Investigation of chromosome 1q reveals differential expression of members of the S100 family in clinical subgroups of intracranial paediatric ependymoma

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Gain of 1q is one of the most common alterations in cancer and has been associated with adverse clinical behaviour in ependymoma. The aim of this study was to investigate this region to gain insight into the role of 1q genes in intracranial paediatric ependymoma. To address this issue we generated profiles of eleven ependymoma, including two relapse pairs and seven primary tumours, using comparative genome hybridisation and serial analysis of gene expression. Analysis of 656 SAGE tags mapping to 1q identified CHI3L1 and S100A10 as the most upregulated genes in the relapse pair with de novo 1q gain upon recurrence. Moreover, three more members of the S100 family had distinct gene expression profiles in ependymoma. Candidates (CHI3L1, S100A10, S100A4, S100A6 and S100A2) were validated using immunohistochemistry on a tissue microarray of 74 paediatric ependymoma. In necrotic cases, CHI3L1 demonstrated a distinct staining pattern in tumour cells adjacent to the areas of necrosis. S100A6 significantly correlated with supratentorial tumours (P < 0.001) and S100A4 with patients under the age of 3 years at diagnosis (P = 0.038). In conclusion, this study provides evidence that S100A6 and S100A4 are differentially expressed in clinically relevant subgroups, and also demonstrates a link between CHI3L1 protein expression and necrosis in intracranial paediatric ependymoma.

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Identification of cancer-specific molecular alterations has had a major impact on understanding the biology of cancer and improving treatment options in many cancers. For example, therapeutic agents such as Imatinib (Gleevec®) targeting genes altered in the cancer cell, but not in normal cells, are increasingly being tested in the clinical setting. By systematically deciphering the genomes of different cancers, frequent genomic aberrations and gene expression changes have been revealed and correlated with clinical details. However, the biological and clinical relevance of most aberrations in cancer is largely unknown. One such cancer, where the investigation of the tumour-specific genetic aberrations would have a major benefit upon the understanding of the disease that could lead to better treatment choices and patient survival, is ependymoma.

Ependymoma is the third most common brain tumour of childhood, with around 50% occurring in infants younger than 5 years of age (Bouffet et al, 1998). Treatment and prognostication is predominantly currently based on clinical criteria despite many genomic studies identifying common molecular aberrations (Reardon et al, 1999; Zheng et al, 2000; Scheil et al, 2001; Ward et al, 2001; Carter et al, 2002; Dyer et al, 2002; Grill et al, 2002; Jeuken et al, 2002; Gilhuis et al, 2004; Taylor et al, 2005; Suarez-Merino et al, 2005; Mendrzyk et al, 2006; Modena et al, 2006; Sowar et al, 2006; Lukashova-v Zangen et al, 2007). Currently complete tumour resection is the only confirmed independent prognostic marker, indicating a better patient outcome (Bouffet et al, 1998; Sala et al, 1998). Despite complete resection, local recurrence is reported in up to 50% of paediatric cases (McLaughlin et al, 1998; van Veelen-Vincent et al, 2002). Some improvements in survival rates have been seen over the last 30 years, with some 50% of patients now obtaining 5-year survival (Gatta et al, 2003, 2005). However, when compared with other cancers such as acute lymphoblastic leukaemia, where more than 80% of children are long-term survivors, these improvements lag far behind. There is a need to identify robust biological markers and to better understand the biology of ependymoma to improve therapeutic strategies and patient survival.

Gain of 1q is one of the most common genomic aberrations in cancer (Struski et al, 2002) and is frequently gained in ependymoma, occurring at an incidence of >20% (Reardon et al, 1999; Scheil et al, 2001; Ward et al, 2001; Dyer et al, 2002; Grill et al,
2002). The gain of the whole of the q-arm of chromosome 1 has been associated with a poor prognosis in ependymoma (Dyer et al., 2002), and has also been shown to adversely affect patient survival in other paediatric cancers, including Wilms’ tumour and Ewing’s sarcoma (Hirai et al., 1999; Hing et al., 2001). The region-specific amplicon, 1q25, has been demonstrated as an independent prognostic marker, indicating a poor prognosis (Mendrzyk et al., 2006). However, the mechanisms by which 1q, or 1q25, confer adverse biological behaviour in ependymoma is unclear and a more detailed analysis of 1q is necessary.

