

Loss of neuronatin expression is associated with promoter hypermethylation in pituitary adenoma

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Abstract

The imprinted gene, neuronatin (*NNAT*), is one of the most abundant transcripts in the pituitary and is thought to be involved in the development and maturation of this gland. In a recent whole-genome approach, exploiting a pituitary tumour cell line, we identified hypermethylation associated loss of *NNAT*. In this report, we determined the expression pattern of *NNAT* in individual cell types of the normal gland and within each of the different pituitary adenoma subtypes. In addition, we determined associations between expression and CpG island methylation and used colony forming efficiency assays (CFE) to gain further insight into the tumour-suppressor function of this gene. Immunohistochemical (IHC) co-localization studies of normal pituitaries showed that each of the hormone secreting cells (GH, PRL, ACTH, FSH and TSH) expressed *NNAT*. However, 33 out of 47 adenomas comprising, 11 somatotrophinomas, 10 prolactinomas, 12 corticotrophinomas and 14 non-functioning tumours, irrespective of subtype failed to express either *NNAT* transcript or protein as determined by quantitative real-time RT-PCR and IHC respectively. In normal pituitaries and adenomas that expressed *NNAT* the promoter-associated CpG island showed characteristics of an imprinted gene where ~50% of molecules were densely methylated. However, in the majority of adenomas that showed loss or significantly reduced expression of *NNAT*, relative to normal pituitaries, the gene-associated CpG island showed significantly increased methylation. Induced expression of *NNAT* in transfected AtT-20 cells significantly reduced CFE. Collectively, these findings point to an important role for *NNAT* in the pituitary and perhaps tumour development in this gland.

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Introduction

Pituitary tumours are classified as either functional (hormone secreting) or non-functional and comprise ~10% of diagnosed intracranial neoplasms (Asa & Ezzat 2002, 2005). Each of the differentiated cell types within the gland has the potential to develop a tumour and although the majority will remain benign, approximately one-third will show invasive and/or recurrent growth and a smaller proportion will become metastatic. Adenomas are frequently associated with significantly reduced life quality and irrespective of their hormone status morbidity can ensue through

either compressive effects on brain structures and/or excessive hormone secretion (Ezzat *et al.* 2004).

Methylation-mediated or -associated gene silencing is a frequent finding in pituitary adenomas where most studies, with limited exceptions, have adopted candidate gene approaches of primary human adenomas (Zhang *et al.* 2003, Bahar *et al.* 2004, Zhao *et al.* 2005). Recently, our laboratory using a whole-genome approach found that the imprinted gene, neuronatin (*Nnat*), is expressed in normal mouse pituitary, but is not expressed in the mouse pituitary adenoma cell line At-T20. However, targeted, siRNA-mediated

knockdown of DNA methyltransferase 1 (*Dnmt1*) in At-T20 cells induced re-expression in a time-dependent manner (Dudley et al. 2008). Re-expression was associated with partial demethylation of the *Nnat* genes CpG island, implying that silencing is mediated through DNA methylation (Dudley et al. 2008). Among several other genes identified in this murine model system, we also showed that *NNAT* was inappropriately silenced in association with CpG island methylation in a proportion of primary human pituitary tumours relative to normal human pituitary (Dudley et al. 2008).

In the pituitary, serial analysis of gene expression has identified *NNAT* as the fifth most abundant transcript after GH, pro-opiomelanocortin, PRL and an unknown transcript, and this and other studies also show that this transcript is not expressed at significant levels in most other adult tissues (Usui et al. 1997, Nishida et al. 2005, Suh et al. 2005). In the brain, *NNAT* is thought to participate in maintaining segment identity and the overall structure of the nervous system (Wijnholds et al. 1995). In addition, *NNAT* is also thought to be involved in both pituitary development and maturation (Aikawa et al. 2003). Taken together, these findings suggest an important role for *NNAT* in the adult and developing pituitary gland and that loss may have implications for pituitary tumorigenesis and perhaps that of other tumour types. Indeed, and in this context, decreased *Nnat* expression was identified in a screening study of rat cells in the progression from immortalization to transformation (Yoshioka et al. 2000). Although this study did not report the functional significance of *Nnat* loss in this transition, other studies of different tumour types have shown that *NNAT* may exhibit either a tumour suppressing or in some cases a tumour promoting role (Takeuchi et al. 1995, Asimakopoulos et al. 1996, Usui et al. 1997, Hu et al. 2002, Kuerbitz et al. 2002, Wood et al. 2002, Becker et al. 2005, Zhong et al. 2006).

Our initial findings of methylation associated silencing of *NNAT* in a small number of pituitary adenomas (Dudley et al. 2008) prompted us to address the unanswered questions of frequency of loss across different pituitary adenoma subtypes and expression patterns of *NNAT*, at the protein and transcript level, in individual cell types that comprise the normal human pituitary. These studies, in addition to allow us to determine the cell-type specificity of *NNAT* expression patterns in normal and tumour cells, also allowed us to determine associations between expression and CpG island methylation and finally, to gain further insight into the tumour suppressor function of this gene in a pituitary context.

Materials and methods

Cell line and tissue samples

The mouse pituitary adenoma cell line AtT-20/D16v-F2 (AtT-20) was purchased from the European collection of cell cultures (Salisbury, UK). Semi-confluent (70–80%) cells were split 1:5 using 0.25% trypsin/0.53 mM EDTA (Sigma-Aldrich), into high glucose DMEM with glutamine and pyruvate (Biosera, Ringmer, UK), 10% foetal bovine serum (Biosera), 4 µg/ml gentamycin (Sigma-Aldrich) and 2 µg/ml ampicillin (Sigma-Aldrich). Monolayer cultures were incubated at 37 °C in a humidified atmosphere containing 5% CO₂.

