Epigenetic deregulation of multiple S100 gene family members by differential hypomethylation and hypermethylation events in medulloblastoma

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Deregulated expression of genes encoding members of the S100 family of calcium-binding proteins has been associated with the malignant progression of multiple tumour types. Using a pharmacological expression reactivation approach, we screened 16 S100 genes for evidence of epigenetic regulation in medulloblastoma, the most common malignant brain tumour of childhood. Four family members (S100A2, S100A4, S100A6 and S100A10) demonstrated evidence of upregulated expression in multiple medulloblastoma cell lines, following treatment with the DNA methyltransferase inhibitor, 5'-aza-2'-deoxycytidine. Subsequent analysis revealed methylation of critical CpG sites located within these four genes in an extended cell line panel. Assessment of these genes in the non-neoplastic cerebellum (from which medulloblastomas develop) revealed strong somatic methylation affecting S100A2 and S100A4, whereas S100A6 and S100A10 were unmethylated. Assessed against these normal tissue-specific methylation states, S100A6 and S100A10 demonstrated tumour-specific hypermethylation in medulloblastoma primary tumours (5 out of 40 and 4 out of 35, respectively, both 12%) and cell lines (both 7 out of 9, 78%), which was associated with their transcriptional silencing. Moreover, S100A6 hypermethylation was significantly associated with the aggressive large cell/anaplastic morphophenotype (P = 0.02). In contrast, pro-metastatic S100A4 displayed evidence of hypomethylation relative to the normal cerebellum in a significant proportion primary tumours (7 out of 41, 17%) and cell lines (3 out of 9, 33%), which was associated with its elevated expression. In summary, these data characterise complex patterns of somatic methylation affecting S100 genes in the normal cerebellum and demonstrate their disruption causing epigenetic deregulation of multiple S100 family members in medulloblastoma development. Epigenetic events affecting S100 genes have potential clinical utility and merit further investigation as molecular biomarkers for this disease.

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Epigenetic alterations play an important role in the development of medulloblastoma, an embryonal tumour of the cerebellum, which accounts for ~20% of childhood brain tumours (Giangaspero et al, 2000; Lindsey et al, 2005). The S100 protein family is a large family of EF hand calcium-binding proteins of approximately 20 members (Donato, 2003; Marenholz et al, 2004), involved in the regulation of a variety of cellular processes including cell growth and cell cycle regulation, differentiation, transcription and motility. Sixteen members of the gene family (S100A1–S100A16) map within a 1.65 Mb region at chromosome 1q21.3 (Figure 1A) (Marenholz et al, 2004).

Members of the S100 gene family show divergent patterns of cell and tissue-specific expression, and the expression of specific family members is disrupted in a range of diseases including cancer (Heizmann et al, 2002; Marenholz et al, 2004; Heizmann, 2005). Overexpression of several of the S100 proteins is thought to be involved in tumour progression, such as S100A4, which is upregulated in many cancers including medulloblastoma and promotes angiogenesis and metastasis (Hernan et al, 2004; Emberley et al, 2004; Helfman et al, 2005; Garrett et al, 2006) and S100P which is associated with metastasis in breast and pancreatic cancer (Arumugam et al, 2005; Wang et al, 2006). Other S100 proteins are downregulated in tumours and have putative tumour suppressor roles, including S100A2 and S100A6 in prostate cancer (Rehman et al, 2005).

Expression of several members of the S100 family, including S100A2, S100A4, S100A6 and S100P, is known to be regulated epigenetically, by methylation of key CpG sites within the genes or their promoters (Wicki et al, 1997; Rosty et al, 2002; Sato et al, 2004; Rehman et al, 2005). This regulation has been postulated to be important in controlling the cell type-specific expression of S100 genes, as methylation associated with transcriptional silencing of these genes in normal somatic tissues has been found to occur in a tissue-specific manner (Lesniak et al, 2000; Rosty et al, 2002; Sato et al, 2004). Accordingly, the normal regulation of
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MATERIALS AND METHODS

Cell lines

Nine independently derived medulloblastoma cell lines (DAOY, D283 Med, D425 Med, MHH-MED-1, MEB-MED8A, D341 Med, D384 Med, D425 Med, D556 Med and UW228-3) were studied. All cells were grown under recommended culture conditions provided by suppliers (see acknowledgements) and cell line identity was confirmed before use by karyotyping (data not shown). Cell line DNA was extracted using the Qiagen DNeasy kit (Qiagen, Crawley, UK).