Chromosome 1q gain has also been shown to be the most common global genetic change in ependymoma recurrent tumours, seen in 67% of cases, with de novo gain of 1q frequently occurring in the relapse sample (Dyer et al., 2002). The region-specific amplicon 1q21.1–q32.1 has been associated with tumour recurrence in intracranial ependymoma (Mendrzyk et al., 2006). Several other region-specific amplicons frequently gained in ependymoma include 1q21.3–q23.1, 1q21–q31 and 1q22–q31, 1q31.1–q31.3, 1q31–q32 and 1q41–qter (Kramer et al., 1998; Scheit et al., 2001; Ward et al., 2001; Mendrzyk et al., 2006; Modena et al., 2006). The 1q32 amplicon contains two genes laminin and GAC1 that are overexpressed in ependymoma and the candidate gene DUSP12 is located within the frequently gained ‘hotspot’ 1q23.3 (Suarez-Merino et al., 2005; Mendrzyk et al., 2006). Despite these observations the role of these amplicons remain unclear and, to date, no specific genes on 1q have been shown to directly relate to ependymoma tumorigenesis, relapse or patient outcome.

Taken together this evidence implicates the gain of 1q as a marker for adverse clinical behaviour. However, the underlying biology and the gene(s) involved remain to be elucidated. To address these issues we used a combination of comparative genome hybridisation (CGH) and serial analysis of gene expression (SAGE) to identify candidate genes on 1q. Candidates were validated using immunohistochemistry (IHC) on a tissue microarray and the protein expression levels correlated with clinicopathological data to determine their potential role in intracranial paediatric ependymoma.

**MATERIALS AND METHODS**

**Sample cohort**

For SAGE and CGH analysis, 11 fresh-frozen tumour samples were obtained from the Duke Brain Tumour Bank, USA and Birmingham Children’s Hospital, UK (Table 1). Five normal brain libraries (white matter, cerebral cortex, paediatric frontal cortex and two cerebellum) and six other brain tumour types (one astrocystoma grade I, eight astrocytoma grade II, 11 astrocytoma grade III, 10 glioblastoma, two oligodendroglioma and 20 medulloblastoma) were downloaded from the SAGE Genie website (http://cgap.nci.nih.gov/SAGE; Boon et al., 2002).

For immunohistochemistry, a tissue microarray (TMA) was constructed using formalin-fixed paraffin-embedded (FFPE) tumour material from 74 primary tumours. The samples were obtained from the Histopathology Department at the Birmingham Children’s Hospital and further Neuropathology Departments of the Children’s Cancer Leukaemia Group (CCLG) Centres. The histology of each tumour was verified, representative areas were identified by a pathologist (MAB) and a minimum of three cores were taken. Haematoxylin and Eosin smears of corresponding frozen material were used to confirm viable tumour. Clinical information was obtained from the CCLG Data Centre, West Midlands Children’s Tumour Registry and case notes.

**CGH and SAGE libraries**

Comparative genome hybridisation was performed as described by Dyer et al. (2002). Serial analysis of gene expression libraries were constructed using the RNA isolated from 11 frozen tissue samples as described by Boon and Riggins (2003). SAGE2000 software (http://www.sagenet.org) was used to extract tags from the original sequence files and processed to remove duplicate ditags, linker sequences and repetitive tags. Tag counts and library information for nine SAGE libraries have been posted to CGAP’s SAGE Genie website (http://cgap.nci.nih.gov/SAGE) using the HUGO gene symbol. All SAGE libraries were normalised to tags per 200 000 to enable cross-library comparison.