Human normal pituitaries and adenomas were obtained with informed consent and institutional ethical approval. Five normal pituitaries were obtained within 12 h of death, freeze fractured using the BioPulveriser 59013N (Biospec, Bartlesville, OK, USA) to achieve a homogenous mixture of cells. The pulverized homogenate was stored at –80 °C.

The tumour cohort comprised a total of 47 adenomas. Subtypes were determined on the basis of clinical and biochemical findings before surgery, and on morphological and immunohistochemical (IHC) characterization of the tumour specimen. Out of the 47 adenomas included in the study, there were 11 somatotrophinomas, 10 prolactinomas, 12 corticotrophinomas and 14 non-functioning pituitary adenomas (NFPAs).

Nucleic acid extraction

Total RNA was extracted using the guanidinium thiocyanate–phenol–chloroform method as previously described (Chomczynski & Sacchi 2006). DNA was extracted from tissue using the Nucleon DNA extraction kit (Anachem, Bedfordshire, UK) according to the manufacturer's instructions. DNA extraction from whole blood was achieved using the Wizard Genomic DNA Purification Kit as recommended by the manufacturer (Promega). Nucleic acid purity and concentrations were assessed using a NanoDrop ND-1000 spectrophotometer (Labtech, Ringmer, East Sussex, UK).

cDNA synthesis and RT-PCR

M-MLV Reverse Transcriptase (Promega) was used to prepare cDNA template as previously described (Dudley et al. 2008). Quantitative real-time RT-PCR (RT-qPCR) using the relative standard curve method was performed on the ABI PRISM 7300 HT Sequence Detection System (Applied Biosystems, Cheshire, UK)

using brilliant SYBR Green QPCR Master Mix solution (Stratagene, Amsterdam, The Netherlands). One microlitre cDNA was amplified under default conditions: 96 °C for 10 min followed by 40 amplification cycles of 95 °C for 30 s, 60 °C for 1 min and 72 °C for 1 min. Hypoxanthine-guanine phosphoribosyl transferase (*HPRT*) was amplified in parallel and used as an internal control to normalize the results and to allow for quantitative analysis of *NNAT* transcript levels. The primers used to amplify *NNAT* were 5'-CAACAGCGGACTCCGAGA-3' and 5'-CATTCAGGAACACCTGCA-3'. The primers used to amplify *HPRT* were 5'-CGGCTCCGTTATGGCG-3' and 5'-GGTCATAACCTGGTCATCATCAC-3'. The calibrated RT-qPCR values for samples relative to the mean of five normal pituitaries were plotted as log to the base 2. An adenoma was deemed negative for *NNAT* expression in those cases where a threefold reduction in expression was observed.

IHC staining

To identify specific cell types within normal pituitary, sections were stained with antibodies directed against each of the major hormones secreted by this gland. Thus, as previously described (Simpson *et al.* 1999) with minor modifications, slides of formalin fixed, wax embedded normal human pituitary tissue were first re-hydrated and washed, serum blocked for 15 min at room temperature then incubated at room temperature for 30 min with antibodies directed against ACTH (mouse monoclonal, 1:2000, DAKO Cytomation, Ely, UK), GH (rabbit polyclonal, 1:6000, DAKO), FSH (mouse monoclonal, 1:5000, Serotec, Oxford, UK), PRL (mouse monoclonal, 1:2000, Serotec) or TSH (mouse monoclonal, 1:20 000, Serotec). Sections were then washed and incubated with biotinylated secondary antibody (Vector, Southgate, UK) for 30 min at room temperature. Antibodies were detected using the avidin–biotin complex (Vector) method using vector blue-stain as the chromogen.

To observe co-localization of *NNAT* with each pituitary hormone, the slides were then taken through an antigen retrieval step, serum blocked for 15 min at room temperature and then incubated with an antibody directed towards *NNAT* (rabbit polyclonal, 1:1000, Abcam, Cambridge, UK). *NNAT* antibody was detected using the EnVision DAB system (DAKO Cytomation) with Dako REAL 3,3-diaminobenzidine tetrahydrochloride (DAB; DAKO Cytomation) as the chromogen. Finally, the slides were haematoxylin counterstained with a 5 min immersion in 5% copper sulphate to intensify the brown DAB stain, then

de-hydrated and mounted with Pertex substitute (BDH, Lutterworth, UK) and visualized using the Nikon Eclipse e600 camera. Specificity of the antibody to *NNAT* was assessed by incubation with 25 µg *NNAT* immunizing peptide (Abcam) prior to the staining procedure described above.

Co-localization of *NNAT* with each of the individual pituitary hormone antibodies used in this study was observed as a merging of the blue-(hormone) and the *NNAT*-(brown) specific stains to produce an intensity of colour that varied between dark blue and dark brown.

A total of 21 adenomas, that included specimens of each of the pituitary adenoma subtypes, were stained for *NNAT* immunoreactivity using the EnVision DAB system as described above.