Primary tumours and normal control samples

A cohort of 41 primary medulloblastomas was analysed including representatives of all the major histopathological subtypes (25 classic, six large cell/anaplastic and 10 nodular/desmoplastic) (Giangaspero et al, 2000). Patients (15 females and 26 males) ranged in age from 1.3 to 19 years (seven infants <3 years, 30 children 3–16 years and four adults >16 years). Six cerebellar samples were analysed consisting of post-mortem material from three fetuses (18-19- and 22-weeks gestational age), one infant (newborn) and two adults (60 and 67 years) who had died of non-neoplastic conditions. DNA was extracted from frozen tissues using standard methods and from formalin-fixed, paraffin-embedded tissue using a Nucleon hard tissue kit (Amersham Biosciences, Little Chalfont, UK). Local Ethical Committee and Institutional Review Board approval was obtained for the collection, storage and biological study of all material.

5′-Aza-2′-deoxycytidine (5-aza CdR) treatment

Four cell lines (DAOY, D283 Med, D425 Med and MEB-MED8A) were grown in the presence or absence of the demethylating agent 5-aza CdR (5 μM) (Sigma-Aldrich, Poole, UK) for 3 days. Medium was renewed daily.

Microarray analysis

Total RNA was extracted from cell lines D283 Med, D425 Med and MEB-MED8A, which had been grown in parallel cultures with or without 5-aza CdR treatment. These cell lines were selected for analysis as they exhibit characteristic chromosomal defects representative of primary medulloblastomas (Langdon et al, 2006). RNA was extracted from 10⁷ cells using Trizol reagent (Invitrogen, Paisley, UK) according to manufacturer’s instructions. Microarray expression analysis was performed at the Newcastle University microarray facility; RNA was converted into biotin-labelled cRNA.

Figure 1 Methylation-dependent regulation of S100 genes in medulloblastoma. (A) The S100 gene cluster at 1q21.3, showing the position (vertical lines) and direction of transcription (> or <) of each gene. Physical positions are based on NCBI Assembly 36 of the human genome (see http://www.ncbi.nlm.nih.gov/). Genes not belonging to the S100 family have been omitted for clarity. (B) Methylation-dependent changes in expression of 16 S100 genes in three medulloblastoma cell lines (MEB-MED8A, D283Med, D425Med), following treatment with the DNA methyltransferase inhibitor, 5-aza-CdR (5 μM, 72 h), determined by analysis using the Affymetrix U133A Human expression array (sequence identities of probes indicated were verified independently). Fold-changes in expression detected by each probe are shown, based on signal intensities assessed by MAS5 software (Affymetrix). Four genes (marked**) displayed evidence of positive methylation-dependent regulation in >2 cell lines; S100A2, S100A4, S100A6 and S100A10.
and hybridised to the Human U133A array according to manufacturer’s protocols (Affymetrix, Santa Clara, CA, USA). MAS5 software (Affymetrix) was used for data processing, normalisation and calculation of signal intensities. Signal intensities were compared between treatment conditions using Microsoft Excel software (Microsoft, Reading, UK).

Analysis of S100 gene methylation status

Bisulphite treatment of DNA was carried out using a CpG genome DNA modification kit (Serologicals, Livingston, UK) according to the manufacturer’s instructions. The promoter and exon1 regions of S100A2 and S100A6 and a region within the first intron of S100A4 were amplified following bisulphite treatment using previously published primers and conditions (Rosty et al, 2002; Rehman et al, 2005). The promoter region of S100A10 (Huang et al, 2003) was analysed using primers designed by ‘MethPrimer’ (Li and Dahiya, 2002) to amplify a region of the CpG island 400 – 652 bp relative to the transcriptional start site. Primer sequences were 5’-ATTATTTGTGGATTTTTGAGG-3’ and S100A10R 5’-ACAAAATATAAAATCCCTATTC-3’ (designed to amplify the antisense strand following bisulphite conversion). Thirty nanograms of bisulphite treated DNA was used per reaction. PCR products were amplified using standard conditions with an annealing temperature of 60°C. PCR products were directly sequenced with a CEQ DTCS kit (Beckman Coulter, High Wycombe, UK). Sequenced products were analysed on a CEQ 2000XL DNA analysis System (Beckman Coulter), and the methylation status at each CpG residue determined by assessment of the relative peak intensities.

