Quantitative, genome-wide analysis of the DNA methylome in sporadic pituitary adenomas

Cuong V Duong, Richard D Emes¹, Frank Wessely¹,
Kiren Yacqub-Usman, Richard N Clayton and William E Farrell

Institute of Science and Technology in Medicine, Keele University School of Medicine, Stoke-on-Trent, Staffordshire ST4 7QB. UK

¹School of Veterinary Medicine and Science, University of Nottingham, Sutton Bonington Campus, College Road, Sutton Bonington, Leicestershire LE12 5RD. UK

(Correspondence should be addressed to W E Farrell; Email: w.e.farrell@keele.ac.uk)

Abstract

DNA methylation is one of the several epigenetic modifications that together with genetic aberrations are hallmarks of tumorigenesis including those emanating from the pituitary gland. In this study, we examined DNA methylation across 27 578 CpG sites spanning more than 14 000 genes in the major pituitary adenoma subtypes. Genome-wide changes were first determined in a discovery cohort comprising non-functioning (NF), growth hormone (GH), prolactin (PRL)-secreting and corticotroph (CT) adenoma relative to post-mortem pituitaries. Using stringent cut-off criteria, we validated increased methylation by pyrosequencing in 12 of 16 (75%) genes. Overall, these criteria identified 40 genes in NF, 21 in GH, six in PRL and two in CT that were differentially methylated relative to controls. In a larger independent cohort of adenomas, for genes in which hypermethylation had been validated, different frequencies of hypermethylation were apparent, where the *KIAA1822* (*HHIPL1*) and *TFAP2E* genes were hypermethylated in 12 of 13 NF adenomas whereas the *COL1A2* gene showed an increase in two of 13 adenomas. For genes showing differential methylation across and between adenoma subtypes, pyrosequencing confirmed these findings. In three of 12 genes investigated, an inverse relationship between methylation and transcript expression was observed where increased methylation of *EML2*, *RHOD* and *HOXB1* is associated with significantly reduced transcript expression. This study provides the first genome-wide survey of adenoma, subtype-specific epigenomic changes and will prove useful for identification of biomarkers that perhaps predict or characterise growth patterns. The functional characterisation of identified genes will also provide insight of tumour aetiology and identification of new therapeutic targets.

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Introduction

The genesis and outgrowth of sporadic pituitary adenomas are, in common with most other tumour types, characterised by inappropriate expression of hormone and growth factor receptors, mediators in their associated signal transduction pathways, transcription factors and cell cycle regulators (Melmed 2003, Dudley *et al.* 2009). The progenitor cells that give rise to each of the differentiated cell types within this gland are also the precursor population of their cognate subtype-specific adenomas (Melmed 2003, 2011).

Although important exceptions exist, genetic aberrations leading to inappropriate expression, activation or silencing of regulatory genes are infrequent in pituitary adenomas (Vandeva *et al.* 2010). However, in these tumours, multiple studies have described epigenetic change within gene-associated CpG islands and/or those that lead to modification of histone tails (Ezzat 2008, Yacqub-Usman *et al.* 2012b). In these cases, inappropriate methylation of CpG islands is frequently associated with gene silencing whereas particular combinatorial modifications to histone tails are

associated with either silenced or expressed genes (Dudley *et al.* 2009, Yacub-Usman *et al.* 2012b).

In common with specific genetic aberrations, which frequently show subtype specificity, epigenetic modification termed epimutations also show a degree of specificity (Simpson *et al.* 2000, Zhang *et al.* 2002, Zhao *et al.* 2005). As an example, subtype-specific genetic changes are apparent for the *gsp* oncogene and are also apparent for inappropriate expression of HMGA2 in somatotroph- and lactotroph-derived adenomas respectively (Farrell & Clayton 2000, Hayward *et al.* 2001, Fedele *et al.* 2006). For specific epimutations, methylation-mediated silencing of the *MEG3* gene is a frequent finding in pituitary adenomas of gonadotroph origin whereas methylation-mediated silencing of the *p16* gene (*CDKN2A*) is infrequent in somatotrophinomas but is a common finding in most other pituitary adenoma subtypes (Simpson *et al.* 1999, Zhao *et al.* 2005). Equally, for epigenetic modifications that are manifest as histone tail modification, a degree of subtype specificity is also apparent. In this case, and in a recent report, we showed specific histone modifications to the *BMP4* gene that are indicative of either gene silencing or modifications that are permissive for expression and furthermore that these modifications show adenoma subtype specificity (Yacub-Usman *et al.* 2012a).