DNA methylation analysis

Extracted DNA samples (3 µg) were mutagenized by treatment with sodium bisulphite solution, as previously described (Grunau *et al.* 2001). Following PCR amplification using primers specific for converted DNA, the methylation status of the CpG island associated with exon 1 of *NNAT* was assessed, by several different methodological approaches, including combined bisulphite restriction analysis (COBRA), single molecule sodium bisulphite sequencing or methylation sensitive pyrosequencing. Primers for COBRA and sodium bisulphite sequencing analysis were 5'-TATGGAAAGAGTAGATGGATTAT-3' and 5'-AATTAAAAACCCTCTCCCACC-3'. The primary amplification was followed with a nested PCR with the upstream primer described above and the downstream, nested primer 5'-TCAAACCTACCTACAACAACA-3'. In this way, the derived amplicons encompassed a total of 25 CpG sites.

COBRA

For COBRA, equal amounts of sodium bisulphite converted PCR product were incubated in the presence and absence of the restriction enzyme, *BSTUI* (New England Biolabs, Hertfordshire, UK) at 60 °C for 2 h as previously described (Simpson *et al.* 2004). Digested fragments were visualized by ethidium bromide staining of DNA electrophoresed through a 1.5% agarose gel. Post-digestion of the PCR product, and in the absence of methylation, a 218 bp amplicon was apparent. Methylation, at one or more of the four *BSTUI* (CGCG) sites within the amplicon yielded a profile of fragments, 75, 56, 39, 27 and 21 bp of which the larger fragments, 75 and 56 bp can be visualized on the gel.

Single molecule sodium bisulphite sequencing

JM109 cells were transformed with gel purified sodium bisulphite PCR products using the T-Easy vector system (Promega), according to the manufacturer's instructions. Blue white selection of individual clones was used prior to sequence analysis on the ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Warrington, UK). In each case (normal pituitaries and adenomas), at least five and typically ten discrete clones were subjected to sequence analysis.

Methylation standards

In order to determine the sensitivity of methylation sensitive pyrosequencing, the known standards of methylated DNA were prepared and analysed using this technique. NNAT gene-specific DNA was amplified from blood-derived DNA using primers 5'-GATCTCGGCAAACCCTCTTT-3' and 5'-TTTCAACACAGTAGTGCCCC-3' at an annealing temperature of 55 °C, resolved on a 1% agarose gel, excised and recovered using the Gene Clean II kit (Anachem) according to the manufacturers instructions and divided into two fractions. One fraction, comprising 100 µg PCR amplified DNA in a final volume of 100 µl was *in vitro* methylated with the enzyme *SssI*, 3 µl *SssI* methylase (NEB, Luton, UK) and 10 µl S-adenosyl methionine (NEB). The other fraction was sham *in vitro* methylated by omitting the *SssI* enzyme. The *in vitro* methylated and sham *in vitro* methylated NNAT DNA were mixed in ratios representing 0, 25, 50, 75 and 100% methylation and subjected to methylation sensitive pyrosequencing.

Methylation sensitive pyrosequencing

Prior to pyrosequencing, sodium bisulphite-modified DNA was amplified with primers, 5'-GATCTCGGCAAACCCTCTTT-3' and 5'-TTTCAACACAGTAGTGCCCC-3'. Amplified DNA was subjected to pyrosequencing with primer 1, 5'-TTATTTTTTTT-TATAAATAT-3' and primer 2, 5'-GGTGGGTGGG TATTTAAGG-3' that encompassed 10 CpG dinucleotides. Pyrosequencing was performed as previously described (Dudley et al. 2008).

Loss of heterozygosity analysis

For loss of heterozygosity (LOH) studies, the microsatellite marker *D20S478* was employed that maps proximal (1.08 Mb) to the NNAT gene. Matched blood and tumour DNA from eight samples was extracted as previously described and subjected to PCR. The PCR parameters followed the outline; 94 °C for 5 min,

40 cycles of 94 °C for 30 s, 55 °C for 1 min and 72 °C for 30 s and 72 °C for 5 min. PCRs were performed with 1 µl DNA and 20 pmol oligonucleotides for *D20S478*; 5'-CCAAAGACAAGTTTGTTTATCTG-3' and 5'-TGTTATAGCAGCAATAGAAAATAA-3'. Products were visualized by PAGE and silver staining as previously described (Simpson et al. 2000).

Functional analysis

The complete coding sequence of NNAT (GeneBank ID NM181689) was obtained from the Mammalian Gene Collection (MGC: 3353944, Cambridge, UK) and cloned into a 'TET on' system using the tetracycline-inducible vector, pcDNA 4/TO (Invitrogen) to generate a NNAT expressing construct pcDNA 4/TO-NNAT (TO-NNAT). Empty vector controls (TO-MCS) did not contain the coding sequence for NNAT. Constructs were transfected into AtT-20 cells that had been established in our laboratory to express the Tet repressor construct, pcDNA 6/TR (Invitrogen) and designated, AtT-20TRC1 cells. For selection, the growth media of cells transfected with pcDNA 6/TR were supplemented with 6 µg/ml blasticidin, and for TO-NNAT and TO-MCS, 0.5 µg/ml bleocin. Transfectants were generated as previously described (Frost et al. 1999, Bahar et al. 2004). Stable-transfected AtT-20 cells expressing the Tet repressor (AtT-20-TRC1) and either the NNAT (TO-NNAT) construct or empty vector control (TO-MCS) was designated TR/TO-NNAT and TR/TO-MCS respectively.

