16%)	11/316 (3%)	5/109 (5%)	Mod/strong	95	<1	Moderate	Strong
9204 - 31R2	56	33/538 (6%)	109/538 (20%)	47/204 (23%)	Mod/strong	93	4	Negative	Strong
9204 - 31R3	56	23/448 (5%)	43/448 (10%)	21/198 (11%)	Mod/strong	30	1	Negative	Strong
9204 - 32R1	103	13/113 (12%)	8/113 (7%)	8/113 (7%)	Mod/strong	95	20	Negative	Focal strong
9204 - 33R1	180	Unable to score	Unable to score	Unable to score	Strong	11	<1	Strong	Strong
9204 - 34P	164	27/175 (15%)	29/175 (17%)	29/175 (17%)	Mod/Strong	95	<1	Focal strong	Strong
9204 - 35P	204	15/118 (13%)	12/118 (10%)	12/118 (10%)	Mod/Strong	93	3	Focal strong	Strong
9204 - 36P	83	16/228 (7%)	21/228 (9%)	13/115 (11%)	Mod/strong	93	1	Weak	Moderate
9204 - 36R1	83	Unable to score	Unable to score	Unable to score	Weak	90	11	Weak	Strong
9204 - 37R1	122	15/145 (10%)	5/145 (4%)	5/145 (4%)	Mod/strong	95	x	Negative	Weak
9204 - 38P	197	35/279 (13%)	23/279 (8%)	16/134 (12%)	Mod/strong	85	<1	Weak	Strong
9204 - 39P	18	82/726 (11%)	42/726 (6%)	12/154 (8%)	Mod/Strong	95	8	Strong	Focal strong
9204 - 40R1	72	x	x	x	x	x	x	x	x
9204 - 41R1	190	x	x	x	Strong	95	1	Strong	Strong
9204 - 42P	200	21/253 (8%)	20/253 (8%)	10/132 (8%)	Mod/Strong	95	3	Moderate	Strong
9204 - 42R1	200	10/111 (9%)	7/111 (6%)	7/111 (6%)	Mod/Strong	95	7	Strong	Strong
9204 - 43P	81	Unable to score	Unable to score	Unable to score	Mod/strong	95	3	Weak	Moderate
9204 - 44P	129	14/101 (14%)	1/101 (1%)	1/76 (1%)	Mod/strong	95	1	Weak	Moderate
9204 - 45P	139	31/453 (7%)	78/453 (17%)	31/139 (22%)	Mod/Strong	65	<1	Strong	Weak
9204 - 46P	145	3/112 (3%)	11/112 (10%)	11/112 (10%)	Mod/strong	78	1	Strong	Moderate
9204 - 47P	58	Unable to score	Unable to score	Unable to score	Weak	95	1	Strong	Focal strong
9204 - 48P	112	16/290 (6%)	21/290 (7%)	11/147 (7%)	Weak	78	1	Moderate	Focal strong
9204 - 49P	161	12/104 (12%)	20/104 (19%)	20/104 (19%)	Mod/strong	78	<1	Focal strong	Moderate
9204 - 49R1	161	Unable to score	Unable to score	Unable to score	Weak	95	20	Strong	Strong
9204 - 50P	162	Unable to score	Unable to score	Unable to score	Mod/Strong	90	1	Strong	Moderate
9204 - 51P	176	24/404 (6%)	116/404 (29%)	72/110 (66%)	Mod/Strong	55	<1	Weak	Strong
9204 - 52P	187	34/426 (8%)	45/426 (11%)	15/130 (12%)	Mod/Strong	43	<1	Weak	Strong
9204 - 53P	208	9/112 (8%)	6/112 (5%)	6/112 (5%)	Mod/Strong	93	3	Strong	Weak
9204 - 53R1	208	15/117 (13%)	6/117 (5%)	6/117 (5%)	Weak	95	<1	Strong	Strong