Combined bisulphite and restriction analysis (COBRA (Xiong et al, 1997) of S100A4 was carried out by overnight digestion of the 142 bp PCR product at 37°C with the HpyCH4IV restriction endonuclease (New England Biolabs, Hitchin, UK), which has the recognition sequence 5’-ACGT-3’ and which cuts twice within the product if fully methylated before bisulphite conversion to give 100, 26 and 16 bp fragments. Digested PCR products were separated on a 4% Nusieve 3:1 agarose gel in 1 x TBE and stained with ethidium bromide, before visualisation by transillumination under ultraviolet (UV) light. The unmethylated control for COBRA and bisulphite analysis was a pool of 20 newborn cord blood DNA samples, the methylated control was universal methylated DNA (Serologicals Corporation, Livingston, UK). Representative examples of PCR products showing different digestion patterns were sequenced as described above, to determine relative peak intensities.

Reverse transcription PCR (RT – PCR)

RNA was extracted from 10^2 subconfluent cells using Trizol reagent (Invitrogen, Paisley, UK) according to manufacturer’s instructions. One microgram of total RNA was used to synthesise cDNA using a reverse transcription system (Promega, Southampton, UK). Equivalent amounts of this cDNA were used for PCR amplification of the respective S100 gene, RASSF1A and ACTB transcripts. RT – PCRs for S100A2, S100A4 and S100A6 were carried out using previously published primers and conditions (Rosty et al, 2002; Rehman et al, 2005). RT primers for S100A10 were designed using transcript information obtained from the Ensembl genome browser (www.ensembl.org: Gene ID, ENSG00000197747). Primer sequences were S100A10f (in exon 2) 5’-TTCCAAATTCGAGTTGGGATG-3’ and S100A10r (in exon 3) 5’-AACTGTGATCCTCTTGCTTCTATCTT-3’, which amplified a 264 bp product. PCR products were amplified using standard conditions with an annealing temperature of 54°C. RT – PCR of RASSF1A was used as a positive control and was carried out according to procedures published previously (Lusher et al, 2002).

RT – PCR of ACTB (encoding β-actin, a housekeeping gene) was used as a control for RNA concentration and integrity and was carried out using previously published primers and conditions (Horiikoshi et al, 1992). PCR products were electrophoresed on a 2% agarose gel in 1 x TBE and stained with ethidium bromide, before visualisation by transillumination under UV light.

RESULTS

Re-expression of S100 family members following demethylating treatment of medulloblastoma cell lines: identification of candidate epigenetically regulated genes

To identify S100 genes showing evidence of methylation-dependent transcriptional regulation in medulloblastoma, three medulloblastoma cell lines (D425Med, D283Med and MEB-MED8A) were cultured in the presence or absence of the demethylating agent, 5-aza CdR. Resultant expression changes were assessed using the Affymetrix Human U133A array. The U133A array contains probe sets which recognise 16 S100 genes (S100A1-S100A14, S100B and S100P), for which probe sequence identity and specificity could be verified using the BLAST version 2.0 sequence alignment tool (http://www.ncbi.nlm.nih.gov/BLAST/). Changes in probe signal intensity following 5-aza CdR treatment were calculated and genes showing an expression increase >2-fold were classed as being significantly upregulated, and thus showing evidence of methylation-dependent transcriptional regulation. Four independent S100 transcripts showed evidence of transcriptional upregulation in multiple cell lines (Figure 1B); S100A6 and S100A10 were upregulated in all three cell lines, while S100A2 and S100A4 were upregulated in two cell lines. In addition, S100A3, S100A11 and S100P were each upregulated in one cell line, and the remaining S100 genes were not significantly upregulated in any cell line.