Transfected cells were seeded at 2×10^4 cells/ml in DMEM supplemented with tetracycline reduced FCS in 60 mm Petri dishes with a gridded base (Corning Costar, Koolhovenlaan, The Netherlands) with or without the addition of 0.5 µg/ml tetracycline (Sigma) which was replaced at 2 day intervals. Cells were cultured at 37 °C until distinct colonies could be visualized following which the number of colonies were counted on a Nikon Microscope ($\times 4$ magnification) and photographed using a Leica confocal microscope (Milton Keynes, Buckinghamshire, UK). Colony forming efficiency (CFE) was calculated by counting the number of colonies over 100 squares on each plate.

Statistical analysis

The χ^2 statistical test was used to determine the correlation between loss of NNAT expression as determined by RT-qPCR with IHC and also the association of loss of NNAT expression with promoter hypermethylation. Statistical significance was accepted at $P < 0.05$.

Results

IHC localization of NNAT in normal pituitary

Co-localization of NNAT, in post-mortem normal pituitaries, with individual pituitary hormones as determined by IHC was used to identify the different cell types that expressed NNAT. Within the anterior pituitary gland (AP), somatotrophs comprise ~50% of the cells with the remaining cells, lactotrophs, gonadotrophs, corticotrophs and thyrotrophs comprising ~10–20% each (Horvath & Kovacs 1988, Yeung *et al.* 2006). Irrespective of hormonal status of individual cells, ~40% of cells within the AP-stained positive for NNAT protein (Fig. 1, panel A). The specificity of the NNAT antibody was confirmed in pre-adsorption studies outlined in Materials and Methods and is shown in Fig. 1, panel B.

Staining was apparent within clusters of cells and rarely scattered. Immunopositivity for NNAT was apparent within the cytoplasm and also on the outer cell membranes. Figure 1, panels C–L, shows normal pituitary stained for each of the pituitary hormones and the double staining of pituitary hormone and NNAT. These co-localization studies show that NNAT is expressed by each of the cell subtypes of the AP. NNAT was found to co-localize with ~40% of somatotrophs, ~30% of corticotrophs, ~50% of thyrotrophs, ~30% of lactotrophs and ~20% of gonadotrophs.

NNAT expression status in pituitary adenomas

A total of 47 pituitary adenomas were analysed by real-time RT-qPCR for NNAT transcript expression. Overall, 33 out of 47 adenomas (70%) failed to express

or showed barely detectable NNAT expression relative to the mean of expression in normal pituitaries. By subtype, 12 out of 14 non-functioning adenomas, 5 out of 11 somatotrophinomas, 6 out of 12 corticotrophinomas and 10 out of 10 prolactinomas showed loss or significantly reduced expression of NNAT (Fig. 2A).

In total, 21 adenomas comprising representative numbers of each pituitary adenoma subtype were assessed for the presence of NNAT by IHC (Fig. 2B). Of these, eight out of nine that expressed NNAT, as determined by RT-qPCR, stained positively for NNAT. Eleven out of twelve adenomas that showed loss or barely detectable expression of NNAT, as determined by RT-qPCR, did not express IHC detectable NNAT (Fig. 2B). There was a significant correlation between loss of NNAT expression, as determined by IHC and loss or significantly reduced expression as determined by RT-qPCR, $P=0.002$.

Methylation status of the NNAT genes CpG island in normal pituitaries and pituitary adenomas

The proximal promoter region of the NNAT gene was first identified using the CisRED database of highly conserved sequence elements in the proximal promoters of genes from multiple species (http://www.ensembl.org/Homo_sapiens/Gene/Regulation?g=ENSG00000053438) and we mapped a CpG island to this region with the CpG island Finder program (<http://www.ebi.ac.uk>). Sodium bisulphite sequencing of this CpG island showed, in normal pituitary, a methylation pattern where ~50% of individual molecules were densely methylated and in the

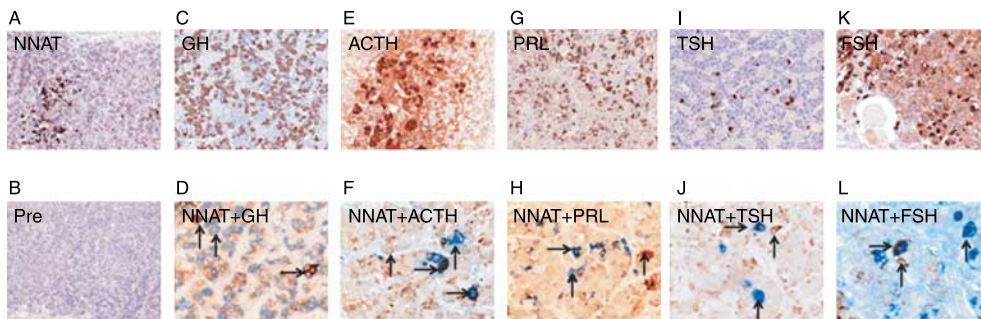


Figure 1 IHC of NNAT and pituitary hormone antibodies in normal anterior human pituitary. (A) Immunoreactivity of NNAT protein is detectable in many cells of the normal anterior human pituitary ($\times 40$ magnification, DAB–HRP, haematoxylin counterstain). (B) Signal specificity is demonstrated using pre-absorbed serum (Pre; $\times 40$ magnification, DAB–HRP, haematoxylin counterstain). (C, E, G, I and K) Immunohistochemical staining of GH, ACTH, PRL, TSH or FSH in normal human anterior pituitary ($\times 200$ magnification, DAB–HRP, haematoxylin counterstain). (D, F, H, J and L) Co-localization of NNAT and GH, ACTH, PRL, TSH or FSH ($\times 400$ magnification). Representative co-localized cells are indicated by horizontal arrows occasionally appears as a dark almost black shade of blue, and for others both blue (NNAT) and brown (pituitary hormone) pigments can be distinguished as overlapping each other. Representative cells staining only for NNAT in brown or hormone in blue are indicated by vertical arrows.