**SAGE analysis**

The SAGE data was analysed in four ways: (1) Tags were identified in relapse pair R1 with a higher tag count in the relapsed sample (E1023) than in the corresponding primary (E628). This data was filtered to determine tags with either the same, or less, count in the...
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RESULTS

Patients and CGH profiles

Genomic profiles were generated for 11 flash-frozen ependymoma (Table 1). Of the nine tumours with a CGH profile, six tumours (four paediatric and two adults) had a balanced genome (i.e., had no detectable genomic losses or gains) and three (two paediatric and one adult) had a structural genome (i.e., had few, mainly partial chromosome gains). Two paediatric relapse pairs, R1 and R2, were included in this study. Comparative genome hybridisation revealed that the recurrent sample (E1023) of relapse pair R1 had gain of 1q whereas the primary (E628) was balanced (i.e., de novo 1q gain). The genomes of the primary (E1p) and recurrent sample (E1r) of relapse pair R2 were both balanced.

SAGE libraries and 1q tags

A total of 801 076 SAGE tags were generated from 11 ependymoma samples with, on average, over 26 000 unique tags per library. The complete libraries for 9 of the 11 ependymoma are available to download from the SAGE Genie website. The unique SAGE tags representing the genes on 1q were identified using the Ensembl genome browser, reducing the number of tags to be analysed to 656.

Upregulated genes associated with 1q gain in recurrent ependymoma

From the filtered dataset of 656 1q tags, 205 were selected that had a higher tag count in the relapse of pair R1 than in the corresponding primary. To identify the genes upregulated on account of gain of 1q, filtering was done to select tags that were specifically upregulated upon relapse in the R1 pair compared with the relapse pair R2. This reduced the number of tags to 149. Once the tags were ranked based on the difference in tag count between the recurrent sample of pair R1 and the primary, CHI3L1, S100A10 and PSMB4 were revealed as the top three genes upregulated as a consequence of the gain of 1q in recurrent ependymoma (Table 2).

Ependymoma-associated 1q transcripts

A comparison of 1q tags in 10 ependymoma with five normal brain libraries revealed S100A10 as the most upregulated gene (125.5 tags; Table 3). A second member of the S100 family, S100A6, was identified with one of the highest differences between ependymoma and normal brain of 27.1. CHI3L1 also ranked highly, with a
difference of 25.7 tags. When the data was then filtered for tags meeting the criteria \( p < 0.5 \) mean tag count in normal brain but \( > 2 \) tags in ependymoma, the uncharacterised gene C1orf192 showed the highest difference in expression of 17.3 (Table 3). S100A4, a third member of the S100 family, was also one of the most upregulated genes in ependymoma with a difference in tag count of 6.6.

**S100 gene expression in ependymoma and other brain tumours**

Our analyses revealed that several members of the same gene family were associated with 1q gain and were also upregulated in ependymoma compared with normal brain tissue. Therefore, to investigate this gene family, the mean tag counts were calculated for all 14 S100 genes located on 1q21.3 represented in SAGE genie in the ependymoma SAGE libraries (Figure 1). Of the 13 S100 genes, S100A4 had the highest mean tag count for ependymoma relative to normal brain with a fold change of 20.7 and S100A10 had the highest mean tag count of 133 in ependymoma. S100A2 was the only S100 gene that was expressed in ependymoma but not in normal brain with mean tag counts of 1.5 and zero, respectively. No expression of five members of the S100 family (A15, A7, A5, A14 and A13) was observed in ependymoma.