Sample ID	Trial Number	Average FISH Signal absence (%)	Average 1q25 Score (%)	Highest focal 1q25 score (%)	Tenascin-C Score	Mean Nucleolin Score (%)	Mean Ki-67 Score (%)	NAV-1 Score	PRUNE Score
9204 - 54P	188	37/350 (11%)	40/350 (11%)	14/107 (13%)	Mod/Strong	70	4	Strong	Moderate
9204 - 55P	196	24/315 (8%)	93/315 (30%)	53/160 (33%)	Weak	90	1	Strong	Strong
9204 - 56P	51	41/507 (8%)	40/459 (9%)	15/171 (9%)	Mod/Strong	17	<1	Focal strong	Negative
9204 - 56R1	51	x	x	x	x	Unable to score	<1	x	Unable to score
9204 - 57P	174	76/797 (10%)	79/797 (10%)	22/174 (13%)	Mod/Strong	95	4	Moderate	Moderate
9204 - 57R3	174	26/235 (11%)	16/235 (7%)	11/132 (8%)	Mod/Strong	90	9	Focal strong	Strong
9204 - 58P	185	28/302 (9%)	24/302 (8%)	13/150 (9%)	Weak	95	4	Strong	Moderate
9204 - 59P	212	26/249 (10%)	82/249 (33%)	47/127 (37%)	Mod/Strong	95	3	x	x
9204 - 60P	116	39/729 (5%)	39/729 (5%)	12/168 (7%)	Mod/Strong	95	6	Strong	Strong
9204 - 60R1	116	24/485 (5%)	24/485 (5%)	8/127 (6%)	Mod/Strong	63	4	Strong	Moderate
9204 - 61P	132	Unable to score	Unable to score	Unable to score	Mod/Strong	95	5	Focal strong	Negative
9204 - 62P	181	22/144 (15%)	12/144 (8%)	12/144 (8%)	Mod/Strong	95	1	Weak	Strong
9204 - 63P	1	30/410 (7%)	22/410 (5%)	9/108 (8%)	Mod/Strong	85	1	Focal strong	Moderate
9204 - 64P	131	10/135 (7%)	13/135 (10%)	11/90 (12%)	Mod/Strong	80	<1	Negative	Weak
9204 - 65P	171	105/724 (15%)	62/724 (9%)	17/159 (11%)	Mod/Strong	95	<1	Moderate	Moderate
9204 - 66P	194	39/435 (9%)	30/435 (7%)	13/154 (8%)	Mod/Strong	70	<1	Weak	Moderate
9204 - 67R1	177	38/384 (10%)	106/384 (28%)	51/136 (38%)	Mod/Strong	65	3	Strong	Strong
9204 - 68P	184	Unable to score	Unable to score	Unable to score	Mod/Strong	95	2	Strong	Moderate
9204 - 68R1	184	Unable to score	Unable to score	Unable to score	Negative	80	3	x	x
9204 - 69P	192	31/320 (10%)	88/320 (28%)	40/144 (28%)	Mod/Strong	95	<1	Moderate	Strong
9204 - 70P	46	40/279 (14%)	17/279 (6%)	8/106 (8%)	Mod/Strong	95	<1	Strong	Moderate
9204 - 71P	166	42/564 (7%)	34/564 (6%)	15/150 (10%)	Mod/Strong	93	<1	Focal strong	Moderate

P = primary, R1 – R5 = 1^{st} – 5^{th} recurrence, x = core loss or intact core less than 50 %, Unable to score = cases of non-viable tumour, Mod/Strong = moderate to strong staining.