The expression changes observed for S100A6, S100A10, S100A2 and S100A4 on array analysis were next validated in an independent series of experiments by RT – PCR (Figure 2A). The increases in expression observed by microarray for these genes following 5-aza CdR treatment were also clearly seen using RT – PCR methods, with good concordance between results obtained using the two techniques (Figure 2A). Reverse transcription – PCR analysis following demethylation treatment was also extended to a fourth medulloblastoma cell line, DAOY, which did not appear to show altered expression of any of the four S100 genes examined following 5-aza CdR treatment, despite clear evidence of re-expression of the RASSF1A control transcript.

The methylation status of S100A2, S100A4, S100A6 and S100A10 in medulloblastoma cell lines is associated with methylation-dependent transcriptional silencing

S100 genes with confirmed evidence of methylation-dependent upregulation in ≥2 medulloblastoma cell lines were selected for direct analysis of their DNA methylation status. The methylation status of CpG sites within the promoter/exon 1 regions of S100A6 and S100A2, which have previously been identified as being involved in their epigenetic regulation (Rehman et al, 2005), was established in a panel of nine medulloblastoma cell lines by bisulphite sequencing. Both genes were frequently methylated (in 7 out of 9 and 8 out of 9 lines, respectively). S100A6 displayed extensive methylation in D283 Med, D425 Med, MEB-MED8A, MHH-MED-1, D341 Med, D384 Med and D556 Med; the methylation of S100A6 in D283 Med, D425 Med, MEB-MED8A is in accordance with its observed methylation-dependent transcriptional silencing and re-activation by 5-aza CdR in these cell lines. In contrast, DAOY and UW228-3 were completely unmethylated, correlating with the higher level of expression and the lack of expression change following demethylating treatment, seen in...
DAOY (Figure 2A and D). S100A2 showed more variable methylation patterns, with methylation ranging from partial methylation at one site (DAOY) to complete methylation at all sites (D283 Med) (Figure 2D). However, the observed methylation patterns again correlated well with methylation-dependent transcriptional silencing (Figure 2A and D), except in the case of D425 Med, which is densely methylated and transcriptionally silenced, but does not undergo significant re-expression following 5azaCdR treatment, suggesting that methylation-independent mechanisms are involved in its silencing.

Methylation analysis of S100A10 has not been performed previously; however, the promoter and associated CpG island have been characterised. (Huang et al., 2003) Primers were designed using MethPrimer (Li and Dahiya, 2002) to assess the methylation status of a region of the S100A10 promoter-associated CpG island (400–652 bp relative to the transcriptional start) site by bisulphite sequencing (see Figure 2B and D). This region was methylated at multiple CpG residues in 7 out of 9 cell lines, including all three cell lines, which showed evidence of transcriptional silencing and methylation-dependent upregulation of S100A10 following treatment with 5-aza CdR (D283 Med, D425 Med, MEB-MED8A), but was not methylated in DAOY, which showed higher endogenous expression levels and no clear change in expression following demethylating treatment (Figure 2A), indicating that methylation of this region is involved in the transcriptional silencing and epigenetic regulation of S100A10.

S100A4 does not contain a promoter-associated CpG island, but has been shown to be epigenetically regulated by methylation of critical intragenic CpG sites (Rosty et al., 2002). We therefore assessed the methylation status of two CpG sites within its first intron, whose methylation has been previously correlated with expression (Rosty et al., 2002). Methylation was assessed by COBRA (Figure 1C), with representative examples (n = 3) of each digestion pattern confirmed by bisulphite sequencing. These two CpG sites were fully methylated in 6 out of 9 cell lines, and their methylation status correlated well with the expression patterns observed; reduced expression and 5-aza CdR-associated upregulation was seen in methylated cell lines (D283 Med, D425 Med), but not in unmethylated cell lines (MEB-MED8A, DAOY) (Figure 1; Figure 2A and D). These data were reinforced by extension of RT–PCR expression analysis of S100A4 to two further cell lines (MHH-MED1, D556 Med), which produced the anticipated expression patterns; unmethylated MHH-MED1 showed higher endogenous expression and no evidence of methylation-dependent regulation, whereas the methylated D556 Med cell line displayed transcriptional silencing and methylation-dependent upregulation following 5-aza CdR treatment (data not shown).