For epimutations that characterise pituitary adenomas, their identification, in most cases, has been through candidate gene approaches (Dudley *et al.* 2009). Less frequently, for identification of novel genes, investigators have used differential display techniques. These approaches have employed either tumour-derived cDNA or DNA that has been subject to prior methylation-sensitive digestion respectively (Bahar *et al.* 2004, Zhao *et al.* 2005). Although these techniques have identified novel genes, a limitation is that identification is frequently confined to a single transcript or gene, that is, on a gene-by-gene basis. Relatively few studies have adopted genome-wide investigation. In cases where these approaches have been used, they have relied upon siRNA knock-down or pharmacological unmasking strategies (Dudley *et al.* 2008, Al-Azzawi *et al.* 2011). However, a current limitation of these techniques is that they are reliant upon actively dividing cells for the effective reversal and/or erasure of epimutations. Moreover, primary pituitary adenomas show limited proliferative potential *in vitro* and investigations are, therefore, reliant upon pituitary adenoma cell lines of mouse or rat origin. While these approaches have uncovered novel genes, they are constrained by the issue of extrapolation across species boundaries (Dudley *et al.* 2008).

Recent technological advances in high-throughput array-based techniques now provide opportunity for the comprehensive genome-wide investigation of DNA methylation in normal and disease states (Irizarry *et al.* 2008, Fryer *et al.* 2011, Wilop *et al.* 2011). For pituitary adenomas, these high-throughput techniques have the potential to not only increase our understanding of the molecular biology of this tumour type but also to identify biomarker for diagnosis, prognosis and for therapeutic intervention. Therefore, and as a first step towards these outcomes, we analysed the methylation profile of 27 578 CpG sites spanning more than 14 000 genes in each of the major pituitary adenoma subtypes relative to post-mortem normal pituitary.

Materials and methods

Human tissue sample

The primary sporadic human pituitary tumours used in this study comprised a discovery and an independent cohort. The discovery cohort comprised three each of the major adenoma subtypes. Tumours in the discovery and independent cohort were classified and graded as we described previously (Yacub-Usman *et al.* 2012a). The investigation cohort comprised seven GH-secreting tumours all of which were grade 2 macroadenomas; 6 corticotrophinomas (CT), four of which were grade 1 microadenomas and two were grade 2 macroadenomas; 6 prolactinomas (PRL) all of which were grade 1 microadenomas and 13 non-functioning (NF) adenomas all of which were grade 3 macroadenomas. Adenoma subtype classifications were on the basis of staining for mature hormone (GH, ACTH, FSH, LH and prolactin but not for α -subunit) as described previously (Yacub-Usman *et al.* 2012a). As tumours were not stained for α -subunit, it was not possible to detect subunit producing gonadotrophinomas or null-cell adenomas; however, none stained for mature LH or FSH and were therefore classified as NF adenomas. As controls, we used four post-mortem normal pituitaries, acquired within 12 h of deaths with no evidence of any endocrine disease. Primary human tissues were stored at -80°C before their use. Tumour tissues were obtained with informed consent and all studies performed with Regional Ethics Committee (South Birmingham Committee: REC reference number: 10/H1207/406) and institutional approval.

Only those adenomas in which tumour cells comprised $\geq 80\%$ of the specimen, as determined at surgery and confirmed by neuropathological assessment, were used in the study. Before the described

extraction protocols, tumours and normal pituitaries were freeze fractured using a biopulveriser (Biospec, Bartlesville, OK, USA) to achieve a homogenous mixture of cells.

DNA extraction and bisulphite modification

High-molecular weight DNA was extracted from adenoma tissue and post-mortem pituitaries (pmPits) using a standard phenol–chloroform extraction procedure. DNA was quantified using a NanoDrop 1000 (Thermo Scientific, Wilmington, NC, USA) and diluted in molecular biology grade water (Sigma). Bisulphite modification of 1 µg DNA was performed using either the EpiTect Plus DNA bisulphite kit (Qiagen) or Zymo EZ DNA Methylation Gold kit (Zymo Research, Cambridge, UK) as described previously (Al-Azzawi *et al.* 2011, Fryer *et al.* 2011). In some cases, where we used whole genome amplification (WGA) of bisulphite-converted DNA, this was performed as described by Mill *et al.* (2006).

Illumina BeadArray analysis

For the discovery cohort, DNA was extracted from three of each of the major pituitary adenoma subtypes (NF, GH, PRL and CT) and from three pmPits. Total DNA (500 ng) from each of the samples was sodium bisulphite converted as described earlier. After conversion, DNA was eluted in 12 µl elution buffer and 4 µl converted DNA was used as template on the Infinium Methylation 27K Arrays (Illumina, San Diego, CA, USA) and was processed as per the manufacturer's instruction as described previously (Fryer *et al.* 2011). This array examines the methylation status of 27 578 CpG sites across 14 496 genes. Data were collected using the Illumina BeadArray (Illumina) reader and analysed with GenomeStudio V2009.1 methylation module 1.1.1 (Illumina). This assigns a ' β value' that is a quantitative measure of methylation for each CpG site and ranges from 0 (no methylation) to 1.0 (100% methylation of both alleles).