Appendix 9: Biological prognostic marker results (FISH and immunohistochemistry) for the SIOP 1999 04 clinical trial cohort

Sample ID	Trial Number	Average FISH Signal absence (%)	Average 1q25 Score (%)	Highest focal 1q25 score (%)	Tenascin-C Score	Mean Nucleolin Score (%)	Mean Ki-67 Score (%)	NAV-1 Score	PRUNE Score
9904 - 1P	75	29/264 (11%)	77/264 (29%)	43/115 (37%)	Weak	55	<1	Strong	Strong
9904 - 1R1	75	18/202 (9%)	67/202 (33%)	42/128 (33%)	Negative	85	1	Focal strong	Strong
9904 - 2P	79	17/272 (6%)	29/272 (11%)	21/158 (13%)	Mod/Strong	48	<1	Weak	Focal strong
9904 - 3P	55	15/123 (12%)	8/123 (7%)	8/123 (7%)	Mod/Strong	80	<1	Negative	Focal strong
9904 - 4P	9	22/304 (7%)	13/304 (4%)	9/157 (6%)	Mod/Strong	88	5	Weak	Moderate
9904 - 5P	3	30/352 (9%)	37/352 (11%)	25/193 (13%)	Mod/Strong	40	<1	Negative	Strong
9904 - 5R1	3	37/212 (17%)	18/212 (9%)	9/103 (9%)	Strong	43	<1	Strong	Strong
9904 - 6P	20	56/618 (9%)	38/618 (6%)	14/168 (8%)	Mod/Strong	95	10	Focal strong	Moderate
9904 - 7P	26	26/294 (9%)	39/294 (13%)	17/120 (14%)	Mod/Strong	95	7	Strong	Strong
9904 - 8P	62	26/181 (14%)	3/181 (2%)	3/181 (2%)	Weak	85	1	Focal moderate	Weak
9904 - 8R1	62	Unable to score	Unable to score	Unable to score	Mod/Strong	Unable to score	x	Moderate	x
9904 - 9P	37	16/173 (9%)	1/173 (1%)	1/51 (2%)	Mod/Strong	95	4	Focal strong	Moderate
9904 - 9R1	37	x	x	x	Negative	70	<1	Strong	Weak
9904 - 10P	41	25/363 (7%)	161/363 (44%)	67/134 (50%)	Weak	32	1	Weak	Strong
9904 - 11P	46	52/574 (9%)	49/574 (9%)	13/135 (10%)	Mod/Strong	94	<1	Focal strong	Focal strong
9904 - 11R1	46	17/142 (12%)	18/142 (13%)	18/142 (13%)	Weak	95	1	Unable to score	Strong
9904 - 11R2	46	x	x	x	Weak	x	<1	Focal strong	Negative
9904 - 11R3	46	Unable to score	Unable to score	Unable to score	Weak	Unable to score	<1	x	Focal strong
9904 - 12P	54	19/136 (14%)	0/136 (0%)	0/136 (0%)	Weak	95	1	Weak	Focal strong
9904 - 13P	56	33/349 (10%)	25/349 (7%)	15/176 (9%)	Weak	85	3	Strong	Moderate
9904 - 14P	24	Unable to score	Unable to score	Unable to score	Unable	x	x	Focal strong	x
9904 - 14R1	24	x	x	x	Weak	95	1	Moderate	Strong
9904 - 15P	6	48/567 (9%)	53/567 (9%)	17/124 (14%)	Weak	95	10	Focal strong	Weak
9904 - 16P	27	29/295 (10%)	20/295 (7%)	14/154 (9%)	Negative	93	4	Weak	Strong
9904 - 17P	52	48/410 (12%)	40/410 (10%)	16/153 (11%)	Negative	95	2	Strong	Strong
9904 - 17R1	52	8/138 (6%)	9/138 (7%)	9/138 (7%)	Weak	95	5	Strong	Strong
9904 - 17R2	52	5/163 (3%)	10/163 (6%)	10/163 (6%)	Weak	90	3	Moderate	Focal strong
9904 - 17R3	52	46/721 (6%)	42/721 (6%)	25/380 (7%)	Weak	95	1	Strong	Weak
9904 - 17R4	52	20/268 (8%)	12/268 (5%)	6/100 (6%)	Weak	95	3	Negative	Strong
9904 - 17R5	52	46/327 (14%)	29/327 (9%)	29/327 (9%)	Weak	95	3	Moderate	Strong
9904 - 18P	35	21/304 (7%)	33/304 (11%)	23/158 (15%)	Mod/Strong	93	3	Strong	Strong