Methylation status of S100 genes in the normal cerebellum
Gene-specific methylation can be a feature of normal somatic tissues (Strathdee et al., 2004). It is therefore crucial to establish the
methylation status of candidate genes in normal non-neoplastic tissues before any role for aberrant methylation patterns in tumourigenesis can be assessed. Six normal cerebellar samples, comprising fetal, infant and adult examples were therefore assessed for the methylation status of all four S100 genes under investigation (Figure 3). For S100A4, S100A6 and S100A10, consistent methylation patterns were observed across all samples. S100A6 and S100A10 were somatically unmethylated, with no evidence of methylation of either promoter-associated CpG island observed, with the exception of one sample which showed minimal methylation (<25%) at 2 out of 13 sites in the S100A10 CpG island. In contrast, S100A4 was somatically methylated, showing evidence of high-level methylation at both CpG residues assessed in all normal cerebellar samples. S100A2 also showed evidence of methylation in the normal cerebellum, although the methylation patterns detected were variable between samples, both in terms of the number of methylated CpG residues and the extent of methylation observed at these residues.

**Aberrant methylation patterns identify roles for S100 genes in medulloblastoma pathogenesis**

S100 gene methylation patterns were next investigated in a series of primary medulloblastomas, and, together with patterns previously observed for medulloblastoma cell lines (Figure 2), were assessed against the normal methylation patterns observed for each gene in the cerebellum, for evidence of altered methylation states. In the majority of cases, methylation patterns observed in medulloblastomas corresponded to the methylation status of the normal cerebellum (Figure 3). For S100A6 and S100A10, the majority of medulloblastomas were unmethylated (35 out of 40 and 31 out of 35 of analysable samples, 87.5 and 88.3% respectively); however five tumours (12.5%) and seven cell lines (78%) showed evidence of hypermethylation of the S100A6 promoter relative to the normal cerebellum. S100A10 was hypermethylated in four tumours (11.4%) and seven cell lines (78%), relative to the normal cerebellum. Direct analysis of expression in tumours was not possible, as RNA was not available for the majority of the cohort; however, the hypermethylation of S100A10 observed in primary tumours is of a comparable level to that seen in the cell lines, consistent with its epigenetic transcriptional inactivation and candidate tumour suppressor role in disease pathogenesis (Figures 2A, D and 3). The hypermethylation observed for S100A6 was less pronounced in primary tumours (affecting a limited number of CpG residues) than cell lines and further work is needed to determine whether this level of methylation leads to transcriptional silencing.

In contrast, all medulloblastomas showed some evidence of methylation of the CpG regions assessed in S100A4 and S100A2. Whereas the normal cerebellum and the majority of primary medulloblastomas (34 out of 41) and medulloblastoma cell lines (6 out of 9) demonstrated complete methylation (>75%) of the S100A4 intronic CpG sites analysed, a subset of primary tumours (17%; 7 out of 41) and cell lines (33%; 3 out of 9) were undermethylated in comparison, displaying either partial methylation or no evidence of methylation at each site (Figures 2D and 3). Considered alongside the methylation-dependent transcriptional silencing observed for S100A4 in medulloblastoma cell lines (Figure 2A), these findings support (i) the somatic hypermethylation-associated transcriptional silencing of S100A4 in the normal cerebellum, and (ii) tumour-specific hypomethylation of S100A4 in a subset of medulloblastomas, consistent with elevated expression and a pro-tumourigenic role.

Patterns of methylation observed for S100A2 were complex; evidence of methylation was observed in all primary tumours tested; however, considerable variation was seen in both the number of CpG residues affected and the extent of methylation observed at individual CpG residues (Figure 3). A comparable incidence and variability of patterns of methylation were also detected in medulloblastoma cell lines and the normal cerebellum, and our data do not therefore provide any clear evidence to indicate the existence of aberrant tumour-specific patterns of S100A2 methylation in medulloblastomas (Figures 2D and 3). In view of the methylation-dependent transcriptional silencing associated with a range of S100A2 methylation states in medulloblastoma cell lines (Figure 2A and D), our data suggest that S100A2 methylation in the normal cerebellum is associated with its somatic epigenetic transcriptional silencing, but more detailed investigations are now required to explore any role for the epigenetic deregulation of S100A2 in medulloblastoma.