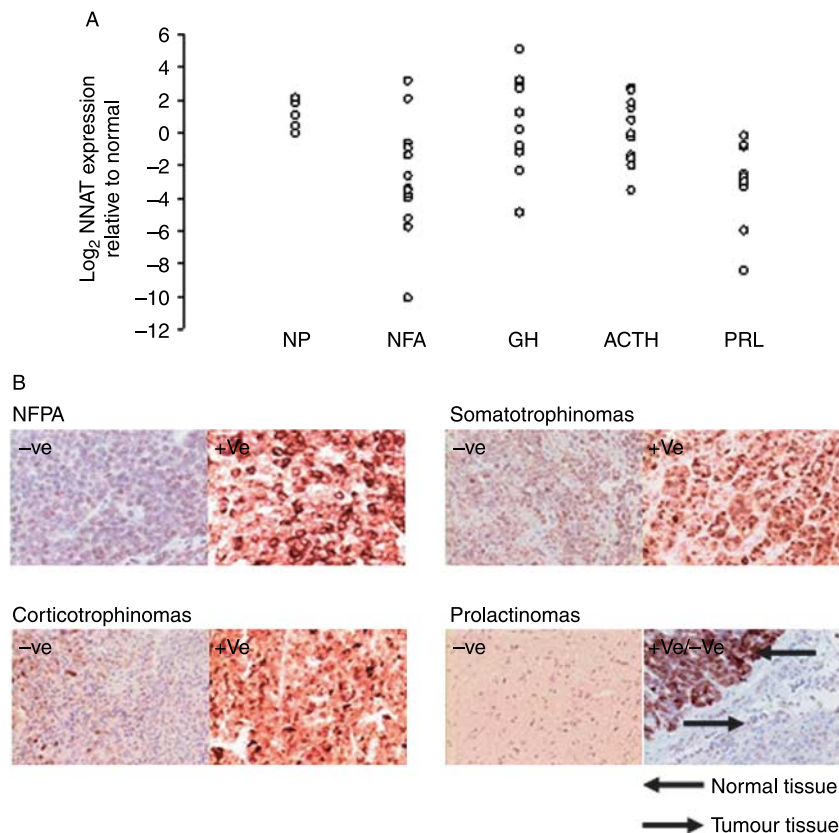


Figure 2 NNAT expression in pituitary adenomas. (A) Transcript expression analysis of each pituitary tumour subtype relative to five normal pituitaries (NP, normal pituitary; NFA, non-functioning adenoma; GH, growth hormone-secreting adenoma (somatotrophinoma); ACTH, corticotrophinoma; PRL, prolactinoma). The mean of five normal pituitaries was used to calibrate the quantitative real-time PCR values from samples of each pituitary adenoma subtype to provide fold difference in expression compared with the mean of normal. These values are plotted as log to the base 2. Adenomas are deemed to be non-expressors of NNAT when there is a threefold reduction in mRNA expression relative to mean normal pituitary. (B) Immunoreactivity of NNAT in each pituitary adenoma subtype: example of negative (–ve) and positive (+ve) staining and an adenoma with focal immunopositive staining of entrapped normal tissue are shown (indicated with arrows).

remaining molecules, this change was infrequent (Fig. 3A). Similar findings of ~50% methylation were apparent using the related technique of COBRA analysis in normal pituitary (Fig. 3B).

In contrast to sodium bisulphite sequencing, COBRA analysis and pyrosequencing require less converted DNA. For adenomas where DNA quantity and quality were frequently limiting, these techniques allowed us to analyse a larger number for their methylation status. COBRA was performed on 14 adenomas, of which 11 did not express and 3 did express NNAT as determined by RT-qPCR. COBRA analysis showed that 10 out of 11 adenomas that did not express NNAT transcript were methylated and apparent as the disappearance of the unmethylated 218 bp amplicons ($P=0.002$). The three tumours that expressed NNAT transcript were not methylated as determined by this technique (Fig. 4A).

A limited number (four) of adenomas from the above COBRA analysis had sufficient DNA available to perform sodium bisulphite sequencing (Fig. 4B). In these cases, in four adenomas that did not express NNAT, a significant increase in methylation relative to that observed in normal pituitary was apparent and thus confirmed the COBRA data.

A total of 26 adenomas provided sufficient converted DNA for pyrosequencing. However, prior to this analysis, we designed experiments to confirm that this method was an accurate way of determining methylation profiles (Fig. 5A). Following this validation, 5 normal pituitaries, 4 adenomas that expressed NNAT and 22 adenomas that did not express NNAT as determined by RT-qPCR were analysed across 10 individual CpG dinucleotides. For seven out of nine specimens, that comprised normal pituitaries and NNAT expressing tumour, a methylation profile