Sample	Trial	Average FISH	Average 1q25	Highest focal	Tenascin-C	Mean Nucleolin	Mean Ki-67	NAV-1	PRUNE

ID	Number	Signal absence (%)	Score (%)	1q25 score (%)	Score	Score (%)	Score (%)	Score	Score
9904 - 18R1	35	20/240 (8%)	40/240 (17%)	20/114 (18%)	Mod/Strong	93	14	Negative	Strong
9904 - 18R2	35	21/253 (8%)	60/253 (24%)	31/120 (26%)	Mod/Strong	95	8	Weak	Strong
9904 - 19P	72	16/166 (10%)	20/166 (12%)	20/166 (12%)	Weak	90	1	Weak	Strong
9904 - 20P	78	Unable to score	Unable to score	Unable to score	Mod/Strong	95	5	Strong	Strong
9904 - 21P	80	Unable to score	Unable to score	Unable to score	Weak	95	1	Strong	Moderate
9904 - 22P	40	7/116 (6%)	7/116 (6%)	7/116 (6%)	Negative	43	<1	Strong	Moderate
9904 - 23P	42	15/245 (6%)	95/245 (39%)	61/141 (43%)	Mod/Strong	44	<1	Strong	Moderate
9904 - 24P	23	26/530 (5%)	42/530 (8%)	15/175 (9%)	Weak	95	<1	Moderate	Weak
9904 - 25P	88	Unable to score	Unable to score	Unable to score	Mod/Strong	90	<1	Strong	Focal strong
9904 - 26P	58	3/168 (2%)	21/168 (13%)	21/168 (13%)	x	95	<1	x	x
9904 - 26R1	58	19/455 (4%)	28/455 (6%)	12/158 (8%)	Negative	95	2	Strong	Moderate
9904 - 26R2	58	x	x	x	Weak	87	4	Strong	Strong
9904 - 27P	81	x	x	x	x	95	2	x	x
9904 - 28P	67	Unable to score	Unable to score	Unable to score	Mod/Strong	80	3	x	x
9904 - 29P	73	26/424 (6%)	240/424 (57%)	96/137 (70%)	Weak	93	1	Focal strong	Strong
9904 - 30P	60	Unable to score	Unable to score	Unable to score	Negative	85	<1	Strong	Focal strong
9904 - 30R1	60	Unable to score	Unable to score	Unable to score	Weak	95	2	x	Negative
9904 - 31P	71	57/461 (12%)	36/461 (8%)	16/157 (10%)	Negative	95	2	Weak	Strong
9904 - 32P	44	15/180 (8%)	56/180 (31%)	33/103 (32%)	Negative	95	1	Focal moderate	Focal strong
9904 - 32R1	44	29/388 (7%)	117/388 (30%)	117/388 (30%)	Weak	95	4	Moderate	Focal strong
9904 - 33P	39	32/342 (9%)	29/342 (9%)	19/184 (10%)	Negative	35	<1	Strong	Focal strong
9904 - 33R1	39	51/356 (14%)	10/356 (3%)	4/119 (3%)	Strong	40	1	Focal strong	Moderate
9904 - 34P	83	9/188 (5%)	27/188 (14%)	27/188 (14%)	Weak	95	2	Moderate	Focal strong
9904 - 35P	76	35/529 (7%)	39/529 (7%)	14/167 (8%)	Mod/Strong	95	4	Focal strong	Strong
9904 - 36P	57	18/277 (7%)	111/277 (40%)	52/122 (43%)	x	60	4	Strong	x
9904 - 36R1	57	21/281 (8%)	74/281 (26%)	38/126 (30%)	Negative	95	1	Strong	x
9904 - 37P	8	15/268 (6%)	29/268 (11%)	18/158 (11%)	Mod/Strong	65	<1	Strong	Moderate
9904 - 38P	18	x	x	x	x	35	<1	Focal strong	x
9904 - 38R1	18	10/140 (7%)	18/140 (13%)	18/140 (13%)	Mod/Strong	90	4	Strong	Strong
9904 - 39P	29	4/62 (6%)	7/62 (11%)	7/62 (11%)	Negative	63	<1	Strong	Strong
9904 - 40P	87	38/480 (8%)	41/480 (9%)	20/179 (11%)	Weak	Unable to score	x	Moderate	Focal moderate
9904 - 41P	17	10/144 (7%)	75/144 (52%)	75/144 (52%)	Weak	70	8	x	x
9904 - 42P	68	14/232 (6%)	18/232 (8%)	9/104 (9%)	Weak	85	1	Negative	Weak