Members of the S100 family have been associated with different cancers, including brain tumours. Therefore, this analysis was extended to six other brain tumour types and the mean SAGE tags were calculated for the 14 S100 genes in each tumour type (Figure 1). S100A10 and S100A6 showed the highest mean expression across the six brain tumour types of 43.6 and 41.7 tags, respectively. In both grade I astrocytoma and glioblastoma (GBM) S100A10 had the highest tag count of 134 and 112.3, respectively. S100A6 had the highest tag counts in oligodendroglioma (15 tags), medulloblastoma (10.4 tags) and grade II and III

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**Table 3** Most highly expressed genes associated with ependymoma

| SAGE TAG | Gene ID | Locus | Gene title | Mean tumour | Mean normal | Difference |
|----------|---------|-------|------------|-------------|-------------|------------|
| (b) Upregulated in ependymoma | | | | | | |
| AGCGAGATCAC | S100A10 | 1q21.3 | S100 calcium-binding protein A10 | 132.9 | 7.4 | 125.5 |
| GTCTGGGCT | TAGLN2 | 1q23.2 | Transgelin 2 | 126.4 | 20 | 106.4 |
| GGCTGGGGCT | ATP1A2 | 1q23.2 | ATPase, Na+/K+ transporting, a 2 (+) polypeptide | 146.3 | 70 | 76.3 |
| TTTTTAAATT | H3F3A | 1q21.12 | H3 histone, family 3A | 96.6 | 24.6 | 72 |
| GGCCTGACCC | CSRP1 | 1q21.2 | Cysteine and glycine-rich protein 1 | 146.4 | 79.2 | 67.2 |
| TGAAGAGGAG | PRDX6 | 1q25.1 | Peroxiredoxin 6 | 47.2 | 6 | 41.2 |
| CCCCCCTGAT | S100A6 | 1q21.2 | S100 calcium-binding protein A6 (calcyclin) | 38.3 | 11.2 | 27.1 |
| GTATGGGCCC | CHI3L1 | 1q32.1 | Chitinase 3-like 1 (cartilage glycoprotein-39) | 26.5 | 0.8 | 25.7 |
| TACATTCTGT | MCL1 | 1q21.2 | Myeloid cell leukaemia sequence 1 (BCL2-related) | 27.4 | 5 | 22.4 |
| TAATCTCTCT | CCT3 | 1q21.2 | Chaperonin containing TCP1, subunit 3 (gamma) | 40.4 | 20.8 | 19.6 |

(c) Upregulated in ependymoma with \( p < 0.5 \) mean tag count in normal brain

| SAGE TAG | Gene ID | Locus | Gene title | Mean tumour | Mean normal | Difference |
|----------|---------|-------|------------|-------------|-------------|------------|
| ATCCACGAC | C1orf192 | 1q23.3 | Chromosome 1 open reading frame 192 | 17.3 | 0 | 17.3 |
| CCCAGATGAT | SLC39A1 | 1q21.3 | Solute carrier family 39 (zinc transporter), member 1 | 8.5 | 0.4 | 8.1 |
| ATTCCTTCC | FMO3 | 1q24.3 | Flavin containing monoxygenase 3 | 7.2 | 0 | 7.2 |
| TAACTCTATA | FCGR2A | 1q23.3 | Fc fragment of IgG, low affinity IIa, receptor for (CD32) | 6.6 | 0 | 6.6 |
| TCCAGATAC | ANPRT | 1q23.5 | Angiopoietin-like 1 | 6.6 | 0 | 6.6 |
| ATGTCTAACG | S100A4 | 1q21.3 | S100 calcium-binding protein A4 | 5.9 | 0.4 | 5.5 |
| AGTCTGGGCT | FMO3 | 1q23.2 | Fibromodulin | 5.7 | 0.4 | 5.3 |
| ATCAGACGAC | LMOD1 | 1q23.2 | Leiomodin 1 (smooth muscle) | 4.4 | 0 | 4.4 |
| TAGGAAGTGGG | C1orf64 | 1q21.2 | Chromosome 1 open reading frame 54 | 4.8 | 0.4 | 4.4 |
| GAAGCCAATGT | DDISP1 | 1q41 | Dispatched homologue 1 (Drosophila) | 3.5 | 0.4 | 3.1 |

All tag counts are normalised per 200 000 tags. Mean tag count per individual tumour SAGE library. Mean tag count per normal brain SAGE library. Difference between mean tag count per ependymoma library and normal brain libraries.