Of the 27 578 sites on the array, and as we described previously, we removed all probes targeted on X and Y chromosomes ($n=1092$) and this resulted in 26 486 probes (Fryer *et al.* 2011). To reduce the number of non-variable sites from subsequent analyses, probes where β values in all samples were ≥ 0.8 or ≤ 0.2 and as described previously by us and other groups (Byun *et al.* 2009, Fryer *et al.* 2011) were excluded ($n=13 945$) from the analysis and this reduced the probe count to 12 541. From this dataset, we also eliminated all sites where one or more of the samples demonstrated i) detection P values of > 0.05 (internal

quality control) or ii) null (missing) β values. We took the decision to remove probes that failed in any one of the samples rather than excluding probe values for individual tumour samples as we deemed this a more robust way to identify probes that varied between tumours in a limited number of samples. This resulted in a final dataset of 10 667 probes for further analysis.

Technical validation of array-generated data

For the technical validation of the BeadArray, four single CpGs (cg08047457, cg20289949, cg06197492 and cg24739326) were selected that had been interrogated on the array and that demonstrated a range of β values across the tumour samples. The tumour samples, post-sodium bisulphite conversion, were subject to pyrosequence analysis (as described in a subsequent section). Primer sequences are provided in supplemental data (Table S1, see section on supplementary data given at the end of this article).

DNA pyrosequencing analysis of sodium bisulphite-converted DNA

The pyrosequencing experiments were first performed on the discovery adenoma cohort for the validation of the BeadArray-generated data. Subsequent experiments were performed on the larger investigation cohort of adenoma. The dataset initially used in this study comprised genes that showed an increase in β value of ≥ 0.4 in two or more adenomas within a subtype (as described in the Results section) relative to the mean β values of the pmPits. From the BeadArray dataset, we first selected 22 genes at random (Table 1). For each of these genes, their promoter region-associated CpG islands were identified and downloaded from the UCSC Genome Browser (<http://genome.ucsc.edu/>) and imported into PyroMark Assay Design 2.0 Software for primer design of sodium bisulphite-converted DNA (Qiagen, Crawley, UK). Dependent on the specific gene and the density of CpGs within their promoter region, the amplicons encompassed between 5 and 11 CpGs (Table S1, supplementary data).

In these cases, 2 µl sodium bisulphite-converted and WGA DNA were used as template in the first round of a nested PCR reaction. The product of the first round was diluted 100-fold in dH₂O and 2 µl used as template in a second round nested PCR reaction using the same cycling condition. After initial denaturation at 98 °C for 10 min, we employed touch-down cycling for 14 cycles in which each cycle was touched down at 0.5 °C. The subsequent 35 amplification cycles include denaturing at 98 °C for 30 s, annealing at 55 °C for

Table 1 Identity of 22 randomly selected genes from the BeadArray dataset. 16 of the genes were selected on the basis that they had two CpG within the gene (in at least two of three specimens of an adenoma subtype) with a β value of ≥ 0.4 in one of two CpGs and ≥ 0.25 in the second, relative to the values seen in pmPits. 6 of the genes were selected on the basis that they had a single CpG within the gene (in at least two of three specimens of an adenoma subtype) with a β value of ≥ 0.4 in one CpG and ≤ 0.25 in the second, relative to the values seen in pmPits. Genes where pyrosequencing validated their methylation status are shown in bold

First CpG β value	Second CpG β value	Gene
≥ 0.4	≥ 0.25	EML2
≥ 0.4	≥ 0.25	RHOD
≥ 0.4	≥ 0.25	HOXB1
≥ 0.4	≥ 0.25	FLJ46380 (RNF207)
≥ 0.4	≥ 0.25	MT1G
≥ 0.4	≥ 0.25	HAAO
≥ 0.4	≥ 0.25	KIAA1822 (HHIPL1)
≥ 0.4	≥ 0.25	KCNE3
≥ 0.4	≥ 0.25	TFAP2E
≥ 0.4	≥ 0.25	BCL9L
≥ 0.4	≥ 0.25	PGLYRP1
≥ 0.4	≥ 0.25	COL1A2
≥ 0.4	≥ 0.25	ST6GALNAC6
≥ 0.4	≥ 0.25	UBTD1
≥ 0.4	≥ 0.25	KIAA0676 (TBC1D9B)
≥ 0.4	≥ 0.25	PDLIM4
≥ 0.4	≤ 0.25	CXCL14
≥ 0.4	≤ 0.25	FEM1C
≥ 0.4	≤ 0.25	MAL
≥ 0.4	≤ 0.25	RAMP1
≥ 0.4	≤ 0.25	D4ST1 (CHST14)
≥ 0.4	≤ 0.25	SAMD11

30 s and elongation at 72 °C for 30 s. Additional elongation was set at 72 °C for 10 min.