**Clinicopathological significance of S100 methylation events in medulloblastomas**

Tumour-specific methylation patterns detected for the S100A4, S100A6 and S100A10 genes were next assessed against basic

![Figure 3](Image 557x751 to 572x766)
clinical and pathological information available for all tumours (age, sex, histopathological subtype), to make a preliminary assessment of any clinicopathological significance in disease development. S100A6 methylation showed a statistically significant association with the large cell/anaplastic histopathological morphotype (50%; 3 out of 6) of large cell/anaplastic tumours vs 8% (2 out of 25) of classic and 0% (0 out of 9) of nodular/desmoplastic tumours; P = 0.026 by Fisher’s exact test). No further significant associations were found for any gene.

**DISCUSSION**

This study demonstrates a role for the epigenetic deregulation of three members of the S100 gene family, S100A4, S100A6 and S100A10, in medulloblastoma tumourigenesis. Furthermore, our data provide evidence for the control of S100 gene expression in the normal cerebellum by gene-specific somatic methylation events, and highlight the importance of assessing epigenetic events in tumour development in the context of normal tissue-specific methylation patterns.

Epigenetic deregulation of gene expression plays an important role in the development of medulloblastoma, and studies of DNA methylation have identified a series of putative tumour suppressor genes for this disease; however, analysis has so far been restricted to a limited number of candidate genes (Lindsey et al., 2005). In this study, we therefore used a pharmacological expression reactivation approach, to allow a more global analysis of gene expression changes using expression microarrays. Using this analysis to assess members of the S100 gene family for evidence of methylation-dependent regulation, we have successfully identified four S100 family members which showed upregulation in multiple cell lines following demethylation treatment, and were subsequently shown to be associated with specific DNA methylation events. Our findings thus strongly support the value of this experimental approach for the identification of novel methylated genes relevant to both normal development and oncogenesis, and provide important precedent for the epigenetic deregulation of S100 gene expression in medulloblastoma. Moreover, the role of methylation events in the regulation of S100 gene expression in the normal and malignant cerebellum may yet be more extensive than currently recognised. Our investigations of S100 gene DNA methylation status were limited to four genes that displayed evidence of methylation-dependent regulation in multiple cell lines. Additional S100 genes either (i) showed evidence of methylation-dependent regulation in a more limited number of cell lines (<2; S100A3, S100A11, S100P and S100G), and were therefore not investigated further in the present study, or (ii) were not represented on the U133A expression microarray (e.g., S100A15), suggesting that more systematic investigations are now required to investigate any roles for DNA methylation events in the epigenetic regulation of further S100 family members in medulloblastoma.

Two of the genes identified by this approach, S100A6 and S100A10, were each hypermethylated in a proportion of both medulloblastoma cell lines and primary tumours (each in ~12% of cases), but not in the normal cerebellum. The higher frequency of hypermethylation observed for both genes in cell lines than tumours may reflect either a limited representation of the clinical and pathological diversity of primary medulloblastomas by these cell lines (Langdon et al., 2006), or an involvement of culture-related de novo methylation events (Jones et al., 1990; Pantoja et al., 2005). Nonetheless, for both genes a significant frequency of tumour-specific methylation was observed in primary tumours, which was associated with transcriptional silencing in medulloblastoma cell lines, indicating epigenetic inactivation and candidate tumour suppressor roles for S100A6 and S100A10 in primary medulloblastoma development. S100A6 has been shown to be expressed in a cell type-specific manner in the brain, with expression present in subsets of neurons including granule cells of the cerebellum (Filipke et al., 1993). Consistent with this observation, the S100A6 promoter was unmethylated in the normal cerebellum in the present study. In other tumour types, S100A6 has also been reported to be hypermethylated in prostate cancer (Rehman et al., 2005). S100A10 is also expressed in the brain (Zimmer et al., 2005). To our knowledge, the present study is the first to demonstrate that S100A10 expression can be deregulated by methylation of its promoter-associated CpG island in tumourigenesis. The finding that S100A10 was hypermethylated in medulloblastoma cell lines and tumours was unexpected, as its overexpression has previously been associated with plasminogen-degrading activity in cellular and/or secreted matrix metalloproteases (reviewed in Helfman et al., 2005). Previously we have shown evidence of partial methylation of CASP8, HIC1 and EDNRB (Lindsey et al., 2004) in non-neoplastic cerebella. As less than 50 genes have been analysed for methylation in medulloblastoma and the normal cerebellum (Lindsey et al., 2005); this raises the possibility that somatic methylation in the normal cerebellum may be a feature of a significant number of genes, although some may be restricted to specific cell types within the cerebellum. In addition to cell type-specific methylation (Lesniak et al., 2000; Rosty et al., 2002; Sato et al., 2004), members of the S100 gene family have been shown to undergo temporal methylation changes during development, for example S100β, becomes demethylated during astrocyte development in the mouse brain correlating with the time when its expression commences at E14 (Komisar et al., 2004). This raises the possibility that aberrant methylation in embryonal tumours such as medulloblastoma may reflect a failure of developmentally regulated methylation changes. The identification of normal tissue-specific methylation in the non-neoplastic cerebellum emphasizes the importance of including relevant somatic tissue controls in medulloblastoma methylation studies (Lindsey et al., 2004; Lindsey et al., 2005).