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**Figure 1** Summary of the mean SAGE tag counts for 14 S100 genes in ependymoma and six other brain tumour types (astrocytoma grade I, II, III, glioblastoma, oligodendroglioma and medulloblastoma). The S100 genes are in genomic order and the start and end positions on chromosome 1 are given in megabases (Mb). Mean of the 52 SAGE libraries from the six brain tumour types. A red star marks the genes selected for further investigation.
Differential expression of S100 proteins in intracranial paediatric ependymoma

Four members of the S100 family were selected for further investigation based on their distinct gene expression profiles in ependymoma. Protein expression levels of S100A10, S100A6, S100A4 and S100A2 were determined by immunohistochemistry using an independent cohort of seventy-four primary paediatric ependymoma arrayed on a tissue microarray (Figure 2A–H). One of the eleven ependymoma samples used to create SAGE libraries, ER1p, was represented on the TMA. In this sample, no gene or protein expression was observed for S100A2 and S100A4 with SAGE tag counts of 0 per 200 000 tags and negative protein staining observed by IHC. For S100A6 the SAGE tag count was 15 per 200 000 tags and immunostaining determined as negative/weak. Both gene and protein expression was observed for S100A10, where the SAGE tag count was 76 per 200 000 tags and moderate protein expression was observed by IHC.

Univariate analysis was performed to explore possible associations between the S100 protein expression levels and clinical variables in primary ependymoma (Table 4). S100A6 significantly correlated with tumours arising in the supratentorial region of the brain (P < 0.001) and S100A4 correlated with age at diagnosis under 3 years (P = 0.038). No significant correlations were found for S100A10 or S100A2. Kaplan–Meier survival analysis of the clinical parameters and S100 protein expression showed that resection status and tumour location were the only indicators of prognosis, with complete resection and supratentorial tumours indicating a better patient outcome (P < 0.001 and P = 0.020, respectively). Similarly, multivariate analysis revealed extent of resection and tumour location as independent prognostic markers (P = 0.007 and P = 0.001, respectively).

CHI3L1 expression in ependymoma

Forty-eight primary tumours were scored for CHI3L1 protein expression. Tumours were categorised as having negative (0%), weak (< 25%) or strong (≥ 25%) expression levels. Twenty-eight (58%) were negative for CHI3L1 protein expression, 14 (29%) showed weak and six (13%) demonstrated strong expression. CHI3L1 protein expression was determined for sample ER1p, which had a SAGE tag count of 30 per 200 000 tags. No significant correlations with the clinical parameters investigated were found. Histopathological review revealed that in five cases, where areas of necrosis were visible, CHI3L1 protein expression was restricted to the cytoplasm of viable tumour cells adjacent to the areas of necrosis (Figure 2I and J).

DISCUSSION

Little is known about the genes and genetic mechanisms underlying ependymoma tumorigenesis, patient relapse and survival. To address these issues we focused our study on chromosome 1q, one of the most commonly gained regions in ependymoma. Using CGH and SAGE profiling we identified CHI3L1 and members of the S100 family as candidate genes in ependymoma. Immunohistochemical analysis on a large cohort of paediatric ependymoma revealed that CHI3L1 protein expression is associated with necrosis and that members of the S100 family are differentially expressed in clinically relevant subgroups. S100A6 is significantly associated with paediatric ependymoma arising in the supratentorial compartment and S100A4 strongly correlates with patients aged less than 3 years at diagnosis.