The capture of the single-stranded and biotin-labelled DNA was carried out using a Pyromark Q96 ‘Vacuum Prep’ workstation as recommended by the manufacturer and pyrosequencing was performed using a PSQ96A Pyrosequencer using Pyro Q CpG Software (version 1.0.9; Qiagen). For each set of independent bisulphite modification reactions, a control DNA sample was run to ensure efficient sodium bisulphite-mediated conversion. The pyrosequencing data from the control DNA sample were used to standardise levels across experimental runs.

Quantitative RT-PCR

Total RNA was extracted from primary human tumour tissue and pmPits, as described previously (Bahar et al. 2004). cDNA was synthesised using 200 U M-MLV reverse transcriptase (Promega) according to the manufacturer’s instructions and as described

previously (Bahar et al. 2004). The primer sequences used for real-time quantification are shown in Table S1 (supplementary data) using conditions described previously (Al-Azzawi et al. 2011). The target genes were normalised to an endogenous control and relative quantification was carried out using relative standard curve and $2^{-\Delta\Delta}$ cycle thresholds (CT) methods, where $-\Delta\Delta CT = CT^{(\text{gene of interest of tumour} - GAPDH \text{ of tumour})} - CT^{(\text{gene of interest of normal pituitary} - GAPDH \text{ of normal pituitary})}$.

Loss or significantly reduced transcript expression in individual adenomas was assigned using the following criteria: we first determined the mean and s.d. of expression in four normal pituitaries. We next set limits of four times that s.d., where expression in individual adenomas at or less than the lower limit was deemed loss or significantly reduced.

Results

Methylation profiling of sporadic pituitary adenoma

To evaluate whether sporadic human pituitary tumours display subtype-specific methylation profiles, we used Illumina Infinium BeadArray technology to determine the methylation status of 27 578 CpG sites that span more than 14 000 genes. The discovery cohort comprised adenomas that represented each of the major pituitary adenoma subtypes, non-functioning (NF), somatotrophinoma (GH), prolactinoma (PR) and corticotrophinoma (CT). Quantitative differential methylation was assessed relative to post-mortem normal pituitaries.

Before determining differential methylation, we first excluded probes in chromosomes X and Y and low-quality probes (detection probability > 0.05). In addition, we removed individual probes where the β values were either ≤ 0.2 or ≥ 0.8 across the 15 samples as we and others have previously described (Byun et al. 2009, Fryer et al. 2011). Through application of these criteria, a total of 10 667 probes spanning 7956 genes were included in the analysis. Within this probe set, 5287 CpG were within and 5280 were outside of CpG islands. Analysis of the CpG methylation profiles across individual CpG sites showed these to be different between adenoma subtypes; however, individual adenomas, within a subtype, showed similar methylation profiles to each other (data not shown). A Kolmogorov–Smirnov test was used to compare the distribution of β values between samples using the NIMBL Software (Wessely & Emes 2012 (Roche NimbleGen, Madison, WI, USA)). A single pmPit sample was identified as having a significantly different ($P < 0.05$) distribution to the

other samples and was excluded from further analysis. The remaining pmPits showed different methylation profiles to those apparent in each of the adenoma subtypes (data not shown).

To characterise the differential methylation profiles of the adenoma subtypes, we first performed a class comparison between each of the adenoma subtypes and relative to that apparent in the pmPits. For the comparison, we initially filtered for a minimum increase of $\Delta\beta$ of 0.4 relative to pmPits at single gene-specific CpG sites in at least two of three subtype-specific adenomas. This analysis identified 326 genes in the NF adenomas, 49 in PRL, 97 in GH and 4 in CT that were hypermethylated relative to pmPits supplemental data (Table S2, see section on [supplementary data](#) given at the end of this article). In the majority of cases and irrespective of subtype, the CpGs were within CpG islands.