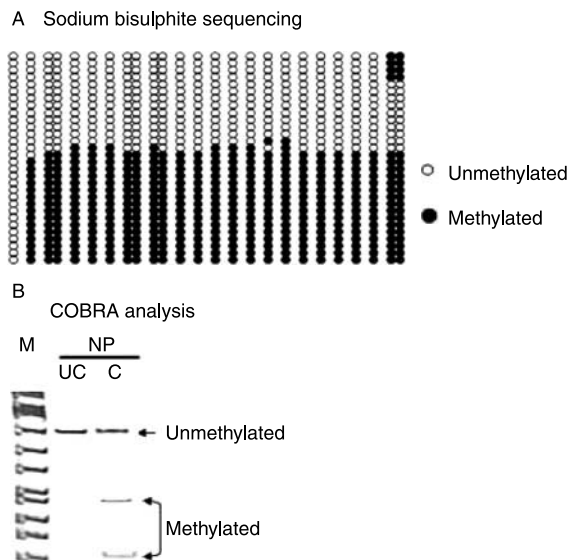


Figure 3 Imprinted status of *NNAT* in normal pituitary. (A) Single molecule sodium bisulphite sequencing of *NNAT* in normal human pituitary tissue represented by 'beads on a string' with each bead representing a CpG site and each string a single molecule. Filled circles are methylated CpGs and unfilled circles are unmethylated CpGs. (B) Detection of *NNAT* CpG island methylation by COBRA analysis in normal human pituitary tissue (NP). UC, uncut; C, cut. Unmethylated molecules remain uncut at 218 bp, methylated molecules cut to yield fragment sizes of 75 and 56 bp and smaller fragments from the digestion are not visible.

consistent with that of an imprinted gene was apparent ($\sim 50\%$ methylation; $P=0.015$ and Fig. 5B). However, 20 out of 22 tumours that fail to express *NNAT* showed a significant increase in methylation that showed some variation between 75 and 100% ($P=0.007$ and Fig. 5B).

Taken together, each of these techniques showed in a large number of adenomas that gene silencing was associated with CpG island methylation. In addition, where methylation was determined by more than one technique, the findings were concordant.

Loss of *NNAT* expression in pituitary tumours is not associated with LOH

The possibility that loss of *NNAT* expression was through the loss or deletion of the unmethylated allele and that the methylation patterns that we observed represented preferential retention of the imprinted, methylated, allele was investigated through LOH analysis in a limited number of specimens. Ten adenomas relative to matched blood DNA were screened using the polymorphic microsatellite marker, *D20S478*, closely linked to *NNAT* on chromosome 20q. Eight adenomas (three expressing and five

non-expressing) were informative. Using previously published criteria (Miller & Yuan 1997), we found no evidence for loss (data not shown).

Functional studies

The robust expression of *NNAT* in normal human pituitary and the methylation associated silencing in the majority of human pituitary adenomas investigated, suggested an important function for the protein product of this gene in pituitary tumorigenesis. Therefore, we assessed the tumour suppressor activity of this gene/protein in At-T20 cells by CFE assays. These experiments were performed on TR/TO-*NNAT* cells relative to empty vector control TR/TO-MCS cells in the presence and absence of tetracycline. Tetracycline induced expression of *NNAT* in At-T20 cells reduced CFE by $\sim 60\%$ (Fig. 6). These findings reinforce those from our previous study, where IPTG (isopropyl β -D-1-thiogalactopyranoside) induced expression of *NNAT* reduced proliferation as determined by growth curve analysis (Dudley *et al.* 2008).

Discussion

Despite data that show *NNAT* to be one of the most abundant transcripts present in the mature pituitary and its expression in the different cell types that characterize this gland had not thus far been described (Nishida *et al.* 2005). Employing IHC co-localization, we show that each of the discrete hormone secreting cell types within the anterior gland express *NNAT*. In contrast to these findings, expression of *NNAT* as determined by RT-qPCR showed that a significant proportion of primary tumours, irrespective of subtype, showed barely detectable expression of the *NNAT* transcript relative to normal pituitaries. In addition, IHC *in situ* detection of *NNAT* in representative examples of each adenoma subtype, confirmed these observations. In some cases, tumour and infiltrating or juxtaposed normal pituitary cells were apparent on single sections and in these, IHC frequently revealed the absence (tumour cells) and presence of *NNAT* protein (normal cells) respectively providing further confidence in our findings. The expression of *NNAT* in each of the differentiated cell types of the AP and loss in a substantial proportion of adenomas suggests an important role for this protein within this gland and most likely in tumour evolution.

Only a single previous study has reported expression of *NNAT* in pituitary adenomas and in this case, these investigators employed northern blotting