In this study, different approaches were taken to mine the SAGE data to identify the ependymoma-associated genes on
Table 4  Patient demographics, univariate and multivariate analysis of clinical and biological factors of 74 primary paediatric intracranial ependymoma

| S100 protein | Gender | Tumour location | WHO grade | Age <3 years at diagnosis* | Resection status |
|--------------|--------|-----------------|-----------|---------------------------|-----------------|
|              |        | M F             | ST PF     | II III <3 >3 C IC         |
| Patient demographics |        | No. | 41 33 | 19 55 | 49 35 | 34 40 | 26 44 | 37.1 62.9 |
| % | 41 33 | 19 55 | 49 35 | 34 40 | 26 44 | 37.1 62.9 |
| > Univariate analysis | | | | | | | |
| S100A2 | | | | | | |
| Positive | 14 21 | 0.598 | 0.252 | 0.788 | 0.038 |
| Negative | 51 79 | 65 | | | |
| Total scored | | | | | | |
| S100A4 | | | | | | |
| Negative/weak | 41 64 | 0.549 | | 1.000 | 0.236 |
| Moderate | 23 36 | | | 1.000 | |
| Total scored | 64 | | | | |
| S100A6 | | | | | | |
| Negative/weak | 55 81 | 0.589 | 0.12 | 0.783 | 0.788 |
| Moderate/strong | 13 19 | | | |
| Total scored | 68 | | | |
| S100A10 | | | | | | |
| Negative/weak | 21 35 | 0.905 | 0.219 | 1.01 | 1.124 | 5.357 |
| Moderate/strong | 39 65 | | | 0.072–0.662 | 0.448–2.278 | 0.561–2.251 | 2.080–13.792 |
| Total scored | 60 | | | |
| > Multivariate analysis | | | | | | |
| Hazard ratio | | | | | | |
| 95% CI | | | | | | |
| P-value | | | | | | |
| 0.078 | 0.007 | 0.981 | 0.742 | 0.001 |

C = complete resection; IC = confidence interval; F = female; IC = incomplete resection; M = male; No. = number; PF = posterior fossa; Sc = samples scored; ST = supratentorial.

*The median age at diagnosis of the primary tumours was 3 years (range, 8 months to 14.5 years). Significant P-values (<0.05) are given in bold.
necrosis is a characteristic, both CHI3L1 expression and necrosis suggested that it has a function in a number of pro-survival pathways. The role of CHI3L1 in cancer is unknown, but it has been reported to be overexpressed in a number of different cancers, including glioma, and has been proposed as a new therapeutic target (Pelloski et al., 2005; Johansen et al., 2007). In glioblastoma (GBM), where necrosis is a characteristic, both CHI3L1 expression and necrosis are associated with poor prognosis (Burger and Green, 1987; Raza et al., 2002; Pelloski et al., 2005; Homma et al., 2006; Kleihues et al., 2007). In ependymoma, we did not find a correlation with prognosis, thus, raising the possibility that CHI3L1 is a marker of necrosis rather than of adverse biology per se. These observations in GBMs and our findings in ependymoma suggest a link between CHI3L1 and necrosis in brain tumours. As the cores represented on the TMA are selected to avoid regions of necrosis, our findings maybe an under-representation and further investigation of CHI3L1 expression on whole tissue sections is necessary.

Previously we identified gain of 1q as one of the most common gains in primary and recurrent ependymoma and demonstrated a tendency that patients with gain of 1q have a poorer outcome (Dyer et al., 2002). The aim of this study was to investigate 1q in ependymoma to gain insight into the role of genes located in this region. In this study, we have identified members of the S100 family located within the commonly gained amplicon 1q21.3 and provide evidence of their differential expression in clinical subgroups of paediatric ependymoma: S100A4 is associated with patients of a very young age at diagnosis and S100A6 with supratentorial tumour location. We also demonstrated a link between CHI3L1 protein expression and necrosis. However, we are yet to elucidate the underlying mechanism by which 1q gain confers adverse biological behaviour in paediatric ependymoma. We are now extending this study to a larger tumour cohort to further unravel the underlying biology of 1q in this complex tumour.

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