Technical validation of BeadArray by pyrosequencing

To validate the BeadArray-generated data, we performed pyrosequence analysis of 48 CpG sites, in four gene-associated CpG islands from the 12 samples (discovery cohort) that had been interrogated on the array. The analysis (Fig. 1) for the quantitative measurement of CpG methylation showed the two techniques to be significantly correlated (Spearman's r , 0.85; $P \leq 0.0001$). Importantly, the correlation involves samples across the range of β values and hence is unlikely to be an artefact due to dominance of extreme values (Roessler *et al.* 2012). In addition, inspection of the 20 imprinted genes interrogated on the array showed a mean β value of 0.51 ± 0.1 across a total of 40 CpG sites. These β values are consistent with monoallelic methylation apparent in most normal tissues including pituitaries and providing further confidence in the methodological approach.

Figure 1 Correlation between BeadArray-derived β values (expressed as a percentage) and pyrosequencing across a range on β values from 12 adenomas for four CpGs (cg08047457, cg20289949, cg06197492 and cg24739326). Spearman's r , 0.85; $P \leq 0.0001$.

CpG island methylation profiles in primary adenoma subtypes

To determine the relationship between methylation at array-interrogated CpG sites and methylation across the gene-associated CpG islands, we used pyrosequence analysis that encompassed, in each case, between 5 and 11 CpGs. We initially selected 22 genes on the basis that they each showed a $\Delta\beta$ of ≥ 0.4 (in at least two of the three NF adenomas) relative to pmPits and where the array-interrogated CpG are within promoter-associated CpG islands (Table 1). The analysis was initially performed in the discovery cohort of adenomas and relative to four pmPits. Mean methylation across these CpGs, as determined by pyrosequencing, was able to confirm the BeadArray methylation status for only 12 of the 22 genes (55%). However, we noted that where concordance between the techniques is observed (12 of 22 genes), it was for genes that were represented by more than one island-associated CpG on the array, and where the $\Delta\beta$ for the second CpG also showed increase. For the 22 genes that we had selected for pyrosequence analysis, on the basis of a single CpG site, 16 genes also showed increase in a second CpG site ($\beta \geq 0.25$), in at least two of the three NF adenomas that had also been interrogated on the array (Table 1). Of these 16 array-identified genes, pyrosequence analysis confirmed increase in mean methylation (across 5 to 11 CpG sites) for 12 of 16 (75%) genes in the discovery cohort of NF adenomas.

On the basis of the finding described earlier, we modified our filter criteria to include genes where one of the two CpGs showed a $\Delta\beta$ of ≥ 0.4 and a second CpG a $\Delta\beta$ of ≥ 0.25 and where these criteria were fulfilled in at least two of the three adenomas (within a subtype) interrogated on the array. The revised, more stringent, filtering criteria identified 40 genes in NF adenomas, 21 in GH, 6 in PRL and 2 in CT that showed increase in methylation-relative pmPit (Table 2). Table 2 also shows genes that are methylated in common across adenoma subtypes and those that segregate with one or more pituitary adenoma subtypes. In addition, and for two of the adenoma subtypes, NF and GH, where these criteria were met in multiple genes, we generated a heatmap displaying β values across individual adenomas within these subtypes (Fig. 2).

Gene-specific methylation frequencies in NF adenomas

Through constraints imposed by methodologies (sodium bisulphite conversion, pyrosequencing and transcript expression analyses), the availability of some adenoma subtypes (number and amount of

Table 2 Genes identified on the BeadArray as hypermethylated on the basis of one of two CpGs (in two of three adenomas within a subtype) showing a β value of ≥ 0.4 and a second CpG showed a $\beta \geq 0.25$ relative to the mean of the pmPits. The table shows the genes fulfilling these criteria in each of adenoma subtypes and where the CpG is within a CpG island. Genes shown in bold and underlined were subject to the additional analyses described in Fig. 3 and Table 3

Gene	GH	NF	PRL	CUSH
SEPT9				
ACTA1				
ADRA1A				
ALX4				
BCL9L				
BIK				
BMP8A				
C1QTNF5				
C6orf150 (Mb21d1)				
CHST8				
CNFN				
COL1A2				
CRIP1				
DLX5				
EFEMP1				
EFS				
ELN				
EML2				
ENTPD2				
ERBB2				
FLJ32569 (PM20D1)				
FLJ46380 (RNF207)				
FLJ90166 (APCDD1L)				
HAAO				
HAST				
HDAC11				
HOXB1				
KCNE3				
KCNQ1				
KIAA0676 (TBC1D9B)				
KIAA1822 (HHIPL1)				
LDHC				
MT1G				
NPPB				
PDLIM4				
PGLYRP1				
PON3				
RAB34				
RAC2				
RASSF1				
RHOD				
SECTM1				
SIPA1				
SLC5A1				
SLITL2 (VASN)				
SOCS1				
SOCS2				
ST6GALNAC6				
TCF15				
TFAP2E				
TNFRSF10D				
UBTD1				
VILL				
WFIKKN2				
Total	21	40	6	2