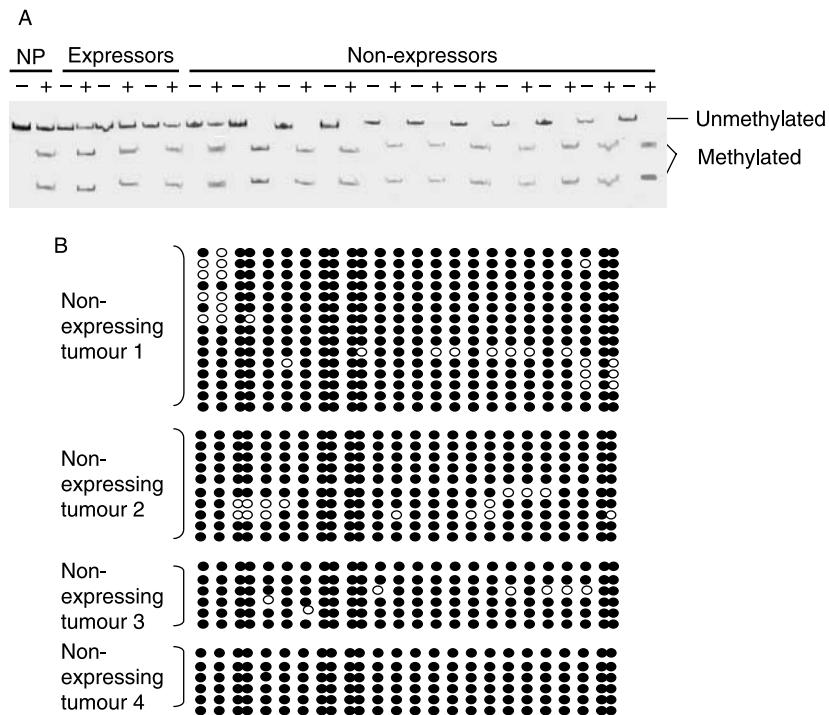


Figure 4 Methylation status of NNAT in pituitary adenomas. (A) COBRA analysis of 3 expressing adenomas and 14 non-expressing adenomas. Lanes where DNA has not been subjected to restriction digest are denoted by a minus sign and lanes showing DNA subjected to digestion are denoted by a plus sign. Unmethylated molecules remain uncut at 218 bp, methylated molecules cut to 75 and 56 bp. (B) Single molecule sodium bisulphite sequencing of four non-expressing adenomas. Black circles represent methylated individual CpG dinucleotides, unfilled circles, unmethylated CpGs.

(Usui *et al.* 1997). In contrast to our own findings, each of the major pituitary adenoma subtypes, with the exception of prolactinomas, but including, non-functional, ACTH and GH secreting adenomas expressed *NNAT*. The differences between these and our own observations are not entirely clear; however, this previous report only investigated two adenomas of each subtype, whereas our own study comprised a considerably larger number of each. In addition, a further explanation might be that northern blotting requires large amounts of RNA and the extraction of this nucleic acid may have included juxtaposed normal pituitary gland with tumour material.

Our initial identification and focus on *NNAT* was on the basis of its demethylation associated re-expression following siRNA mediated knockdown of the maintenance methylase enzyme *DNMT1* in AtT-20 cells (Dudley *et al.* 2008). In addition, in the same study, we also showed in a small number of primary human pituitary adenomas, methylation-mediated silencing of *NNAT* as determined by COBRA analysis and pyrosequencing. However, insufficient adenomas were investigated to determine subtype-specific expression patterns. In this subsequent report, we

have investigated the methylation status of this gene's CpG island in a larger cohort of adenomas that comprises each of the major subtypes. For many of the adenomas included in the investigation, the limited amount of tumour DNA available for study and or its integrity post-sodium bisulphite conversion required use of several different but related methodological approaches. Thus, for normal pituitaries, using either sodium bisulphite sequencing of individual molecules, COBRA analysis or pyrosequencing, we show a methylation pattern similar to that apparent for imprinted genes, which is differential methylation. In these cases, sodium bisulphite sequencing and pyrosequencing of *NNAT* showed that approximately half of the molecules examined were densely methylated and this change was infrequent in the remaining molecules. A similar pattern of methylation in normal pituitary and as determined by sodium bisulphite sequencing has recently described for the *MEG3* gene which is also imprinted (Zhao *et al.* 2005). Using this and related techniques for tumours that failed to express *NNAT* as determined by RT-qPCR and IHC, a significant increase in methylation of this gene's CpG island was apparent,

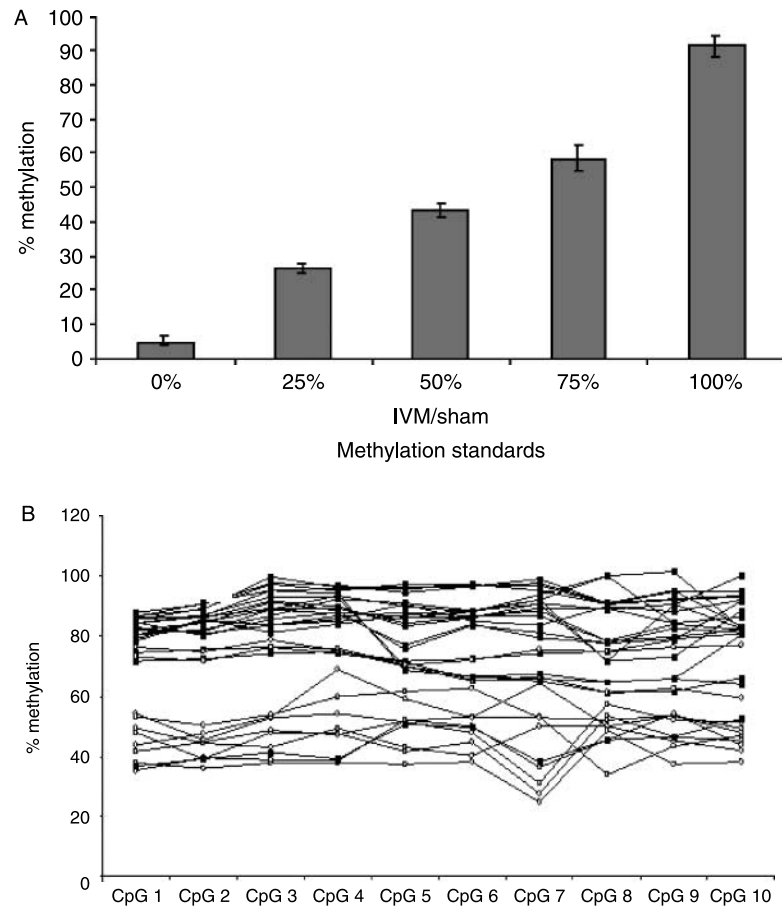


Figure 5 Pyrosequencing analysis of *NNAT* in pituitary adenomas. (A) Methylation-specific pyrosequencing bias study of 0, 25, 50, 75 and 100% methylation standards. (B) Methylation-specific pyrosequencing of non-expressing adenomas and expressing pituitary samples comprising both normal and adenomatous tissue. Tumours expressing *NNAT* as determined by RT-qPCR and/or IHC analysis are shown using unfilled circles and non-expressing tumour by filled squares.

whereas tumours that expressed *NNAT* showed a methylation pattern similar to that observed in normal pituitaries.