Sample	Trial Number	Average FISH Signal absence (%)	Average 1q25 Score (%)	Highest focal 1q25 score (%)	Tenascin-C Score	Mean Nucleolin Score (%)	Mean Ki-67 Score (%)	NAV-1 Score	PRUNE Score

9904 – 43P	31	122/1795 (7%)	171/1795 (10%)	22/162 (14%)	Mod/Strong	93	<1	Moderate	Strong
9904 – 44P	53	17/177 (10%)	23/177 (13%)	23/177 (13%)	Negative	95	2	Negative	Strong
9904 – 45P	64	26/129 (20%)	8/129 (6%)	8/129 (6%)	Negative	93	3	Strong	Focal strong
9904 – 46P	89	Unable to score	Unable to score	Unable to score	Weak	93	8	Weak	Weak
9904 – 47P	65	45/398 (11%)	35/398 (9%)	18/155 (12%)	Weak	67	1	Focal strong	Focal strong

P = primary, R1 – R5 = 1^{st} – 5^{th} recurrence, x = core loss or intact core less than 50 %, Unable to score = cases of non-viable tumour, Mod/Strong = moderate to strong staining.

Appendix 10 (CD-ROM):

This is not available on the e-thesis. Please contact the author if requiring access to the information documented below (e-mail: mgzjk@exmail.nottingham.ac.uk or jpkilday@hotmail.com).

A. CNAG derived copy number data for all of the probes analysed across the SNP array cohort of 63 paediatric ependymomas, together with a filtered version of the Affymetrix® 500K annotation file incorporating selected probe classification features (Netaffx file build 07.12.07). Data presented using a Microsoft Excel 2007 data sheet.

B. The R computer script enabling array quality assessment (including BASH) and probe background correction using the Bioconductor® *beadarray* program for data from the Illumina® GoldenGate® Cancer Panel I assay for methylation. Courtesy of Dr. Edward Schwalbe and Dr. Steve Clifford at the Northern Institute for Cancer Research, Newcastle.

C. The R computer script enabling Mann-Whitney testing with Benjamini-Hochberg False Discovery Rate correction of grouped data from the Illumina® GoldenGate® Cancer Panel I assay for methylation. Courtesy of Dr. Edward Schwalbe and Dr. Steve Clifford at the Northern Institute for Cancer Research, Newcastle.

D. Sheet 1 – The calculated number of SNP probes within each cytoband of the 22 autosomes analysed in the SNP array analysis. Sheet 2 – CNAG derived copy number data for each cytoband of the 22 autosomes analysed across the SNP array cohort of 63 paediatric ependymomas. Cytobands demonstrating gain were defined as those where 80 % or more of interrogated SNP probes had a copy number greater than two. Such loci were labelled with the number three. Cytobands demonstrating loss were those where 80 % or more of interrogated SNP probes had a copy number less than two. These were labelled with the number one. All other genomically stable cytobands were identified by the number two to reflect a diploid state. Data presented using a Microsoft Excel 2007 data sheet.