tumour tissue) with the exception of NF adenomas was limited. We, therefore, focused this part of the study primarily, but not exclusively, on the NF adenoma cohort. Through analysis of the 12 genes identified by BeadArray and confirmed by pyrosequencing in the discovery cohort, we determine the frequency of aberrant methylation across an independent cohort of 13 NF adenomas relative to four pmPits. **Figure 3** shows the analysis where each gene in the pmPits, with the exception of *HOXB1*, shows low but variable levels of methylation. We set a stringent cut-off criterion for increased methylation in individual adenomas of ≥ 4 s.d. higher than the s.d. of the mean (for each of the genes) that was apparent in the four pmPits. The proportion of adenomas showing increase, above each of the gene-specific thresholds, is shown in **Fig. 3** and is summarised in **Table 3**. Within the NF cohort, the number of adenomas that show gene-specific increase in methylation is variable, where the CpG island methylation of the *KIAA1822* (*HHIPL1*) and *TFAP2E* is found in 12 of 13 NF adenomas whereas the *COL1A2* CpG island shows increased methylation in only two of 13 adenomas.

Subtype-specific gene methylation frequencies

Of the 12 genes subject to validation in the NF adenomas, six of these genes also fulfilled the $\Delta\beta$ criterion in two or more of the other adenoma subtypes (**Table 2**). To gain insight into the subtype specificity of hypermethylation, we performed pyrosequence analysis for three of these genes where, in addition to the NF adenomas, we included each of the other subtypes (**Fig. 4**). For *EML2*, and confirming the BeadArray finding, methylation is confined to NF adenomas. Cross subtype-specific methylation is apparent for the *RHOD* and *KIAA1822* genes where the array (**Table 2**) and pyrosequencing shows increased methylation in a proportion of the NF- and GH-secreting adenomas and in the NF-, GH- and PRL-secreting adenomas respectively (**Fig. 4**).

Methylation-associated gene silencing

For the 12 genes with pyrosequencing-confirmed methylation status, we used Quantitative RT-PCR (RT-qPCR) to determine association between CpG island methylation and gene silencing. In these cases, loss or significantly reduced transcript expression was assigned on the basis of the stringent criterion described for gene-specific methylation. However, in these cases, it was ≤ 4 s.d. of the s.d. on the mean of the pmPits. In the 13 NF adenomas, an inverse relationship between increased methylation and reciprocal

Figure 2 Heatmap of genes in individual NF and GH adenomas that fulfilled the criteria of elevated β in two gene-associated CpGs relative to the pmPits (NP). For each gene that satisfied the criteria, the sum of the two β values is used and displayed as a graded colour scale that is shown in the figure.

significant decrease in transcript expression is apparent for three of the 12 genes examined. Figure 5 shows that relative to pmPit, all the methylated tumours (filled circles) show significantly reduced transcript expression. Conversely, in adenomas that were not hypermethylated (unfilled circles), expression levels are similar to or in some cases higher than that apparent in the pmPits. A single exception to these findings is apparent for the *HOXB1* gene, where a single adenoma that was not methylated also shows reduced transcript expression. In addition, as multiple studies using candidate gene approaches have shown frequent methylation-associated loss of *CDKN2A* (Woloschak *et al.* 1997, Simpson *et al.* 1999, Vandeve *et al.* 2010), we included RT-qPCR analysis of this transcript. These studies show significantly reduced expression of this transcript in 12 of 13 adenomas relative to pmPits (data not shown) further confirming and reinforcing the robustness of our approach.

Gene ontology analysis of hypermethylated genes

Gene ontology analysis of the hypermethylated genes was performed to determine the biological processes where these genes are known to contribute together

with their molecular function and finally their associated KEGG pathways. These analyses are shown in Supplemental Tables S3, S4 and S5, see section on supplementary data given at the end of this article respectively. The aberrantly methylated, promoter-associated CpG islands were distributed across various categories of biological processes, molecular function and pathways. However, multiple genes associated with particular biological processes showed frequent methylation in the analysis. In this context, we noted that 11 genes in intracellular signalling cascades (Supplemental Table S4) were hypermethylated in pituitary adenomas. Interestingly, among these genes, the majority (*SOCS2*, *RAC2*, *ERBB2*, *RASSF1*, *SOCS1* and *COL1A2*) have also been shown to exhibit tumour suppressor properties in multiple other tumour types (Qian *et al.* 2005, Mottok *et al.* 2007, Sasi *et al.* 2010, Bonazzi *et al.* 2011, Mizukawa *et al.* 2011, Walter *et al.* 2012).