The *MEG3* gene represents the first imprinted gene identified as inappropriately methylated in pituitary tumours that showed loss or significantly reduced

expression (Zhao *et al.* 2005). In addition, a previous study suggested through functional analysis, *MEG3* to be a *bona fide* tumour suppressor (Zhang *et al.* 2003). Thus, *NNAT* represents a further example of an imprinted gene that is silenced in association with methylation of the normally expressed and

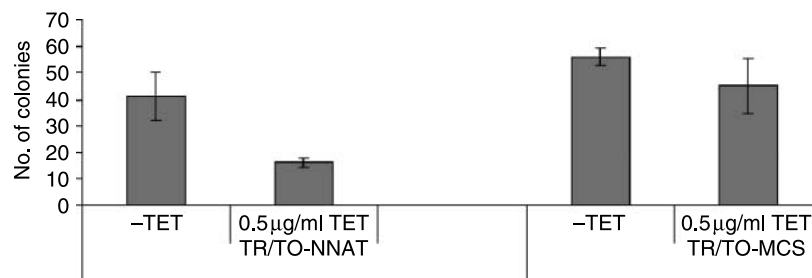


Figure 6 Functional studies. Colony forming efficiency (CFE) of AtT-20 cells transiently transfected with *NNAT* indicates that enforced expression of *NNAT* significantly affects the survival of these cells. AtT-20 cells harbouring the tetracycline repressor plasmid pcDNA 6/TR were transfected with an inducible expression vector containing *NNAT* to give TR/TO-*NNAT* or with an empty vector to give TR/TO-MCS. Triplicate CFE assays were counted following the formation of discrete colonies.

unmethylated allele in pituitary tumours and which appears to act as a tumour suppressor gene. In contrast to *MEG3*, where methylation associated silencing appears to be confined, predominantly to NFPA in the gonadotroph lineage (Zhao *et al.* 2005), methylation associated silencing of *NNAT* does not appear to show particular subtype specificity.

Previous reports, in this case of various primary leukaemias and their derived cell lines, show that they may harbour either, genetic or epigenetic aberrations that target the chromosome 20q12 region. This region, that also harbours the *NNAT* gene within a micro-imprinted domain, has been shown, in this tumour type, to incur either LOH or, more frequently, epigenetic (CpG island methylation) aberration (Kuerbitz *et al.* 2002 and references therein). Indeed, our own results of aberrant methylation, that presumably targets the expressed paternal allele of *NNAT*, could be explained by LOH in the 20q12 region that specifically targets this allele. Microsatellite analysis, employing a marker in close proximity to *NNAT*, albeit in a small number of informative tumours, relative to matched blood samples, did not show losses and thus suggest that genetic mechanisms are not responsible for loss of *NNAT* in these adenomas. Our search for informative single nucleotide polymorphisms in the *NNAT* gene was not successful (data not shown) and, therefore, precluded a more definitive exclusion of genetic aberrations.

Genome-wide identification of genes silenced through epigenetic mechanisms, and in particular through CpG island methylation, has been investigated in multiple different tumour types (Kuerbitz *et al.* 2002, Liang *et al.* 2002, Suzuki *et al.* 2002, Foltz *et al.* 2006, Ibanez de Caceres *et al.* 2006). These studies frequently employ pharmacological strategies to ‘unmask’ silent genes through inhibition of the DNA maintenance methylase enzyme, DNMT1. Our own genome-wide studies that led to preliminary identification of multiple genes including *NNAT* used a genetic (siRNA) ‘unmasking’ strategy that targeted the maintenance methylase, DNMT1. Interestingly, *NNAT* was also recently identified in a genome-wide pharmacological ‘unmasking’ approach in non-small cell lung carcinoma (NSCLC) cell lines following drug treatment with a DNA methylase inhibitor (Zhong *et al.* 2006). Similar to our own studies, these investigators also reported loss of *NNAT* in primary tumours although the methylation status of the *NNAT* genes CpG island in these tumours was not investigated. In addition, this study also showed that ectopic expression of *NNAT* in a NSCLC cell line inhibited growth in colony forming assays (Zhong *et al.* 2006).

Our own studies, in this case of tetracycline induced expression of *NNAT* in AtT-20, also showed a significant reduction in CFE and reinforce our previous report that IPTG-induced *NNAT* expression inhibited cell proliferation as determined by growth curve analysis (Dudley *et al.* 2008).

The present study is the first report that describes the co-localization of *NNAT* to each of the major hormone secreting cell types of the anterior human pituitary gland. We also show that *NNAT* expression is decreased in a significant proportion of pituitary adenomas in association with promoter hypermethylation and that loss is irrespective of adenoma subtype. Although our functional analysis shows that re-expression in a pituitary cell line has a significant effect on the ability of these cells to form colonies, it is likely that this gene and its protein product will have multiple other functions within the pituitary.

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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