E. The R computer script enabling a PCA cluster plot for the Affymetrix® 500K SNP array cytoband imbalance data, using three principal components. Courtesy of Dr. Edward Schwalbe and Dr. Steve Clifford at the Northern Institute for Cancer Research, Newcastle.

F. The corrected Beta scores for 1,421 GoldenGate® Cancer Panel I CpG probes across all 98 tumours comprising the ependymoma methylation cohort, together with a filtered version of the methylation annotation file incorporating selected probe classification features (Illumina® GoldenGate® Cancer Panel I CpG List 2007). Data presented using a Microsoft Excel 2007 data sheet.

G. The R computer script enabling bootstrapped unsupervised hierarchical clustering and PCA clustering array of data from the Illumina® GoldenGate® Cancer Panel I assay for methylation. Courtesy of Dr. Edward Schwalbe and Dr. Steve Clifford at the Northern Institute for Cancer Research, Newcastle.

H. Acquired uniparental disomy data for all Affymetrix® 500K array SNP probes across the aUPD cohort of 44 paediatric ependymomas, together with a filtered version of the 500K annotation file incorporating selected probe classification features (Netaffx file build 07.12.07). All tumours were analysed against patient-matched constitutional DNA. Probes demonstrating aUPD were labelled with the number one, while probes not demonstrating aUPD were labelled zero. Data presented using a Microsoft Excel 2007 data sheet.

I. Real time quantitative PCR results for 15 selected genes of interest from the SNP array analysis. Data presented using a Microsoft Excel 2007 data sheet.

J. CNAG derived copy number data from the Affymetrix® 500K SNP array, identifying focal regions of maintained gain across eight patient-matched primary and recurrent ependymomas sets (A – H), together with a filtered version of the 500K annotation file incorporating selected probe classification features (Netaffx file build 07.12.07). Regions of gain were labelled with the number three. All other regions were labelled with the number two.

K. CNAG derived copy number data from the Affymetrix® 500K SNP array, identifying focal regions of maintained gain across eight patient-matched primary and recurrent ependymomas sets (A – H). Regions of loss were labelled with the number one. All other regions were labelled with the number two.

L. CNAG derived copy number data from the Affymetrix® 500K SNP array, identifying focal regions of acquired gain across eight patient-matched primary and recurrent ependymomas sets (A – H). Regions of gain were labelled with the number three. All other regions were labelled with the number two.

M. CNAG derived copy number data from the Affymetrix® 500K SNP array, identifying focal regions of acquired loss across eight patient-matched primary and recurrent ependymomas sets (A – H). Regions of loss were labelled with the number one. All other regions were labelled with the number two.

Appendix 11:

Copy number gain of 1q25 predicts poor progression-free survival for paediatric intracranial ependymomas in an age and treatment dependent manner: a European clinical trial cohort analysis on behalf of the Children's Cancer Leukaemia Group (CCLG), *Société Française* **d'Oncologie** *Pédiatrique (SFOP) and International Society for Paediatric Oncology (SIOP).*

Kilday JP, Mitra, B, Domerg, C, Ward J, Andreiuolo F, Varlet P, Lowe J, Ellison, D, Gilbertson, R, Coyle B, Grundy R. Submitted to *Clinical Cancer Research*, October 2011.

Supratentorial and spinal ependymomas display a hypermethylated phenotype which includes the loss of tumor suppressor genes involved in the control of cell growth and death

Rodgers H/Kilday JP, Mayne CE, Barrow J, Schwalbe E, Clifford S, Adamowicz-Bryce M, Coyle B, Grundy R. Submitted to *Acta Neuropathologica*, September 2011.

Paediatric Ependymoma: Biological Perspectives

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