Discussion

The study of the epigenome in normal and disease states, and in particular as it relates to tumorigenesis, is an area of growing interest and research focus. In this

Figure 3 Pyrosequence analysis of promoter-associated CpG island methylation of genes first identified as hypermethylated by BeadArray analysis. The figure shows methylation in an independent cohort of 13 NF adenomas relative to the mean methylation for each gene examined in the four pmPits. For each gene, in the pmPits, the mean percentage methylation and four times the s.d. of the mean are shown as horizontal bars, either without or with arrows respectively. Only adenomas equal to or greater than this upper threshold were deemed hypermethylated and are shown as filled circles. Adenomas in which methylation is not increased relative to pmPits are shown as unfilled circles. The pmPits are shown as diamonds to the left of the adenomas (circles) in each case.

report, the role of changes in DNA methylation at CpG dinucleotides has been analysed in each of the major pituitary adenoma subtypes. Through use of an Infinium BeadArray approach, we performed a genome-wide analysis, thus providing a quantitative measure of DNA methylation at CpG sites across the genome. The analysis revealed aberrant methylation in each of the adenoma subtypes. Furthermore, it showed that hypermethylation, at CpG sites within gene-associated CpG islands, is more frequent compared with CpG sites outside of CpG islands and similar findings in other tumour types have been reported elsewhere (Kim *et al.* 2011). The epigenetic changes were more frequent in NF adenomas than other pituitary adenoma subtypes; however, we recognise that the absolute numbers of sites identified as hypermethylated is dependent on the cut-off criteria employed.

As in our previous studies and those of other groups, the technical validation of the BeadArray approach shows it to be a robust approach for the quantitative determination of methylation (Fryer *et al.* 2011, Lima *et al.* 2011). Furthermore, and although not reported by others, we found, in pmPits, that the imprinted genes show β values that reflect silencing of one of two alleles and provides further confidence in the derived data.

For the majority of genes interrogated by the 27K BeadArray technology, most are represented by one or two CpG sites on the array (Irizarry *et al.* 2008, Wilop *et al.* 2011). In these cases, although the number of CpG sites interrogated, as representations of a CpG islands, are few in number, the *a priori* of the technology is that CpG sites in close proximity are often co-methylated (Irizarry *et al.* 2008, Ogoshi *et al.* 2011). However, our validation studies on the basis of an elevated β value at a single CpG site failed to show a robust correlation. In these cases and by pyrosequence

analysis, we had determined mean methylation across 5–11 CpGs within the promoter-associated CpG islands. Using modified and more stringent criteria, which considered two promoter-associated CpGs on the BeadArray, we found significant concordance between the techniques. In this case, 12 of 16 (75%) genes were validated by pyrosequencing. Other studies, where they report validation of BeadArray data, frequently use techniques such as methylation-sensitive PCR (Yoon *et al.* 2010, Wilop *et al.* 2011). A limitation of this technique is that they determine methylation at a limited number of CpG sites. Equally, this approach is reliant upon an amplification step and may not necessarily accurately reflect the methylation status of the CpG islands that is representative of the majority of cells within the specimen (Irizarry *et al.* 2008, Wilop *et al.* 2011, Zeller *et al.* 2012).

Table 3 Frequencies of gene-specific methylation in an independent cohort of NF adenomas. Genes were first identified in the discovery cohort and using the criteria described in the text and in Figs 2 and 3

Gene	Number of hypermethylated adenoma	Number of adenomas showing methylation similar to pmPits
BCL9L	4	9
COL1A2	2	11
EML2	7	6
FLJ46380	9	4
HAAO	11	2
HOXB1	7	6
KCNE3	10	3
KIAA1822	12	1
MT1G	11	2
PGLYRP1	7	6
RHOD	8	5
TFAP2E	12	1

Figure 4 Adenoma subtype specificity of hypermethylation for three genes in which pyrosequencing confirmed their methylation in NF adenomas is shown in Fig. 3. For subtype specificity, in addition to the 13 NF adenomas, the methylation status of each gene was also determined in seven GH, six PRL and six CT (CUSH) adenomas. Symbols and cut-off criteria are as described in Fig. 3. The pmPits are shown as diamonds on the right side of each of the gene investigated.