S100A4 showed complete methylation of the intronic CpG sites analysed in all normal cerebellar samples, consistent with its epigenetic transcriptional silencing (see above). S100A4 has been previously shown to be methylated in other normal tissues, including pancreatic tissue (Rosty et al., 2002). Three medulloblastoma cell lines were unmethylated for S100A4, and were associated with higher gene expression unaffected by demethylation treatment (in contrast to methylated lines, which were associated with methylation-dependent transcriptional silencing), suggesting that they had undergone selection for hypomethylation and overexpression of S100A4 during the tumourigenic process. No tumour samples were completely unmethylated at S100A4; however, 7 out of 41 (17%) of tumours displayed reduced levels of methylation compared to the normal cerebellum. S100A4 hypermethylation is also a feature of other cancers, including pancreatic and colon adenocarcinoma (Nakamura and Takenaga, 1998; Rosty et al., 2002). S100A4 promotes angiogenesis and extracellular matrix degradation through its upregulation of specific matrix metalloproteases (reviewed in Helfman et al., 2005) and high levels
of S100A4 expression are associated with metastatic progression in a wide range of cancers including medulloblastoma (Hernan et al., 2003). Current findings are therefore consistent with a role for the oncogenic activation of S100A4 by hypomethylation-associated expression upregulation in medulloblastoma tumourigenesis.

S100A2 has been postulated in certain studies to play a suppressor role in tumour development, as it shows reduced expression in a range of cancer types, while other investigators have been unable to show any clear difference in methylation patterns between tumours and normal tissue (Rehman et al., 2005). S100A2 methylation was detected in all normal cerebella, cell line and tumour samples tested; however, significant variability in methylation patterns and levels were observed in all sample groups. While these methylation patterns were associated with transcriptional silencing in medulloblastoma cell lines, suggesting that S100A2 is epigenetically silenced by hypermethylation in the normal cerebellum, no clear evidence of aberrant tumour-specific epigenetic regulation in medulloblastoma development was found, and more detailed examination of extended tumour cohorts are now required to further discern any role in medulloblastoma.

The majority of the S100 genes, including S100A2, S100A4, S100A6 and S100A10 are organised in a cluster on chromosome 1q21.3 (Figure 1). Recent studies have provided precedent for epigenetic silencing across an entire chromosome band in colorectal cancer (Frigola et al., 2006); however, this does not appear to be the case for the S100 gene cluster in medulloblastoma. Microarray analysis of S100 gene expression changes following demethylation treatment of medulloblastoma cell lines (Figure 1) shows that there is no positional clustering of upregulated genes and the expression of many genes in the cluster remains unchanged. Furthermore, analysis of DNA methylation changes shows that some medulloblastoma cell lines (e.g., MHH-MED1, MEB-MED8A) show both hypermethylation (e.g., of S100A6) and hypomethylation (e.g., of S100A4) of genes in close physical proximity, suggesting that epigenetic remodelling appears to be localised within the S100 cluster, involving gene-specific hypermethylation and hypomethylation events. Furthermore, our findings indicate that S100 gene methylation patterns may have clinical significance and potential utility as medulloblastoma biomarkers. Based on an initial analysis in our limited cohort, methylation of S100A6 was significantly associated with the more aggressive large cell/anaplastic histopathological variant (Giangaspero et al., 2000; McManamy et al., 2003), and investigations in large uniformly treated clinical trials cohorts are now required to investigate these relationships further.

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