The gene ontology analysis, on the basis of the more stringent cut-off criteria, shows the genes to be distributed across various categories of biological processes, molecular function and pathways. Although no biological processes are specifically enriched, we found that multiple genes involved in intracellular signalling pathways are hypermethylated relative to pmPit. Interestingly, many of these genes are silenced in association with CpG methylation in other tumour types (Qian *et al.* 2005, Mottok *et al.* 2007, Sasi *et al.* 2010, Bonazzi *et al.* 2011, Mizukawa *et al.* 2011, Walter *et al.* 2012) and interestingly the *RASSF1* gene has been identified in earlier studies using a candidate gene approach of sporadic pituitary adenomas (Qian *et al.* 2005).

Although the BeadArray allowed us to examine each of the major pituitary adenoma subtypes in a single experiment, which is on the same array, we were aware of the constraints and limitations of any conclusions reached through examining a limited number of each of the adenoma subtypes. Equally the amount and absolute number of each adenoma subtype were also a limitation for all the analyses we wished to undertake. However, through focusing our studies, post-BeadArray analysis, principally but not exclusively on NF adenomas, we gained further insight into the aspects of epigenetic change. In this case, in an independent cohort that comprised 13 NF adenomas, and for 12 genes (identified in the discovery cohort), we determined methylation frequencies relative to pmPits. Again using stringent criteria (four times the s.d. apparent in pmPits), we determined the frequencies of differential methylation across the cohorts for each of these genes. The genes show variable frequencies of methylation and have not been previously described in

pituitary adenomas. Indeed, for many of the genes identified in our discovery cohort, as example, *SOCS1*, *SEPT9*, *PDLIM4*, *TFAP2E*, *MT1G*, *HAAO*, *TFAP2E*, *CRIP1* and *COLIA2* methylation-mediated gene silencing, together with known or putative tumour suppressor characteristics has been reported in other tumour types (Qian *et al.* 2005, Mottok *et al.* 2007, Sasi *et al.* 2010, Bonazzi *et al.* 2011, Mizukawa *et al.* 2011, Walter *et al.* 2012).

Although the absolute number and amount of tumour tissue, for the other adenoma subtypes, was

Figure 5 Quantitative RT-PCR analysis for three of 12 gene transcripts that show an inverse relationship between hypermethylation and decrease in transcript expression. For each gene, their methylation status in NF adenomas was first validated in the discovery cohort (Table 2) and in the independent cohort shown in Fig. 3. Expression is shown relative to four pmPits and the same cut-off criteria of four s.d., but in this case lower than the mean level shown in pmPits (horizontal bars with arrow heads). Filled and unfilled circles represent adenomas that are hypermethylated and those that were not for individual NF adenoma respectively. The pmPits are shown as diamonds on the left side of each the gene investigated.

limiting, we considered it important to validate the subtype specificity of aberrant methylation. To that end, we examined hypermethylation of CpG islands that were methylated in common and those where this change is apparent in two or more subtypes as determined by BeadArray analysis. The analysis shows, by an independent technique, namely pyrosequencing, that the identified changes and differences in hypermethylation were indeed subtype specific.

In this first report of unbiased genome-wide changes in methylation, we focused our studies on increase in methylation of CpG islands associated with gene promoter. In these cases, these regions are considered the most relevant for expression of the corresponding gene (Irizarry et al. 2008, Moran et al. 2012). In the 13 NF adenomas and across the 12 genes studied, only three showed an association between hypermethylation and reduced transcript expression. Similar findings have been reported by others and specifically where associations between increased BeadArray-identified CpG methylation and loss or reduced transcript expression has been investigated (Irizarry et al. 2008, Noushmehr et al. 2010, Kim et al. 2011, Wilop et al. 2011, Zeller et al. 2012). Multiple factors might account for this lack of association, including density of methylation across the CpG islands, histone modification or the milieu of transcription factor within a cell or cell type (Irizarry et al. 2008, Wilop et al. 2011). These findings have led some authors to conclude that mRNA expression is more likely to be down-regulated when the promoter region is hypermethylated even through it is not statistically significant (Kim et al. 2011). Therefore, and by extension, this likely reflects the view, put forward by other investigators, that many epigenetic and genetic alterations in cancer are ‘passenger’ as opposed to ‘driver’ events in the evolution and progression of a tumour (Kim et al. 2011, Zeller et al. 2012).

This study provides a first, unbiased survey of the pituitary tumour epigenome across different adenoma subtype. Further studies will be needed to corroborate identification of these CpG islands, characterised on the basis of their hypermethylation and relative to that seen in normal pituitaries. These types of studies will be useful for identification of biomarker that perhaps predict or characterise tumours likely to show aggressive or recurrent growth characteristics. In addition, the functional characterisation of down-regulated genes will provide new understanding of tumour aetiology and biology and perhaps identify novel genes or pathways that provide us with new therapeutic targets or options.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/ERC-12-0251>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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